

RESEARCH ARTICLE

Safety and toxicological evaluation of undenatured type II collagen

Palma Ann Marone¹, Francis C. Lau², Ramesh C. Gupta³, Manashi Bagchi², and Debasis Bagchi^{2,4}

¹*Eurofins/Product Safety Laboratories, Dayton, NJ, USA*, ²*Department of Research and Development, InterHealth Research Center, Benicia, CA, USA*, ³*Toxicology Department, Murray State University, Murray/Hopkinsville, KY, USA*, and ⁴*Department of Pharmacology and Pharmaceutical Sciences, University of Houston College of Pharmacy, Houston, TX, USA*

Abstract

Previous research has shown that undenatured type II collagen is effective in the treatment of arthritis. The present study evaluated the broad-spectrum safety of UC-II by a variety of toxicological assays including acute oral, acute dermal, primary dermal irritation, and primary eye irritation toxicity. In addition, genotoxicity studies such as Ames bacterial reverse mutation assay and mouse lymphoma tests, as well as a dose-dependent 90-day sub-chronic toxicity study were conducted. Safety studies indicated that acute oral LD₅₀ of UC-II was greater than 5000 mg/kg in female Sprague-Dawley rats. No changes in body weight or adverse effects were observed following necropsy. Acute dermal LD₅₀ of UC-II was determined to be greater than 2000 mg/kg. Primary skin irritation tests conducted on New Zealand Albino rabbits classified UC-II as slightly irritating. Primary eye irritation tests conducted on rabbits indicated that UC-II was moderately irritating to the eye. UC-II did not induce mutagenicity in the bacterial reverse mutation test in five *Salmonella typhimurium* strains either with or without metabolic activation. Similarly, UC-II did not induce a mutagenic effect in the gene mutation test in mouse lymphoma cells either with or without metabolic activation. A dose-dependent 90-day sub-chronic toxicity study revealed no pathologically significant changes in selected organ weights individually or as percentages of body or brain weights. No significant changes were observed in hematology and clinical chemistry. Therefore, the results from the current study show a broad-spectrum safety profile of UC-II.

Keywords: *Undenatured type II collagen; 90-day toxicity study; acute oral toxicity; acute dermal toxicity; primary dermal toxicity; primary eye irritation; body and selected organ weights; hematology and clinical chemistry; histopathology*

Introduction

Arthritis and its related chronic conditions affect one in every five Americans, thus representing one of the most prevalent causes of disability in the US (Helmick et al. 2008). Indeed, over 46 million US adults suffered from doctor-diagnosed arthritis in 2008. This number is estimated to rise to 67 million by 2030, a massive 46% increase, due in part to increases in obesity and longevity (Helmick et al. 2008). There are more than 100 different types of arthritis and among them osteoarthritis (OA) is by far the most prevalent form, affecting ~60% of all arthritis sufferers (Lawrence et al. 2008). Rheumatoid arthritis (RA) is the second most common form of arthritis, impinging on 1.3 million US adults (Helmick et al. 2008). Arthritis describes chronic conditions characterized by joint pain and difficulty in performing certain tasks resulting in

limited activity (Trentham 1984; 1996; Trentham et al. 1993; 2001; Barnett et al. 1996; 1998). Consequently, arthritis imposes a tremendous socioeconomic burden on the US public health system and diminishes the quality of life of millions of people. OA is the second most common chronic disease leading to Social Security disability payments due to long-term absence from work (Bitton 1999). It is prevalent in the aging population and affects roughly 12% of people aged 60 or older (Felson 2009).

OA is defined by the American College of Rheumatology as a heterogeneous group of conditions characterized by degeneration of articular cartilage and changes in the underlying bone at the joint margins (Altman et al. 1986). The etiopathogenesis of OA is multifactorial, and includes inflammatory, metabolic, and mechanical components. A number of risk

Address for Correspondence: Debasis Bagchi, Department of Pharmacology and Pharmaceutical Sciences, University of Houston College of Pharmacy, Houston, TX, USA. Tel: 707.751.2800. Fax: 707.751.2801. Email: dbagchi@interhealthusa.com

(Received 09 December 2009; revised 19 January 2010; accepted 23 January 2010)

ISSN 1537-6516 print/ISSN 1537-6524 online © 2010 Informa UK Ltd
DOI: 10.3109/15376511003646440

<http://www.informahealthcare.com/txm>

factors such as genetics, dietary intake, muscle weakness, obesity, and trauma may initiate various pathogenic pathways leading to OA (Felson et al. 2000). In spite of considerable medical advances in recent years, there is little effective treatment for OA. Common non-surgical treatments of OA include cyclooxygenase-2 (COX-2) inhibitors and non-steroidal anti-inflammation drugs (NSAIDs) targeting pain and inflammation. Unfortunately, many of these agents show limited efficacy and are associated with serious side-effects and high toxicities (Sarzi-Puttini et al. 2005). These side-effects include renal and upper gastrointestinal adverse events, increased risk for cardiovascular events, and elevated blood pressure (Sarzi-Puttini et al. 2005; Berenbaum 2008). In addition, the recent negative press and the withdrawal of certain COX-2 selective NSAIDs from the market have prompted many OA-sufferers to seek alternative therapies. There is a growing recognition of the important role of nutraceuticals in the maintenance of bone and joint health (Goggs et al. 2005). Among these nutraceuticals, a natural collagen extract known as UC-II has gained considerable attention recently for its demonstrated efficacy in the treatment of OA (Crowley et al. 2009).

UC-II is a undenatured type II collagen derived from chicken sternum cartilage. Animal studies (DeParle et al. 2005; D'Altilio et al. 2007; Peal et al. 2007; Bagchi et al. 2008a; 2009; Gupta et al. 2009a; b) and human trials (Bagchi et al. 2008b; Crowley et al. 2009) have demonstrated UC-II to be effective and safe in treating OA. A quantitative evaluation of the therapeutic efficacy of UC-II for 120 days was assessed in osteoarthritic dogs using a Ground Force Plate (GFP) procedure which objectively measures the peak force and impulse area (Gupta et al. 2009b). Dogs on placebo exhibited no significant change in arthritic conditions. UC-II supplemented dogs exhibited a significant improvement, as indicated by GFP analysis. The peak force was increased by 18% and impulse area was elevated by 44%, suggesting an increase in g-force and a decrease in level of pain.

The beneficial effects of UC-II on OA was also observed in horses (Gupta et al. 2009a). Osteoarthritic horses were supplemented with placebo, UC-II (320, 480, or 640 mg) or a combination of 5400 mg of glucosamine plus 1800 mg of chondroitin for 150 days. Horses receiving 320, 480, or 640 mg of UC-II exhibited significant reduction in arthritic pain. UC-II at a dose of 480 or 640 mg provided equal effects, and, therefore, 480 mg was considered optimal. With this dose, there was an 88% decrease in overall pain and a 78% decrease in pain upon limb manipulation. UC-II was found to be more effective in reducing arthritic pain than glucosamine plus chondroitin (Gupta et al. 2009a).

A recent human clinical trial further demonstrated the safety and efficacy of UC-II in the treatment of OA (Crowley et al. 2009). A randomized, double-blind clinical study was conducted in North America on 52 people with OA of the knee. A daily dose of 40 mg of UC-II was more than twice as effective as 1500 mg of glucosamine plus 1200 mg of chondroitin in promoting joint health after 90 days. UC-II significantly decreased joint pain, discomfort, and immobility compared to baseline, and outperformed the glucosamine

plus chondroitin combination using three standard OA assessments: Western Ontario and McMaster Osteoarthritis Index (WOMAC), Visual Analog Scale (VAS), and Lequesne Functional Index.

The objective of the present study was to determine the safety profile of UC-II and hence acute oral toxicity, acute dermal toxicity, primary dermal irritation, primary eye irritation, mutagenicity, and 90-day sub-chronic toxicity studies were conducted by in vivo and in vitro procedures.

Materials and methods

Study compound

UC-II is a unique, patented natural collagen concentrate containing 25% undenatured type II collagen. UC-II (UC-250, off white powder) was obtained from InterHealth Research Center (Benicia, CA) and used in all the studies reported here.

Animals and treatment

Safety tests were conducted at Eurofins/Product Safety Laboratories (Dayton, NJ) in compliance with the Good Laboratory Practices (GLP) as defined in 21CFR58 by the US Food and Drug Administration (FDA, 1987) and in accordance with the Organization for Economic Cooperation and Development (OECD) guidelines for testing of chemicals (OECD 1998). The mutagenicity studies were performed at Bioservice Scientific Laboratories (Planegg, Germany) in compliance with GLP as defined in the Chemikaliengesetz (Chemical Act) of the Federal Republic of Germany (BGB1. I Nr. 50 S. 2407), and in accordance with the Environmental Directorate published by OECD in the Series on Principles of Good Laboratory Practice and Compliance Monitoring (OECD 1998). Animals were cared for in accordance with the most recent Guide for the Care and Use of Laboratory Animals DHEW (NIH). Detailed animal protocols are provided in individual toxicological assessments.

Acute oral toxicity

The acute oral toxicity evaluation (Up and Down Procedure) was conducted in rats to determine the potential of UC-II to produce acute oral toxicity from a single dose through the oral route. Six healthy young adult female, nulliparous, and non-pregnant albino Sprague Dawley rats (aged 9–10 weeks old, initial body weight 188–197 g) were obtained from Ace Animals, Inc. (Boyertown, PA). Female rats were selected for the test because they are frequently more sensitive to the toxicity of test compounds than males. The female rats were singly housed in suspended stainless steel cages with mesh floors conforming to the size recommendations in the most recent Guide for the Care and Use of Laboratory Animals DHEW (NIH). Litter paper was placed beneath the cage and was changed at least three times per week. The rats had free access to standard rat chow (Purina Rodent Chow# 5012) and filtered tap water ad libitum, and were maintained at controlled temperature (20–24°C) and light cycle (12 h light/12 h dark). The animals were acclimated to

laboratory conditions at least 10–14 days prior to initiation of dosing.

UC-II was administered in sequence to the animals, as described in Table 1. The decision to proceed with the next animal was based on the survival of the previous animal following dosing. Before each dosing, rats were fasted overnight, examined through the fasting period for health, and weighed (initial). Individual doses were calculated based on initial body weights at a dose level of 5000 mg/kg. UC-II was administered as a 14% w/w suspension in distilled water using a stainless steel ball-tipped gavage needle. Following administration, each animal was returned to its designated cage and the feed was replaced 3–4 h after the final dosing. Individual body weights were recorded again on days 7 and 14 (termination) following dosing. The animals were observed for mortality, signs of gross toxicity, and behavioral changes during the first several hours post-dosing and at least once daily thereafter for 14 days after dosing. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, and behavioral pattern. Particular attention was directed to observations of tremors, convulsions, salivation, diarrhea, and coma. All rats were euthanized by CO₂ inhalation at the end of the 14-day observation period and gross necropsies were performed on all animals. Tissues and organs of the thoracic and abdominal cavities were examined.

Acute dermal toxicity

The acute dermal toxicity evaluation was conducted in rats to determine the potential for UC-II to produce toxicity from a single topical application. Five healthy young adult albino Sprague Dawley male rats (aged 10–11 weeks old, initial body weight 290–307 g) and five young adult female, nulliparous, and non-pregnant albino Sprague Dawley rats (aged 10–11 weeks old, initial body weight 200–215 g) were obtained from Ace Animals, Inc. (Boyertown, PA). The rats were singly housed in suspended stainless steel cages with mesh floors. Litter paper was placed beneath the cage and was changed at least three times per week. The rats had free access to standard rat chow (Purina Rodent Chow# 5012) and filtered tap water ad libitum, and were maintained at controlled temperature (19–23°C) and light cycle (12 h light/12 h dark). The animals were acclimated to laboratory conditions for 21 days.

On the day prior to UC-II application, the five male and five female animals were prepared by clipping (Oster model

#A5 -small) the dorsal area and the trunk. After clipping and prior to application, the animals were examined for health, weighed (initial), and the skin checked for any abnormalities. Individual doses were calculated based on the initial body weights, taking into account the concentration of the test mixture. Prior to application, UC-II was moistened with distilled water to achieve a dry paste by preparing a 50% w/w mixture. UC-II (2000 mg/kg of body weight) was then applied to a 2 × 3-inch 4-ply gauze pad and placed on a dose area of ~ 2 × 3 inches (~ 10% of the body surface). The gauze pad and entire trunk of each animal were then wrapped with 3-inch Durapore tape to avoid dislocation of the pad and to minimize loss of UC-II. The rats were then returned to their designated cages. The day of application was considered as day 0 of the study. After 24 h of exposure of UC-II, the pads were removed, and the test sites were gently cleansed of any residual test substance. Individual body weights of the animals were recorded prior to UC-II application (initial) and again on days 7 and 14 (termination). The animals were observed for mortality, signs of gross toxicity, and behavioral changes during the first several hours after application and at least once daily thereafter for 14 days. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, and behavioral pattern. Particular attention was directed to observations of tremors, convulsions, salivation, diarrhea, and coma. All rats were euthanized via CO₂ inhalation on day 14. Gross necropsies were performed on all animals at terminal sacrifice. Tissues and organs of the thoracic and abdominal cavities were examined.

Primary dermal irritation

The primary dermal irritation test was conducted in two young adult male New Zealand albino rabbits and one young nulliparous non-pregnant female New Zealand albino rabbit to determine the potential for UC-II to cause irritation after a single topical application. The rabbits were obtained from Robinson Services, Inc. (Clemmons, NC), and singly housed in suspended stainless steel cages with mesh floors, which conform to the size recommendations in the most recent Guide for the Care and Use of Laboratory Animals DHEW (NIH). Litter paper was placed beneath the cage and was changed at least three times per week. The rabbits were allowed free access to lab chow (Purina Rabbit Chow # 5326, St. Louis, MO) and filtered tap water ad libitum, and maintained at controlled temperature (20–22°C) and light cycle (12 h light/12 h dark). Animals were acclimated to

Table 1. Acute oral toxicity dosing sequence and observations.

Dosing sequence	Dose level (mg/kg)	Body weight (g)			Cage-side observations (days 0–14)	Necropsy observations (all tissues)
		Initial	Day 7	Day 14		
1	175	182	207	243	Active and healthy	No gross abnormalities
2	550	205	224	253	Active and healthy	No gross abnormalities
3	1750	181	200	246	Active and healthy	No gross abnormalities
4	5000	200	220	257	Active and healthy	No gross abnormalities
5	5000	177	198	244	Active and healthy	No gross abnormalities
6	5000	186	200	246	Active and healthy	No gross abnormalities

laboratory conditions for a period of 28 days prior to initiation of dosing.

On the day before application, rabbits were prepared by clipping (Oster model #A5 -small) the dorsal area and the trunk. On the day of dosing but prior to application, the rabbits were critically examined for health and the skin checked for any abnormalities, and three healthy rabbits without pre-existing skin irritation were selected for the test. Individual doses were calculated based on the initial body weights, taking into account the concentration of the test mixture. On the day of application (day 0), UC-II was moistened with distilled water to achieve a dry paste by preparing a 50% w/w mixture. Five-tenths of a gram of UC-II (1.0 g of test mixture) was placed on a 1 × 1-inch 4-ply gauze pad and applied to one 6-cm² intact dose site on each rabbit. The pad and entire trunk of each rabbit were then wrapped with semi-occlusive 3-inch Micropore tape to avoid dislocation of the pad. Elizabethan collars were placed on each rabbit and they were returned to their designated cages. After 4 h of exposure to UC-II, the pads and collars were removed and the test sites were gently cleansed of any residual test substance.

Individual dose sites were scored according to the Draize scoring system (Table 2) (Draize et al. 1944) at ~ 1, 24, 48, and 72 h after patch removal. The classification of irritancy was obtained by adding the average erythema and edema scores for the 1, 24, 48, and 72-h scoring intervals and dividing by the number of evaluation intervals (four). The animals were also observed for signs of gross toxicity and behavioral changes at least once daily during the test period. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, and behavior pattern. Particular attention was directed to observation of tremors, convulsions, salivation, diarrhea, and coma.

Primary eye irritation

The primary eye irritation test was conducted in rabbits to determine the potential for UC-II to produce irritation from a single installation through the ocular route. Three female, nulliparous and non-pregnant New Zealand albino rabbits were obtained from Robinson Services, Inc. (Clemmons, NC) and singly housed in suspended stainless steel cages with mesh floors, which conform to the size recommendations in the most recent Guide for the Care and Use of Laboratory Animals DHEW (NIH). Litter paper was placed beneath the cage and was changed at least three times per week. The rabbits were allowed free access to lab chow (Purina Rabbit Chow# 5326, St. Louis, MO) and filtered tap water ad libitum, and maintained at controlled temperature (17–24°C) and light cycle (12h

light/12h dark). Animals were acclimated to laboratory conditions for a period of 22 days prior to initiation of dosing.

Prior to instillation, both eyes of rabbits were examined using a fluorescein dye procedure. One drop of 2% ophthalmic fluorescein sodium was instilled into both eyes of each rabbit. The eyes were rinsed with physiological saline (0.9% NaCl) ~ 30 s after installation of the fluorescein. Using an ultraviolet light source, the eyes were checked for gross abnormalities according to the 'Scale for Scoring Ocular Lesions' (Draize et al. 1944). Three healthy animals without pre-existing ocular irritation were selected for the test. One-tenth of a milliliter (0.06 g) of UC-II was instilled into the conjunctival sac of the right eye of each rabbit by gently pulling the lower lid away from the eyeball. The upper and lower lids were then gently held together for ~ 1 s before releasing to minimize loss of the test substance. The left (control) eye of each animal remained untreated and served as a control. The rabbits were then returned to their designated cages. Ocular irritation was evaluated macroscopically using a high-intensity white light in accordance with Draize et al. (1944) at 1, 24, 48, and 72 h, and 4 days post-instillation. The fluorescein eye evaluation was used at 24h to verify the absence of corneal damage. Individual irritation scores were recorded for each animal. In addition to observations of the cornea, iris, and conjunctivae, any other lesions were noted. The average score for all rabbits at each scoring period was calculated to aid in data interpretation. Time intervals with the highest mean score (Maximum Mean Total Score; MMTS) for all rabbits were used to classify the test substance (UC-II) by the system of Kay and Calandra (1962).

The animals were also observed for signs of gross toxicity and behavioral changes at least once daily during the test period. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, and behavior pattern. Particular attention was directed to observation of tremors, convulsions, salivation, diarrhea, and coma.

Mutagenicity test: Ames' bacterial reverse mutation assay

The *Salmonella typhimurium* reverse mutation test (Maron and Ames 1983) was conducted to determine the ability of UC-II to induce reverse mutation. UC-II was evaluated in the Ames/Salmonella plate incorporation assay to determine its potential to induce reverse mutation at selected histidine loci in five tester strains of *Salmonella typhimurium* viz. TA 1535, TA 97a, TA 98, TA 100, and TA 102 in the presence and absence of a metabolic activation system (S9) (Ames et al. 1977). Suspensions of bacterial cells were exposed to UC-II in triplicate cultures at concentrations of 10.1, 31.6, 100, 316, 1000, 2500, and 5000 µg/plate in the presence and absence of an exogenous metabolic activation system (S9). The suspensions were mixed with an overlay agar and plated immediately onto minimal medium. After 48 h incubation, revertant colonies were counted using a ProtoCOL counter (Meintrup DWS Laborgerate, GmbH) and compared to the number of spontaneous revertant colonies on vehicle (negative) control plates.

Table 2. Primary dermal irritation index (PII) and classification.

Primary Dermal Irritation Index (PDII)	Classification
0	Non-irritating
> 0-2.0	Slightly irritating
2.1-5.0	Moderately irritating
> 5.0	Severely irritating

Mutagenicity test: Mouse lymphoma assay

The mutagenic potential of UC-II was evaluated by in vitro mammalian cell gene mutation assay (Thymidine Kinase Locus/TK⁺) in mouse (*Mus musculus*) lymphoma cell line L5178Y. The assay was performed in both the presence and absence of an exogenous metabolic activation system at the gene locus coding for the enzyme thymidine kinase (TK) in mouse lymphoma cells.

UC-II was investigated at the following concentrations: Experiment I with and without metabolic activation, 200, 400, 600, 800, 1000, 1200, 1500, and 2000 µg/ml; Experiment II with metabolic activation, 300, 500, 700, 1100, 1400, 1800, and 2000 µg/ml; and Experiment II without activation, 4.4, 17.6, 39.6, 70.4, 110, 264, 330, and 440 µg/ml. The selection of concentrations was based on data from the pre-experiment. In experiment I, 2000 µg/ml (with and without metabolic activation) was selected as the highest concentration. In experiment II, 2000 µg/ml (with metabolic activation) and 440 µg/ml (without metabolic activation) were selected as the highest concentration. Experiment II without metabolic activation was performed as a 24 h long-term exposure assay. Ethylmethanesulfonate (EMS), methylmethanesulfonate (MMS), and benzo[a]pyrene (B[a]P) were used as positive controls. Each trial consisted of duplicate cultures of the negative (vehicle) and positive controls, and single cultures treated at each of the dosage levels of UC-II described above. Treatment consisted of 11 ml of the appropriate treatment medium (with or without exogenous activation), designated concentrations of UC-II and 1×10^7 cells in a 25cm² flask, and incubated at 37°C in 5% CO₂/95% humidified air. After 4 h incubation, the test compound was removed by centrifugation (200 x g, 10 min) and the cells were washed twice with phosphate buffered saline (PBS). The cells were suspended in 30 ml complete culture medium and incubated for an expression and growth period of 72 h. For the long-term exposure experiment, 1×10^7 cells were suspended in 50 ml cell culture medium in a 175-cm² flask. After expression and growth period, the relative cloning efficiency (RCE; percentage cloning efficiency of the test group in relation to the negative control) of the cells was determined as previously described (Clive and Spector 1975; Clive 1983; Clive et al. 1983; Mitchell et al. 1997).

Dose-dependent 90-day sub-chronic toxicity study

A 90-day oral toxicity study was conducted in male and female rats at Eurofins/Product Safety Laboratories (Dayton, NJ) to determine the potential of UC-II to produce toxicity. A no-observed-adverse-effect level (NOAEL) was also sought for each sex. Eighty healthy rats (40 males and 40 females) were selected for the test and equally distributed into four groups (10 males and 10 females per dose level) according to Table 3.

Animal selection

After acclimating to the laboratory environment for 7 days, the rats were examined for general health and weighed. Only those rats free of clinical signs of disease or injury and having

a body weight range within $\pm 20\%$ of the mean were selected for test. The animals weighed in the range of 195–219 g for males and 148–174 g for females, and were ~ 7 –8 weeks of age at test initiation. The 40 male and 40 female rats were randomly distributed, stratified by body weight, among the dose and control groups on the day prior to study start.

Dose preparations

The test substance was administered as a 0.4% (low dose), 4.0% (intermediate dose), or 10.0% (high dose) weight/weight dilution in distilled water. On each dosing day and for each concentration, an appropriate amount of the test substance was accurately weighed into a 150 mL glass beaker and distilled water was added until the desired total weight was obtained. The dose preparations were used at room temperature within ~ 2 h, and maintained on a magnetic stir plate during administration.

Dose calculations

Individual doses were calculated based on the most recent weekly body weights and were adjusted each week to maintain the targeted dose level for all rats. All doses were administered volumetrically after correcting for dilution. Doses were administered to all groups at a constant dose volume of 10.0 mL/kg. The control group received the vehicle only (distilled water) at the same volume as the test animals.

Dose administration

Each animal was dosed by oral intubation to the stomach using a ball-tipped gavage needle attached to an appropriate syringe. Dosing was 7 days per week for a period of 92 days for males and 93 days for females. The first day of administration was considered Day 1 of the study. Dosing was at approximately the same time each day ± 2 h, with an exception on the days the hematology and/or clinical chemistry samples were collected. On the days of blood collection, food was returned to the fasted animals for a minimum of 2 h prior to test substance administration.

Ophthalmologic evaluations

Prior to study initiation, the eyes of a group of rats considered for study were examined by focal illumination and indirect ophthalmoscopy. Mydriasis was achieved with 1% tropicamide and the eyes were examined in subdued light. Subdued light was maintained in the animal room for the remainder of the day. This procedure was repeated on Day 91 for all surviving test animals.

Clinical observations

All animals were observed at least twice daily for viability. Cage-side observations of all animals were performed daily during the study or until death occurred. On Day 1 (prior to first treatment with the test substance) and approximately weekly thereafter, a detailed clinical observation test was conducted while handling the animals, generally on days that the animals were weighed and food consumption measurements taken. Potential signs noted included, but were not

Table 3. Dose levels and assignment of animals.

Group	Number/group	Number/sex	Oral gavage dose (mg/kg/day)	Dose volume (ml/kg/day)	% UC-II
1	20	10	Control (0)	10.0	0
2	20	10	Low dose (40)	10.0	0.4
3	20	10	Intermediate dose (400)	10.0	4.0
4	20	10	High dose (1000)	10.0	10.0

See Materials and methods section for details.

limited to changes in skin, fur, eyes, and mucous membranes, occurrence of secretions, excretions, and autonomic activity (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern). Likewise, changes in gait, posture, and response to handling, as well as the presence of clonic or tonic movements, stereotypies (e.g. excessive grooming, repetitive circling), or bizarre behavior (e.g. self-mutilation, walking backwards) were also recorded.

Body weight, organ weight, and body weight gain

Individual body weights were recorded twice during the acclimation period, on Day 0 (the day of study start) and approximately weekly thereafter (7 day intervals \pm 1). Mean daily body weight gains were calculated for each sex and dose level at each interval and for the overall (Days 1–92) testing interval. Animals were also weighed prior to sacrifice (fasted body weight) for the calculation of organ-to-body weight and organ-to-brain weight ratios. The following organs were weighed wet as soon as possible after dissection to avoid drying: liver, kidneys (combined), adrenals (combined), brain, heart, thymus, spleen, ovaries (combined) or testes (combined), epididymides, and uterus and fallopian tubes.

Food consumption and food efficiency

Individual food consumption was measured and was recorded weekly adjusting for spillage. Mean daily food consumption was calculated for each sex/dose level during each weekly interval and overall (Days 1–92) testing interval. Mean daily food efficiency was also calculated for each sex/dose level based on body weight gain and food consumption data. Animals were allowed ad libitum access to food throughout the study. Animals were fasted overnight prior to blood collection on Day 90, and prior to terminal sacrifice on Day 92 (males) or Day 93 (females).

Functional observational battery

A Functional Observational Battery (FOB) was performed on all animals on Day 86 (females) and Day 87 (surviving males). Each rat was evaluated during handling and while in an open field for excitability, autonomic function, gait and sensorimotor coordination (open field and manipulative evaluations), reactivity and sensitivity (elicited behavior), and other abnormal clinical signs including but not limited to convulsions, tremors, unusual or bizarre behavior, emaciation, dehydration, and general appearance. In addition to the above observations, forelimb and hind limb grip strength and foot splay measurements were obtained and recorded. The grip strength was measured with a digital force gauge (Wagner Force Five, Model #FDMV). Triplicate measurements of grip

strength and duplicate measurements for foot splay were taken for each animal and the means for each group were calculated.

Motor activity

Motor Activity (MA) was evaluated on all surviving animals on Day 86 (males) and Day 87 (females). This assessment was done at approximately the same period during the study as the FOB. Activity was monitored using an automated Photobeam Activity System[®] (San Diego Instruments, Inc.). An approximate equal number of animals from each dose group were assigned to the MA assessment for each session. Each animal was placed into a polycarbonate solid bottom cage, room lights were turned off, and a white noise generator was used. The evaluation phase began immediately for that animal. Each animal was evaluated for a single 1-h phase, with photobeam counts accumulated over six 10-min intervals. Total movements (consisting of fine movements and active movements) were considered an appropriate measure for the assessment of potential behavioral effects in this study.

Clinical pathology

All surviving animals were fasted overnight prior to each blood collection. Blood samples for hematology (except coagulation samples) and clinical chemistry were collected via the sub-lingual vein under isoflurane anesthesia during the 12th week of exposure for males and females. Approximately 500 μ l was collected in a pre-calibrated tube containing EDTA for hematology assessments. The whole blood samples were stored under refrigeration and shipped on cold packs. Approximately 1000 μ l was collected into tubes containing no preservative for clinical chemistry assessments. These samples were centrifuged in a refrigerated centrifuge and the serum was transferred to a labelled tube. Serum samples were stored in a -80°C freezer and shipped frozen in dry ice. All samples were shipped to DuPont Haskell Global Centers for Health and Environmental Sciences (Newark, DE). Blood samples used to determine the prothrombin time and activated partial thromboplastin time (coagulation) were collected via the inferior vena cava under isoflurane anesthesia at terminal sacrifice. Approximately 1800 μ l were collected in a pre-calibrated tube containing sodium citrate. These samples were centrifuged in a refrigerated centrifuge and the plasma was transferred to a labelled tube. Plasma samples were stored in a -80°C freezer and shipped frozen in dry ice to DuPont Haskell Global Centers for Health and Environmental Sciences. The day before collection of the samples for the clinical pathology evaluation, the animals

were placed in metabolism cages. These animals were fasted after 3 pm (at least 15 h) and urine was collected from each animal. Urine samples were stored under refrigeration and shipped on cold packs to DuPont Haskell Global Centers for Health and Environmental Sciences. All blood samples were evaluated for quality by visual examination. Upon completion of clinical chemistry, remaining serum samples from two randomly selected animals were pooled at DuPont Haskell and sent to Charles River Diagnostics (Wilmington, MA) for serology.

Sacrifice and macroscopic observations

Scheduled sacrifice. At terminal sacrifice, all surviving males (Day 93) and all females (Day 94) were euthanized by exsanguination from the abdominal aorta under isoflurane anesthesia. All animals in the study (including decedents) were subjected to a full necropsy, which included examination of the external surface of the body, all orifices, and the thoracic, abdominal, and cranial cavities, and their contents. Additional tissues were preserved if indicated by signs of toxicity or target organ involvement.

Histopathology. Histological examination was performed on the preserved organs and tissues of the animals from both the control and high dose groups (Groups 1 and 4, respectively) as well as from any animal that died during the course of the study. In addition, gross lesions of potential toxicological significance noted in any test groups at the time of terminal sacrifice were also examined. Due to findings in the males and females of Group 4 high dose, the nasal turbinates were evaluated in the intermediate Group 3 animals. The fixed tissues were trimmed, processed, embedded in paraffin, microtomed, placed on glass microscope slides, stained with hematoxylin and eosin, and examined by light microscopy. Slide preparation and histopathological assessment was performed by Histo-Scientific Research Laboratories (Mt. Jackson, VA).

Statistical analysis

Eurofins/Product Safety Laboratories performed statistical analysis of all data collected during the in-life phase of the study as well as organ weight data. DuPont Haskell Laboratory provided analysis of clinical pathology results to Eurofins/Product Safety Laboratories. The use of the word 'significant' or 'significantly' indicates a statistically significant difference between the control and the experimental groups. Significance was judged at a probability value of $p \leq 0.05$. Male and female rats were evaluated separately.

Statistical methods (in-life and organ weight data)

Group means and standard deviations were calculated for body weight, daily body weight gain, daily food consumption, daily food efficiency, organ weight, and organ-to-body/brain weight ratio, FOB and MA data. Data within groups were compared using a One-Way of Analysis (ANOVA), followed by comparison of the treated groups to control by Dunnett's Multiple Comparisons test. Data were evaluated for homogeneity of variances and normality by the Bartlett's test. Data

that were considered significant by Bartlett's test were further evaluated with a non-parametric method (Kruskal-Wallis or Dunn's test) (INSTAT Biostatistics, Graph Pad Software, San Diego, CA). Motor activity data (overall total movements) were further analyzed using a Two-Way Repeated Measures ANOVA (SigmaStat, Version 2.03).

Statistical methods (clinical pathology)

Means and standard deviations were calculated for clinical pathology quantitative data. Data within groups were initially analyzed using Levene's test for variance homogeneity, and the Shapiro-Wilk test for normality. If variances were considered not significantly different, groups were compared using a One-Way Analysis of Variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. If the Shapiro-Wilk test was not significant but Levene's test was significant, a robust version of Dunnett's test was used. Where variances were considered significantly different by Levene's test, groups were compared using a non-parametric method (Kruskal-Wallis non-parametric analysis of variance followed by Dunn's test). Differences among groups were judged significant at a probability value of $p \leq 0.05$. Male and female rats were evaluated separately.

Results

Acute oral toxicity

A single oral administration of UC-II was provided to female Sprague-Dawley rats to assess its acute toxicity following Up and Down procedure. UC-II, at the limit dose of level of 5000 mg/kg body weight, did not cause any mortality and did not demonstrate any signs of gross toxicity, adverse pharmacologic effects, or abnormal behavior in the treated female rats following dosing and during the observation period of 14 days thereafter. All animals survived, gained normal body weight, and appeared active and healthy during the study. No gross abnormalities or pathological alterations were noted for any of the rats when necropsied at the conclusion of the 14-day observation period (Table 1). Based on these results and under the conditions of this study, the acute oral LD₅₀ of UC-II is greater than 5000 mg/kg of body weight in female rats.

Acute dermal toxicity

Acute dermal toxicity of UC-II was conducted in male and female Sprague Dawley rats to determine the potential for UC-II to cause toxicity from a single topical application. All animals survived, gained normal body weight, and appeared active and healthy during the study. There were no signs of dermal irritation, gross toxicity, adverse pharmacologic effects, or abnormal behavior. No gross abnormalities were noted for any of the animals when necropsied at the conclusion of the 14-day observation period. The findings are summarized in Table 4. Under the conditions of this study, the single dose acute dermal LD₅₀ of UC-II is greater than 2000 mg/kg of body weight in both male and female-rats.

Primary dermal irritation

Primary dermal irritation was investigated in male and female New Zealand albino rabbits to evaluate the potential of UC-II to produce irritation after a single topical application. Following application of UC-II, all animals appeared active and healthy. Apart from the dermal irritation noted below, there were no signs of gross toxicity, adverse pharmacologic effects, or abnormal behavior. One hour after patch removal, very slight erythema was observed at all three treated sites. The overall incidence and severity of irritation decreased with time. All animals were free from dermal irritation within 24 h. A summary of Draize primary dermal irritation scoring criteria for dermal reactions and descriptive rating for mean primary dermal irritation index (PDII) is presented in Table 2. Under the conditions of this study, the PDII for UC-II was calculated to be 0.3, thus classifying UC-II to be slightly irritating to the skin (Table 5).

Primary eye irritation

A primary eye irritation test was conducted in New Zealand albino rabbits to determine the potential for UC-II to cause irritation from a single instillation via the ocular route. All animals appeared active and healthy. There were no signs of gross toxicity, adverse pharmacologic effects or abnormal behavior. No corneal opacity or iritis was observed in any treated eye during the study. One hour following UC-II instillation, all treated eyes exhibited conjunctivitis (Table 6). Individual eye irritation scores are presented in Table 6 in accordance with the Draize Scale for scoring Eye Lesions and the Kay and Calandra Scheme for classifying eye irritants (Draize et al. 1944; Kay and Calandra 1962). The overall severity of irritation decreased with time (Table 7). All animals were free of ocular irritation within 48 h. Under the conditions of this study, the maximum mean total score (MMTS) of UC-II powder was determined to be 37.7 (Table 7), classifying UC-II to be moderately irritating to the eye.

Mutagenicity test: Ames' bacterial reverse mutation assay

No toxic effects of UC-II were noted in any of the five tester strains used up to the highest dose group evaluated (with and without metabolic activation). No biologically relevant

increases in revertant colony numbers of any of the five tester strains were observed following treatment with UC-II at any concentration level, in neither the presence nor absence of metabolic activation. Therefore, UC-II did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used, indicating that UC-II is non-mutagenic.

Mutagenicity test: Mouse lymphoma assay

In experiment I with metabolic activation, the relative total growth (RTG) was 108.55% for the highest concentration (2000 µg/ml) evaluated. The highest concentration evaluated without metabolic activation was 2000 µg/ml with an RTG of 83.73%. In experiment II with metabolic activation, the RTG was 90.38% for the highest concentration (2000 µg/ml) evaluated. The highest concentration evaluated without metabolic activation was 440 µg/ml with an RTG of 10.11%.

No biologically relevant increases of mutants were found after treatment with UC-II (with or without metabolic activation) in both experiments I and II. No dose-response relationship was observed. Additionally, in experiments I and II colony sizing showed no clastogenic effects induced by UC-II. Therefore, under the experimental conditions of this study, no evidence of mutagenic activity was detected for UC-II in the L5178Y mouse lymphoma cell line, and UC-II is concluded to be negative for the induction of mutagenicity in this assay.

Dose-dependent 90-day sub-chronic toxicity study**Ophthalmoscopic examinations**

Both eyes of all animals were examined by focal illumination and indirect ophthalmoscopy prior to study initiation

Table 5. Summary of primary skin irritation scores (average for three animals).

	Time after patch removal			
	30-60 min	24 h	48 h	72 h
Erythema	1.0	0	0	0
Edema	0	0	0	0
Total (PDI*)	1.0	0	0	0

PDI: Primary dermal irritation = average erythema + average edema.

Table 4. Summary of acute dermal toxicity findings.

Sex	Body weight (g)			Cage-side observations (days 0-14)	Necropsy observations (all tissues)
	Initial	Day 7	Day 14		
Male	252	299	341	Active and healthy	No gross abnormalities
Male	248	302	352	Active and healthy	No gross abnormalities
Male	239	322	364	Active and healthy	No gross abnormalities
Male	257	314	369	Active and healthy	No gross abnormalities
Male	251	299	349	Active and healthy	No gross abnormalities
Female	196	204	234	Active and healthy	No gross abnormalities
Female	201	218	232	Active and healthy	No gross abnormalities
Female	210	223	240	Active and healthy	No gross abnormalities
Female	211	216	245	Active and healthy	No gross abnormalities
Female	199	225	249	Active and healthy	No gross abnormalities

Table 6. Individual scores for ocular irrigation.

		I. Cornea			II. Iris		III. Conjunctivas			Total		
		A. Opacity	B. Area	(A×B)×5	A. Values	A×5	A. Redness	B. Chemosis	C. Discharge (A + B + C)×2			
Rabbit 3401 Male	Hours	1	1	3	15	1	5	3	2	3 ^b	16	36
		24	1 ^a	1	5	1	5	3	2	2	14	24
		48	0 ^a	4	0	0	0	2	1	1	8	8
		72	0	4	0	0	0	1	0	0	2	2
	Days	4	0	4	0	0	0	0	0	0	0	0
Rabbit 3402 Female	Hours	1	1	3	15	1	5	3	2	3 ^b	16	36
		24	1 ^a	1	5	1	5	2	2	2	12	22
		48	0 ^a	4	0	0	0	2	1	1	8	8
		72	0	4	0	0	0	1	0	0	2	2
	Days	4	0	4	0	0	0	0	0	0	0	0
Rabbit 3402 Female	Hours	1	1	4	20	1	5	3	2	3 ^b	16	41
		24	1 ^a	2	10	1	5	2	2	2	12	27
		48	0 ^a	4	0	0	0	2	1	1	8	8
		72	0	4	0	0	0	1	0	0	2	2
	Days	4	0	4	0	0	0	0	0	0	0	0

^a 2% ophthalmic fluorescein sodium was used to evaluate the extent or verify the absence of corneal opacity.

^b Discharge was white in color.

Table 7. Summary of mean scores of severity and reversibility of primary eye irritation study.

Time post-instillation	Severity of irritation
1 h	37.7
24 h	24.3
48 h	8.0
72 h	2.0
4 days	0.0

Maximum Mean Total Score (MMTS) was observed at 1 h post-instillation. See Materials and methods section for details.

and near experimental completion (Day 91). Both eyes of all surviving animals were ophthalmoscopically normal. There was no indication that the test substance, as evaluated, was an ocular toxicant.

Mortality and clinical observations

Two male animals (one in Group 3 and the other Group 4) were found dead on study days 86 and 69, respectively. The animal in Group 3 was active and healthy prior to death and died immediately following the Motor Activity assessment. A cause of mortality could not be definitively determined; however, there was no evidence to suggest that mortality was attributable to test substance administration. Necropsy revealed a distended stomach filled with gas and food and the kidneys appeared enlarged. These observations had no histological correlate and there were no other apparent remarkable findings. The agonal change of congestion was the notable microscopic finding in the adrenal glands, kidneys, liver, and lung.

The animal in Group 4 died as a suspected result of a gavage error. Prior to death this animal exhibited hypoactivity, hypothermia, moist rales, and irregular respiration accompanied by a red nasal discharge. Macroscopically, the trachea and esophagus were punctured, the thoracic cavity was filled with a white liquid substance, and the lungs were dark red in color. Puncture of the esophagus noted at

necropsy was associated microscopically with the presence of hemorrhage, inflammation, and myofiber degeneration at the edges of the puncture wound consistent with an ante-mortem incident. Puncture of the trachea noted at necropsy was not observed at the time of trimming. Noted microscopic findings associated with the esophageal puncture were marked lung atelectasis, moderate fibrinous inflammation of the lungs involving the pleura, and slight fibrinous inflammation involving the heart (epicardium). Lymphoid depletion noted in lymph nodes, spleen, and thymus was a secondary alteration related to stress/cachexia and was not a primary finding associated with test substance administration.

There were no test substance-related clinical signs in any test group (see Table 3) that were considered to be of toxicological significance. Transient clinical signs included black ocular discharge for one Group 1 (control) male on Days 15–34, one Group 2 (40 mg/kg/day) male on Days 22–35, and one Group 1 female on Day 39. Red ocular discharges for one Group 2 male on Days 43–45; red stained fur for one Group 2 male on Days 50–81 and 84–92, one Group 4 (1000 mg/kg/day) male on Days 50–92, two Group 3 (400 mg/kg/day) females on Days 59–62 and 60–65, respectively, were observed. Red facial stainings for one Group 1 male on Days 71–77, one Group 2 male on Days 42–50 and 82–83, one Group 4 male on Days 39–50, and one Group 3 female on Days 63–70 were noted. Hyperactivity for two Group 3 males on Days 36 and 64 and Day 50, respectively, and one Group 4 male on Day 50 and one Group 2 female on Day 92 was observed. One Group 1 male was noted with a swollen right hindlimb (Days 22–24, 28), hind end impairment (Days 28–42), and swollen foot pads (Days 29–63). One Group 1 male had a wound on the ventral surface of the head on Days 15–27. One Group 1 male had a wound on the right ear on Days 78–92. One Group 3 male had a small scab on the right side of the face on Days 8–18, and one Group 1 female had a small scab on the top of its head on Days 1–19; one Group 4 male exhibited enophthalmos (right eye)

on Days 50–92. One Group 2 male exhibited variable red nasal discharge, reduced fecal volume, ano-genital staining, soft feces, moist rales, hunched posture, and piloerection on Days 53–72. The above findings did not show any adverse effects and did not appear to be test substance-related because they were found across all test groups, including control animals.

Body weight, organ weights, and body weight gain

Weekly body weights for male and female rats at 40, 400, and 1000 mg/kg/day were comparable with control values. Overall (Days 1–92) and mean daily body weight gain for male rats at 40, 400, and 1000 mg/kg/day were comparable with control values (Table 8). Overall (Days 1–92) and mean daily body weight gain for female rats at 40, 400, and 1000 mg/kg/day were generally comparable with control values with the exception that daily body weight gain was decreased during Week 3 for Group 4 females.

There were no changes in individual organ weights (Table 9) or individual organ-to-brain weights (Table 10). The organ-to-body weight ratios were unaffected except that the kidney-to-body weight ratios were significantly decreased in Group 3 males (Table 11). This finding was not associated with any other clinical finding and did not herald any corresponding pathological changes in the high dose animals. Therefore, this change was deemed incidental and of no toxicological interest.

Food consumption and food efficiency

Overall (Days 1–92) and mean daily food consumption for male rats at 40 and 400 mg/kg/day were comparable with control values. Food consumption was decreased for male rats at 1000 mg/kg/day (Group 4) during Weeks 5, 7–11, 13, and overall. Overall and mean daily food consumption for female rats at 40, 400, and 1000 mg/kg/day were generally comparable with control values with the exception of the following statistically significant findings. Food consumption was decreased in females during Weeks 1, 2, and overall at 400 mg/kg/day, and during Weeks 1, 8, and overall at 1000 mg/kg/day.

Overall and mean daily food efficiency for male rats at 40, 400, and 1000 mg/kg/day were comparable with control

values. Overall and mean daily food efficiency for female rats at 40, 400, and 1000 mg/kg/day were generally comparable with control values with the exception of the following statistically significant findings. Mean daily food efficiency was decreased during Week 3 for females at 40 mg/kg/day and at 1000 mg/kg/day.

In summary, the oral administration of UC-II led to some dose-related decreases in food consumption in males and females; however, body weight, body weight gain, and food efficiency remained generally unaffected. Reductions in food consumption were considered test substance related and may be of some toxicological interest in light of the pathological findings of nasal turbinate eosinophilia at the high dose (see Clinical Pathology section).

Functional observational battery

In general, the functional behavioral results of the test groups of male and female rats were considered comparable to the control groups. Any decreases in quantitative measurements or increases in incidence of open field measurements were minimal and not associated with a constellation of findings that would support a toxicologically significant behavioral change. In males, these findings included normal (sleeping) postures in 5/10 Group 2 males and 2/10 Group 3 males. Enophthalmos for 1/10 Group 4 males, an inactive/alert activity level for 1/10 Group 2 males, 1/10 Groups 3 males, and 1/10 Group 4 males were observed. A slow reaction to right itself for 1/10 Group 1 males; no approach responses for 1/10 Group 2 males and 2/10 Group 3 males; as well as no tactile responses for 2/10 Group 1 males, 1/10 Group 2 males, 2/10 Group 3 males, and 1/10 Group 4 males were noted. In females, these findings included no tactile responses for 1/10 Group 1 females and 1/10 Group 3 females.

Motor activity

The Motor Activity results of the test groups of male and female rats were considered comparable to the control groups. In general, all groups of animals (including control) exhibited a similar level of movement over all

Table 8. Summary of average weekly body weight.

Days	Group (male)				Group (female)			
	1	2	3	4	1	2	3	4
1	207.2±6.1	207.5±6.9	206.8±6.1	205.7±6.3	160.7±8.1	159.4±7.3	157.7±6.9	161.0±7.0
8	252.2±9.2	251.8±11.7	247.7±9.1	247.3±10.9	182.6±8.6	175.6±11.4	174.3±7.4	175.4±9.1
15	278.5±13.6	277.7±13.8	274.2±10.5	271.1±14.6	195.6±7.4	189.8±12.0	189.9±8.7	191.4±9.4
22	302.6±17.9	305.5±18.5	299.4±11.2	294.3±19.0	215.3±10.5	202.6±14.7	208.8±14.0	202.7±10.8
29	318.3±24.4	322.8±18.7	315.9±12.6	311.8±18.4	223.3±13.4	212.3±15.9	211.7±16.5	211.3±12.5
36	332.6±26.5	336.3±20.9	330.0±12.0	326.3±20.5	225.5±12.6	218.0±13.5	215.2±10.9	215.4±10.9
43	349.7±25.2	351.7±23.8	345.7±13.0	324.8±23.1	234.1±14.4	222.4±16.5	225.2±17.0	224.8±14.5
50	364.2±24.3	367.2±25.2	359.4±15.5	352.4±23.6	241.7±16.2	229.3±15.6	229.6±16.8	233.0±17.5
57	373.2±24.3	368.6±37.2	368.9±17.2	358.5±25.2	246.7±18.7	234.5±18.4	233.7±15.3	233.4±13.6
64	379.6±25.0	377.0±36.8	376.4±18.3	360.1±29.5	249.1±16.0	238.2±17.7	235.9±15.1	237.1±15.8
71	386.3±25.1	386.5±28.1	385.5±17.6	371.0±27.9 [†]	250.5±13.6	241.2±16.4	239.8±14.6	241.7±14.9
78	396.2±28.3	397.6±28.0	394.0±18.5	380.8±32.0 [†]	254.5±15.4	245.2±12.9	244.6±18.5	244.8±14.6
85	400.2±27.9	402.4±28.7	399.2±18.6	388.1±29.8 [†]	256.3±15.2	247.5±15.7	246.7±16.2	246.3±13.7
92	394.9±25.2	398.3±29.9	390.9±19.5 [†]	377.7±26.4 [†]	250.7±12.7	243.0±14.8	240.3±17.1	242.1±15.0

Values are the Mean ± SD ($n=10$ except for [†] $n=9$). No significant difference from control was observed. See Materials and methods section for details.

Table 9. Summary of mean organ weight.

Organ	Group (male)				Group (female)			
	1	2	3	4	1	2	3	4
Adrenals	0.066±0.009 [†]	0.071±0.009	0.067±0.006	0.067±0.011	0.074±0.006	0.072±0.011	0.070±0.004	0.072±0.008
Brain	1.99±0.10	1.98±0.07	1.99±0.07	1.95±0.10	1.85±0.05	1.81±0.06	1.81±0.07	1.85±0.10
Heart	1.31±0.13	1.38±0.11	1.29±0.08	1.29±0.16	0.90±0.09	0.95±0.09	0.89±0.09	0.93±0.09
Kidney	2.90±0.28	2.92±0.19	2.65±0.13	2.79±0.30	1.76±0.11	1.70±0.08	1.72±0.13	1.74±0.18
Liver	10.07±0.87	10.51±0.79	9.85±0.49	9.25±0.83	6.01±0.50	5.99±0.33	5.81±0.34	5.96±0.41
Spleen	0.76±0.10	0.81±0.09	0.75±0.08	0.69±0.08	0.60±0.07	0.62±0.06	0.59±0.08	0.63±0.09
Thymus	0.300±0.054	0.366±0.129	0.313±0.059	0.271±0.062	0.260±0.025	0.243±0.058	0.253±0.038	0.233±0.055
Epididymides	1.490±0.175	1.434±0.222 [†]	1.516±0.119	1.569±0.123	—	—	—	—
Testes	3.87±0.31	3.98±0.23	3.81±0.33	3.80±0.37	—	—	—	—
Ovaries	—	—	—	—	0.139±0.018	0.131±0.017	0.134±0.018	0.147±0.023
Uterus/ Fallopian tubes	—	—	—	—	0.78±0.19	0.65±0.24	0.75±0.50	0.67±0.21

Values are the Mean ± SD ($n=10$ except for [†] $n=9$). No significant difference from control was observed. See Materials and methods section for details.

Table 10. Summary of mean organ-to-brain weight ratios.

Organ	Group (male)				Group (female)			
	1	2	3	4	1	2	3	4
Adrenals	0.030±0.011 [†]	0.036±0.004	0.034±0.004	0.035±0.006	0.040±0.003	0.040±0.006	0.039±0.003	0.039±0.006
Heart	0.66±0.07	0.70±0.06	0.65±0.05	0.66±0.09	0.49±0.05	0.53±0.05	0.49±0.04	0.51±0.05
Kidney	1.45±0.13	1.47±0.09	1.33±0.09	1.43±0.15	0.95±0.07	0.94±0.05	0.95±0.06	0.94±0.07
Liver	5.05±0.40	5.31±0.42	4.95±0.32	4.76±0.50	3.25±0.24	3.31±0.25	3.22±0.18	3.23±0.24
Spleen	0.38±0.06	0.41±0.05	0.38±0.04	0.35±0.05	0.32±0.03	0.35±0.04	0.32±0.04	0.34±0.04
Thymus	0.151±0.026	0.185±0.066	0.157±0.030	0.140±0.034	0.140±0.012	0.134±0.032	0.140±0.021	0.126±0.029
Epididymides	0.747±0.069	0.725±0.120 [†]	0.760±0.040	0.807±0.080	—	—	—	—
Testes	1.94±0.10	2.01±0.14	1.91±0.16	1.95±0.24	—	—	—	—
Ovaries	—	—	—	—	0.075±0.009	0.072±0.011	0.074±0.009	0.079±0.011
Uterus/ Fallopian tubes	—	—	—	—	0.42±0.10	0.36±0.13	0.42±0.29	0.36±0.10

Values are the Mean ± SD ($n=10$ except for [†] $n=9$). No significant difference from control was observed. See Materials and methods section for details.

Table 11. Summary of mean organ-to-body weight ratios.

Organ	Group (male)				Group (female)			
	1	2	3	4	1	2	3	4
Adrenals	0.178±0.029 [†]	0.188±0.018	0.180±0.014	0.190±0.038	0.315±0.031	0.317±0.064	0.310±0.031	0.316±0.046
Brain	5.34±0.31	5.27±0.45	5.38±0.31	5.49±0.51	7.86±0.49	7.94±0.56	7.99±0.41	8.11±0.38
Heart	3.48±0.22	3.67±0.41	3.49±0.28	3.62±0.28	3.81±0.29	4.16±0.34	3.91±0.30	4.09±0.41
Kidney	7.72±0.46	7.76±0.62	7.14±0.34*	7.81±0.46	7.47±0.61	7.43±0.50	7.58±0.35	7.62±0.56
Liver	26.84±0.94	27.91±1.91	26.55±0.66	25.92±0.77	25.45±1.43	26.21±1.54	25.65±1.21	26.11±1.50
Spleen	2.04±0.26	2.17±0.30	2.02±0.17	1.92±0.14	2.55±0.32	2.73±0.29	2.58±0.28	2.77±0.38
Thymus	0.801±0.134	0.974±0.374	0.844±0.163	0.766±0.194	1.099±0.086	1.064±0.264	1.119±0.167	1.019±0.229
Epididymides	3.992±0.510	3.876±0.777 [†]	4.089±0.305	4.421±0.444	—	—	—	—
Testes	10.35±0.75	10.59±0.83	10.29±1.11	10.66±0.90	—	—	—	—
Ovaries	—	—	—	—	0.589±0.090	0.570±0.061	0.591±0.059	0.641±0.089
Uterus/ Fallopian tubes	—	—	—	—	3.30±0.90	2.89±1.21	3.32±2.29	2.95±0.86

Values are the Mean ± SD ($n=10$ except for [†] $n=9$). * Statistically significant different from control value ($p<0.05$). See Materials and methods section for details.

intervals. No statistical differences were noted in any male or female group compared to their corresponding control (Table 12).

Clinical pathology

Hematology. Absolute platelet count (PLT) was significantly decreased in males administered 40 mg/kg/day

compared with control animals (86% of control). This change in mean hematology parameters was not adverse and not considered related to exposure to the test substance because the pathological changes did not occur in a dose-related pattern and because they were not accompanied by any other corresponding clinical- or histopathological change.

Table 12. Summary of motor activity assessment.

Interval	Group (male)				Group (female)			
	1	2	3	4	1	2	3	4
1	158.6±30.74	152.5±25.96	154.2±19.93	179.9±41.01	163.8±26.94	168.8±29.90	153.6±23.29	156.2±14.52
2	93.9±19.3	84.5±20.7	93.3±28.1	102.8±21.9	95.2±26.1	98.4±28.4	79.8±12.9	100.4±15.82
3	63.3±14.3	65.7±22.6	72.9±25.5	80.7±20.3	66.1±17.0	87.0±28.0	61.3±22.5	78.1±17.4
4	64.8±23.4	67.9±25.3	70.5±22.6	62.1±15.3	67.8±31.6	62.4±25.6	59.9±18.2	61.2±19.9
5	62.8±14.4	50.2±12.2	60.9±18.9	54.3±27.1	51.6±14.8	62.5±24.5	56.3±12.5	52.4±24.8
6	63.3±15.2	59.7±27.0	59.0±23.1	48.6±26.6	72.8±24.8	57.1±28.9	44.5±10.1	57.0±13.4

Values are the Mean ± SD. No significant difference from control was observed. See Materials and methods section for details.

Absolute eosinophil concentration (AEOS) was significantly increased in males administered 1000 mg/kg/day (200% of control). Absolute eosinophil concentration was also significantly increased in high dose females in a generalized dose-related response. However, values did not reach the level of statistical significance due to high variability within the group. Two females, in particular, showed high eosinophil levels, and this contributed to the overall increase in the group. Given that this finding occurred in more than one animal within the group and occurred as a generalized increase in all the males in Group 4, a test-substance related effect could not be discounted in females.

In addition to the above findings, one high dose female displayed detectable concentrations of absolute neutrophil band (ABAN). This finding, while appearing non-adverse, might be associated with an individual generalized granulocytic increase in response to test substance administration at the high dose.

Coagulation. There were no treatment-related or statistically significant effects in coagulation parameters.

Clinical biochemistry. There were no adverse changes in clinical biochemistry parameters in male or female rats (Table 13). The following statistically significant changes in mean clinical biochemistry results were not adverse and not considered related to exposure to the test substance because they were not dose-related and because they were not accompanied by any other corresponding clinical- or histopathological change. An increase in the aspartate aminotransferase (AST) concentration in males administered 40 mg/kg/day and females administered 400 mg/kg/day (113 and 115% of control, respectively) was observed. A decrease in sorbitol dehydrogenase (SDH) in females administered 40 and 400 mg/kg/day (66 and 75% of control, respectively) occurred. Both aspartate aminotransferase and sorbitol dehydrogenase are hepatocytic enzymes, their leakage suggestive of liver injury. However, only SDH is liver-specific, and is often accompanied by significant loss of liver mass. Given the absence of dose-dependent changes, uncorrelative with any microscopic alterations as well as the small magnitude of the change, there was no evidence to suggest that these changes were toxicologically relevant to test substance administration.

Urinalysis. There were no treatment-related or statistically significant effects in urinalysis parameters.

Serology. There were no detectable titers against the pathogens and antigens tested. In conclusion, there were no

adverse changes in coagulation, clinical chemistry, or urinalysis parameters in male or female rats administered UC-II. The statistically significant increase in eosinophil concentration in high dose males, with increases in high dose females were considered related to exposure to the test substance because this dose-related change was accompanied by potentially adverse histopathological change in the nasal cavity of both male and female high dose animals.

Sacrifice, macroscopic observations, and histopathology. There were no UC-II related macroscopic findings at scheduled sacrifice, and mortality occurring prematurely was deemed unrelated to test substance administration. At termination, test substance-related microscopic findings were observed involving the nasal turbinates in males and females at 1000 mg/kg/day UC-II. An increase in the incidence and intensity of several findings involving the respiratory epithelium were noted in males and females at 1000 mg/kg/day UC-II as compared to their respective controls. Findings included goblet cell hypertrophy/hyperplasia, eosinophilic infiltrates, acute inflammation, and the presence of eosinophilic cytoplasmic droplets. The incidence and intensity of these microscopic findings are presented in Table 14. The presence of eosinophilic droplets in the nasal turbinates of mice has been described as a non-adverse, adaptive response. Similarly, in this instance, their presence was deemed secondary to the other morphologic alterations described above for the nasal turbinates. There were statistically significant increases in absolute eosinophil counts for males at 1000 mg/kg/day and a non-statistically significant increase in mean absolute eosinophil counts for females at 1000 mg/kg/day. These hematologic alterations are likely associated with the eosinophil infiltrates in the nasal turbinates, which may reflect a test substance-related hypersensitivity reaction at the highest dosage tested.

Microscopic findings unrelated to the test-substance administration include: sporadic alterations involving the esophagus attributable to repeated gavage procedures, such as minimal-to-moderate esophageal changes included myofiber degeneration as well as fibroplasia, hemorrhage, inflammation, and pigmented macrophages (consistent with hemosiderin and resolving hemorrhage) involving the esophageal wall. In addition, sporadic findings of minimal chronic inflammation and necrosis involving the Harderian glands were attributable to sequelae of end of study orbital sinus bleeds. The remaining findings were incidental and most commonly developmental,

Table 13. Mean clinical biochemistry values.

Parameter (Units)	Group (male)				Group (female)			
	1	2	3	4	1	2	3	4
Aspartate Aminotransferase (AST, U/L)	91 ± 27	103 ± 15*	96 ± 15	97 ± 19	85 ± 7	97 ± 11	98 ± 17*	85 ± 9
Alanine Aminotransferase (ALT, U/L)	44 ± 7	49 ± 5	44 ± 5	50 ± 10	36 ± 4	41 ± 6	41 ± 6	37 ± 3
Sorbitol Dehydrogenase (SDH, U/L)	9.2 ± 2.5	8.6 ± 3.7	8.2 ± 2.7	9.2 ± 1.7	10.8 ± 2.4	7.1 ± 2.1*	8.1 ± 2.8*	8.6 ± 1.7
Alkaline Phosphatase (ALKP, U/L)	126 ± 32	137 ± 22	139 ± 34	135 ± 27	103 ± 30	103 ± 27	104 ± 23	88 ± 18
Total Bilirubin (BILI, mg/dL)	0.14 ± 0.02	0.14 ± 0.03	0.14 ± 0.003	0.16 ± 0.03	0.18 ± 0.02	0.20 ± 0.04	0.19 ± 0.03	0.18 ± 0.03
Blood Urea Nitrogen (BUN, mg/dL)	21 ± 3	21 ± 3	20 ± 1	21 ± 5	20 ± 2	21 ± 3	22 ± 4	23 ± 2
Creatinine (CREA, mg/dL)	0.29 ± 0.03	0.31 ± 0.03	0.31 ± 0.02	0.31 ± 0.04	0.39 ± 0.04	0.39 ± 0.06	0.41 ± 0.05	0.39 ± 0.04
Cholesterol (CHOL, mg/dL)	79 ± 10	82 ± 10	80 ± 9	80 ± 8	90 ± 18	84 ± 10	84 ± 13	85 ± 18
Triglycerides (TRIG, mg/dL)	49 ± 9	45 ± 8	45 ± 12	38 ± 8	28 ± 5	31 ± 6	28 ± 6	27 ± 7
Glucose (GLUC, mg/dL)	159 ± 24	160 ± 36	155 ± 23	160 ± 30	119 ± 15	120 ± 12	125 ± 14	116 ± 15
Total protein (TP, g/dL)	6.3 ± 0.2	6.3 ± 0.3	6.4 ± 0.2	6.4 ± 0.3	6.5 ± 0.3	6.8 ± 0.5	6.8 ± 0.2	6.8 ± 0.2
Albumin (ALB, g/dL)	3.2 ± 0.2	3.2 ± 0.1	3.2 ± 0.1	3.3 ± 0.2	3.5 ± 0.2	3.6 ± 0.1	3.5 ± 0.2	3.6 ± 0.2
Globulin (GLOB, g/dL)	3.1 ± 0.2	3.2 ± 0.3	3.1 ± 0.2	3.1 ± 0.2	3.0 ± 0.2	3.2 ± 0.4	3.3 ± 0.3	3.2 ± 0.2
Calcium (CALC, mg/dL)	9.5 ± 0.5	9.6 ± 0.5	9.7 ± 0.2	9.6 ± 0.3	9.8 ± 0.4	9.9 ± 0.5	9.9 ± 0.3	10.0 ± 0.3
Inorganic Phosphorus (IPHS, mg/dL)	6.3 ± 0.7	6.6 ± 1.0	6.5 ± 0.5	6.5 ± 0.5	6.0 ± 0.9	5.8 ± 0.7	6.2 ± 0.5	5.5 ± 0.5
Sodium (NA, mmol/L)	144.6 ± 6.0	144.8 ± 3.9	145.0 ± 6.6	145.0 ± 4.1	146.1 ± 5.5	145.4 ± 7.7	146.7 ± 4.4	148.0 ± 7.6
Potassium (K, mmol/L)	6.06 ± 0.60	5.99 ± 0.75	5.86 ± 0.47	6.14 ± 0.32	5.17 ± 0.44	5.37 ± 0.45	5.33 ± 0.47	5.32 ± 0.54
Chloride (CL, mmol/L)	103.5 ± 3.1	103.8 ± 3.2	104.2 ± 4.6	103.2 ± 1.7	105.8 ± 2.6	105.3 ± 4.4	105.7 ± 3.1	107.1 ± 4.5

Values are the mean ± SD ($n \geq 8$). * Statistically significant different from control values ($p < 0.05$).

Table 14. Incidence and severity of microscopic nasal turbinate findings.

Group	1		3		4	
	0		400		1000	
Sex	Male	Female	Male	Female	Male	Female
Goblet cell hypertrophy/hyperplasia: respiratory epithelium	1	0	0	1	9	9
Grade 1	0	0	0	0	0	0
Grade 2	1	0	0	1	5	8
Grade 3	0	0	0	0	4	1
Eosinophil infiltrates: respiratory epithelium	1	0	1	1	9	9
Grade 1	1	0	1	1	5	2
Grade 2	0	0	0	0	4	7
Acute inflammation: respiratory epithelium	0	0	0	0	4	1
Grade 1	0	0	0	0	3	1
Grade 2	0	0	0	0	1	0
Eosinophil droplets: respiratory epithelium cytoplasmic	0	0	1	1	9	7
Grade 1	0	0	0	1	4	4
Grade 2	0	0	1	0	5	3

See Materials and methods section for details.

inflammatory, or degenerative changes that can be seen in the age and strain of rat used in this study. Examples included, but were not limited to, nephropathy, pulmonary alveolar histiocytosis, pituitary gland cyst, and ectopic thymus in thyroid gland. UC-II related microscopic findings were observed involving the respiratory epithelium of the nasal turbinates in males and females at 1000 mg/kg/day UC-II. Salient microscopic observations included eosinophil infiltrates, goblet cell hypertrophy and hyperplasia, and acute inflammation. Therefore, under the conditions of this study, the anatomic pathology no-observed-adverse-effect level (NOAEL) for UC-II was 400 mg/kg/day following daily oral gavage to male and female Sprague-Dawley rats for at least 90 days.

Discussion

Given that OA is the most prevalent form of arthritis and that the number of persons affected with OA will increase significantly in the near future, finding alternative, safer pharmacological therapies for OA is of considerable importance. With the continued growth of the elderly population in the US, OA is becoming a major medical and financial concern. In the last few years, various nutritional supplements including chondroitin, glucosamine, avocado/soybean unsaponifiables, and diacerein have emerged as new treatment options for osteoarthritis. Among these nutraceuticals, the efficacy of UC-II was repeatedly demonstrated in animal (DeParle et al. 2005; D'Altilio et al. 2007; Peal et al. 2007; Bagchi et al. 2008a; 2009; Gupta et al. 2009a; b) and human (Bagchi et al. 2008b; Crowley et al. 2009) studies without any significant adverse events.

The current study demonstrated the broad-spectrum safety of UC-II in animals over the dose levels and routes of administration tested. Acute oral toxicity did not reveal any significant changes for all examined tissues. Based on these results, the oral LD₅₀ of UC-II was concluded to be > 5000 mg/kg in female rats. Acute dermal toxicity study conducted with a single 2000 mg/kg dose of UC-II applied directly to the skin of male and female rats for 24 h revealed no dermal irritation, adverse pharmacological effects, or abnormal behavior. Based on these results, the acute dermal LD₅₀ of UC-II was > 2000 mg/kg. The primary dermal irritation assay using a single 1000 mg dose of UC-II applied directly to the skin of rabbits for 4 h caused an initial redness of the skin. The overall incidence and severity of irritation decreased with time and irritation completely subsided by 24 h. Based on these results, UC-II was classified as slightly irritating to the skin. There were no other signs of gross toxicity, adverse pharmacologic effects, or abnormal behavior. Primary eye irritation was tested in rabbits using a single dose of 60 mg. One hour after UC-II application, treated eyes exhibited corneal opacity, iritis, and positive conjunctivitis. The overall incidence and severity of irritation decreased gradually with time. All animals were free of ocular irritation within 96 h. Based on these results, UC-II was classified as moderately irritating to the eye.

Ames' Bacterial Reverse Mutation Assay using five strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537, and TA102) was used to evaluate the mutagenic potential of UC-II in the presence and absence of metabolic activation. UC-II was determined to be non-mutagenic. Cell gene mutation assay in mouse lymphoma cells was conducted to test the mutagenic potential of UC-II in the L5178Y mouse lymphoma cell line. UC-II did not induce mutagenic effects either with or without metabolic activation.

The results from the 90-day sub-chronic toxicity study did not show any adverse effects in individual body weight or individual organ weight after 90 days of UC-II administration in increasing doses. No significant changes in organ-to-body weight ratios were observed except for the kidney-to-body weight ratio, which was significantly decreased in Group 3 males. This finding was not associated with any other clinical findings, and did not indicate any corresponding pathologic changes in the high dose animals. Therefore, this change was deemed incidental and of no toxicological interest. Mortality of a single Group 3 male and a single Group 4 male were not associated with test substance administration. Test substance-related microscopic findings were observed involving the respiratory epithelium of the nasal turbinates in males and females at 1000 mg/kg/day UC-II. Salient microscopic observations included eosinophil infiltrates, goblet cell hypertrophy and hyperplasia, and acute inflammation. Therefore, under the conditions of this study, the anatomic pathology no-observed-adverse-effect level (NOAEL) for UC-II was 400 mg/kg/day following daily oral gavage to male and female Sprague-Dawley rats for at least 90 days.

Overall, results from the current study combined with the animal (DeParle et al. 2005; D'Altilio et al. 2007; Peal et al. 2007; Bagchi et al. 2008a; 2009; Gupta et al. 2009a; b) and human (Bagchi et al. 2008b; Crowley et al. 2009) data demonstrate the broad-spectrum safety of UC-II.

Declaration of interest

This study was supported by a research grant from InterHealth Nutraceuticals Inc. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

- Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, Christy W, Cooke TD, Greenwald R, Hochberg M, Howell D, Kaplan D, Koopman W, Longley III S, Mankin H, McShane DJ, Medsger Jr T, Meenan R, Mikkelsen W, Moskowitz R, Murphy W, Rothschild B, Segal M, Sokoloff L, Wolfe F. 1986. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. *Arthritis Rheum* 29:1039-1049.
- Ames BN, McCann J, Yamasaki E. 1975: Methods for the Salmonella Mutagenicity Test. In: Kilbey BJ, editor. *Handbook of mutagenicity test procedures*. Amsterdam: Elsevier. pp 1-17.
- Bagchi M, Gupta RC, Lindley J, Barnes M, Canerdy TD, Goad JT, Bagchi D. 2009. Suppression of Arthritic Pain in Dogs by Undenatured Type-II Collagen (UC-II) Treatment Quantitatively Assessed by Ground Force Plate Dresden, Germany: EUROTOX.

- Bagchi M, Lau FC, Bagchi D. 2008b. Beneficial effects of oral administration of undenatured type II collagen on osteoarthritis: a human clinical trial. *Am Coll Nutr*, 27: 603.
- Bagchi M, Skaggs P, Gupta RC, Canerdy TD, Goad JT, Barnett D, Wegford K, Burke R, Bagchi D. 2008a. Therapeutic Efficacy of Undenatured Type II Collagen (UC-II) In Comparison To Glucosamine Plus Chondroitin in Arthritic Horses San Diego, CA: FASEB. pp LB28.
- Barnett ML, Combitchi D, Trentham DE. 1996. A pilot trial of oral type II collagen in the treatment of juvenile rheumatoid arthritis. *Arthritis Rheum* 39:623-628.
- Barnett ML, Kremer JM, St Clair EW, Clegg DO, Furst D, Weisman M, Fletcher MJ, Chasan-Taber S, Finger E, Morales A, Le CH, Trentham DE. 1998. Treatment of rheumatoid arthritis with oral type II collagen. Results of a multicenter, double-blind, placebo-controlled trial. *Arthritis Rheum* 41:290-297.
- Berenbaum F. 2008. New horizons and perspectives in the treatment of osteoarthritis. *Arthritis Res Ther* 10(Suppl 2):S1
- Bitton R. 2009. The economic burden of osteoarthritis. *Am J Manag Care* 15:S230-S235.
- Clive D. 1983. Viable chromosomal mutations affecting the TK locus in L5178Y/TK+/- mouse lymphoma cells: the other half of the assay. *Ann NY Acad Sci* 407:253-257.
- Clive D, McCuen R, Spector JF, Piper C, Mavournin KH. 1983. Specific gene mutations in L5178Y cells in culture. *Mutat Res* 115:225-251.
- Clive D, Spector JF. 1975. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. *Mutat Res* 31:17-29.
- Crowley DC, Lau FC, Sharma P, Evans M, Guthrie N., Bagchi, M. Bagchi, D., Dey, D. K. Raychaudhuri, S. P. 2009. Safety and efficacy of undenatured type II collagen in the treatment of osteoarthritis of the knee: a clinical trial. *Int J Med Sci* 6:312-321.
- D'Altilio M, Peal A, Alvey M, Simms C, Curtsinger A, Gupta RC, Canerdy TD, Goad JT, Bagchi M, Bagchi D. 2007. Therapeutic efficacy and safety of undenatured type II collagen singly or in combination with glucosamine and chondroitin in arthritic dogs. *Toxicol Mech Methods* 17:189-196.
- Deparle LA, Gupta RC, Canerdy TD, Goad JT, D'Altilio M, Bagchi M, Bagchi D. 2005. Efficacy and safety of glycosylated undenatured type-II collagen (UC-II) in therapy of arthritic dogs. *J Vet Pharmacol Ther* 28:385-390.
- Draize JH, Woodward G, Calvery HO. 1944. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membrane. *J Pharm Exp Ther* 82:377-390.
- FDA. 1987. Good Laboratory Practices (GLP) for Non-Clinical Laboratory Studies. In: Services. D. o. H. a. H. Washington, DC. US Food and Drug Administration. pp297-310.
- Felson DT. 2009. Developments in the clinical understanding of osteoarthritis. *Arthritis Res Ther* 11:203.
- Felson DT, Lawrence RC, Dieppe PA, Hirsch R, Helmick CG, Jordan JM, Kington RS, Lane NE, Nevitt MC, Zhang Y, Sowers M, McAlindon T, Spector TD, Poole AR, Yanovski SZ, Ateshian G, Sharma L, Buckwalter JA, Brandt KD, Fries JF. 2000. Osteoarthritis: new insights. Part 1: the disease and its risk factors. *Ann Intern Med* 133:635-646.
- Goggs R, Vaughan-Thomas A, Clegg PD, Carter SD, Innes JF, Mobasheri A, Shakibaei M, Schwab W, Bondy CA. 2005. Nutraceutical therapies for degenerative joint diseases: a critical review. *Crit Rev Food Sci Nutr* 45:145-164.
- Gupta RC, Canerdy TD, Skaggs P, Stocker A, Zyrkowski G, Burke R, Wegford K, Goad JT, Rohde K, Barnett D, DeWees, W, Bagchi M, Bagchi D. 2009a. Therapeutic efficacy of undenatured type-II collagen (UC-II) in comparison to glucosamine and chondroitin in arthritic horses. *J Vet Pharmacol Ther* 32:577-584.
- Gupta RC, Lindley J, Barnes M, Minniear J, Goad JT, Canerdy TD, Bagchi M, Bagchi D. 2009b. Pain reduction measured by ground force plate in arthritic dogs treated with type-II collagen Baltimore, MD: Society of Toxicology.
- Helmick CG, Felson DT, Lawrence RC, Gabriel S, Hirsch R, Kwoh CK, Liang MH, Kremers HM, Mayes MD, Merkel PA, Pillemer SR, Reveille JD, Stone JH. 2008. Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part I. *Arthritis Rheum* 58:15-25.
- Kay JH, Calandra JC. 1962. Interpretation of eye irritation tests. *J Soc Cos Chem* 13:281-289.
- Lawrence RC, Felson DT, Helmick CG, Arnold LM, Choi H, Deyo RA, Gabriel S, Hirsch R, Hochberg MC, Hunder GG, Jordan JM, Katz JN, Kremers HM, Wolfe F. 2008. Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part II. *Arthritis Rheum* 58:26-35.
- Maron DM, Ames BN. 1983. Revised methods for the Salmonella mutagenicity test. *Mutat Res* 113:173-215.
- Mitchell AD, Auletta AE, Clive D, Kirby PE, Moore MM, Myhr BC. 1997. The L5178Y/TK+/- mouse lymphoma specific gene and chromosomal mutation assay a phase III report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat Res* 394:177-303.
- OECD. 1998. Paris: Committee, C. G. a. M. Principles of Good Laboratory Practice Organization for Economic Cooperation and Development.
- Peal A, D'Altilio M, Simms C, Alvey M, Gupta RC, Goad JT, Canerdy TD, Bagchi M, Bagchi D. 2007. Therapeutic efficacy and safety of undenatured type-II collagen (UC-II) alone or in combination with (-)-hydroxycitric acid and chromemate in arthritic dogs. *J Vet Pharmacol Ther* 30:275-278.
- Sarzi-Puttini P, Cimmino MA, Scarpa R, Caporali R, Parazzini F, Zaninelli A, Atzeni F, Canesi B. 2005. Osteoarthritis: an overview of the disease and its treatment strategies. *Semin Arthritis Rheum* 35:1-10.
- Trentham DE. 1984. Immunity to type II collagen in rheumatoid arthritis: a current appraisal. *Proc Soc Exp Biol Med* 176:95-104.
- Trentham DE. 1996. Evidence that type II collagen feeding can induce a durable therapeutic response in some patients with rheumatoid arthritis. *Ann NY Acad Sci* 778:306-314.
- Trentham DE, Dynesius-Trentham RA, Orav EJ, Combitchi D, Lorenzo C, Sewell KL, Hafler DA, Weiner HL. 1993. Effects of oral administration of type II collagen on rheumatoid arthritis. *Science* 261:1727-1730.
- Trentham DE, Halpner AD, Trentham RA, Bagchi M, Kothari SC, Preuss HG, Bagchi D. 2001. Use of undenatured type II collagen in the treatment of rheumatoid arthritis. *Clin Pract Altern Med* 2: 254-259.