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# A 90-day dietary study of a (2*R*,4*R*)-monatin salt in Beagle dogs

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ABSTRACT

(2*R*,4*R*)-Monatin salt (Na/K) [sodium/potassium (2*R*,4*R*)-2-amino-4-carboxy-4-hydroxy-5-(3-indolyl) pentanoate, hereafter "*R,R*-monatin"] was administered in the diets of groups of Beagle dogs (4/sex/group) at concentrations of 0 (basal diet), 5000, 20,000, or 35,000 ppm for 13 weeks. There were no effects on survival, clinical observations, body weight and body weight gain, feed consumption and feed efficiency, functional observational battery, ophthalmic examination, and electrocardiographic evaluation. No adverse effects on hematology, serum chemistry, and urinalysis parameters were reported. A statistically significant decrease in testicular weights associated with germ cell hypocellularity and reduced luminal sperm in the epididymides was reported in all treated male groups. Based on these findings, the dietary no-observed-adverse-effect level (NOAEL) of *R,R*-monatin for 90 days was considered 35,000 ppm for female dogs (approximately 1101 mg/kg bw/day) and <5000 ppm for male dogs (approximately <151 mg/kg bw/day).



## 1. Introduction

A high-potency sweetener, (2*R*,4*R*)-monatin, which has been found to be approximately 3000 times sweeter than sucrose (Fry et al., 2012) was originally identified in the root bark of a South African shrub, *Sclerochitin ilicifolius* (Archibald et al., 1956; Vleggaar et al., 1992). Four possible stereo-isomers (*R,R*-, *S,S*-, *R,S*- and *S,R*-) have been identified (Bassoli et al., 2005), but the *R,R*-isomer (Fig. 1) has been determined to be the most sweet. Traditionally, the root bark was used by indigenous populations in South Africa as a sweetening agent in food and medicines without apparent adverse effects (Vahrmeijer, 2010).

In recent animal studies, the only consistent findings related to ingestion of enzymatically sourced (2*R*,4*R*)-monatin salt (hereafter "*R,R*-monatin") by rats and mice up to 35,000 ppm in the diet for a period of 90 days were increased serum chloride levels, decreased body weights and body weight gains resulting in a no-observed-adverse-effect level (NOAEL) for female rats of 20,000 ppm (approximately 1544 mg/kg bw/day), the second highest dietary concentration tested, and a NOAEL for male rats, and male and female mice of 35,000 ppm (approximately 2368 mg/kg bw/day for male rats and approximately 5764 and 8013 mg/kg bw/day for male and female mice, respectively), the highest dietary concentration tested (Hlywka et al., 2011, 2013). Additionally, decreased serum potassium levels were observed in the rats. A NOAEL of 30,000 ppm (equivalent to 2564 mg/kg bw/day) was determined for maternal and embryo/fetal developmental toxicity based on the reduced body weight changes at the highest dietary concentration tested of 50,000 ppm *R,R*-monatin during gestation days 6–21 (Brathwaite et al., 2013).

*Abbreviations:* AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOVA, analysis of variance; APTT, activated partial thromboplastin time; ECG, electrocardiogram; FDA, US Food and Drug Administration; FOB, functional observational battery; GGT, gamma-glutamyl transferase; GLP, Good Laboratory Practice; LUC, large unstained cell; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; NOAEL, no-observed-adverse-effect level; OECD, Organisation for Economic Co-operation and Development; PT, prothrombin time; RBC, red blood cell; RDW, red cell distribution width; SD, standard deviation; SDH, sorbitol dehydrogenase; SE, standard error; SOP, standard operating procedure.

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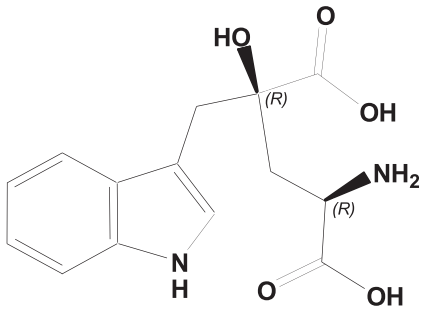


**Fig. 1.** The 2*R*,4*R*-isomer of 2-hydroxy-2-(indol-3-ylmethyl)-4-aminoglutaric acid.

*R,R*-Monatin was not mutagenic in the Ames assay and did not induce forward mutations in the L5178Y/TK$^{+/-}$ mouse lymphoma mutagenesis assay with or without metabolic activation (Casterton et al., 2014a). No significant increase in the incidence of micronucleated polychromatic erythrocytes (MPCEs) was observed in the male ICR mouse bone marrow erythrocyte assay following a single gavage dose of up to 2000 mg/kg bw *R,R*-monatin.

In an absorption, distribution, metabolism and excretion (ADME) study, [$^{14}$C]-labeled *R,R*-monatin was shown to be rapidly absorbed and eliminated primarily unchanged in the urine and feces of male and female Beagle dogs and one bile duct-cannulated male dog within 48 h following a single gavage dose of 10 mg/kg bw (Casterton et al., 2014b).

As part of a program that evaluated the safety of *R,R*-monatin, the 90-day dietary study described herein was conducted using Beagle dogs and in compliance with the United States (U.S.) Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations (FDA, 1987) and the Organisation for Economic Co-operation and Development (OECD) Principles of GLP (OECD, 1997).

## 2. Materials and methods

This study was conducted at WIL Research, Ashland, Ohio, USA with animal facilities that are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The study was performed in accordance with the FDA Redbook 2000 guidelines (FDA, 2000) and the OECD Test Guideline 409 (OECD, 1998).

### 2.1. Test article

Enzymatically sourced (2*R*,4*R*)-monatin salt [sodium/potassium (2*R*,4*R*)-2-amino-4-carboxy-4-hydroxy-5-(3-indolyl) pentanoate] used in this study was an off-white powder produced with a purity of 96.4% (corrected for diastereomeric purity) and supplied by Cargill, Incorporated (MN, USA). The test article, hereinafter referred to as "*R,R*-monatin," was administered orally *via* the diet.

### 2.2. Test animals

Eighteen male and 18 female 5- to 6-month-old Beagle dogs were received in good health from Ridglan Farms, Inc., Mt. Horeb, WI. During the 14-day acclimation period, animals were observed twice daily for mortality and changes in general appearance or behavior, and body weights, feed consumption, clinical pathology, electrocardiographic, ophthalmic examination, and functional observational battery (FOB) data were recorded.

Animals were housed individually in clean, stainless steel cages elevated above stainless steel cage pans at a room temperature of 20 ± 3 °C with a relative humidity of 50 ± 20% and a 12-h light/12-h dark photoperiod. Reverse osmosis-treated drinking water was provided *ad libitum* throughout the study. Studies were conducted under an institutionally approved protocol and complied with all applicable sections of the Final Rules of the Animal Welfare Act regulations (9 CFR), the *Guide for Care and Use of Laboratory Animals* (NRC, 1996), and the PHS Policy on Humane Care and Use of Laboratory Animals.

### 2.3. Diet preparation

PMI Nutrition International, LLC, Certified Canine LabDiet® 5007 (meal) was used in the weekly preparation of the control (untreated) and the *R,R*-monatin/dietary admixes (test) diets. For test diets, appropriate amounts of test article and basal diet were weighed and mixed together in a Hobart mixer to provide the correct final formulation concentrations of 0.5, 2.0, and 3.5% (w/w) *R,R*-monatin. Diets were stored at room temperature until feeding.

### 2.4. Analysis of R,R-monatin in the diet

Samples for homogeneity and concentration determinations were collected from the first test diet formulations and at approximately 30, 60, and 90 days from all group diet preparations for the confirmation of the test article concentration using a validated high performance liquid chromatography tandem mass spectrometry (HPLC-MS) method in the positive electrospray ionization mode. Samples collected from the control group (basal diet) formulation were analyzed to confirm the absence of *R,R*-monatin. Portions from the lowest and highest concentration diet preparations were stored at room temperature and protected from light for 10 days for the evaluation of stability.

### 2.5. Assignment of animals to treatment groups

Animals were assigned to control and test groups (4/sex/group) using a computerized randomization procedure based on body weight stratification in a block design. The animals were approximately 6–7 months old with body weights ranging from 7.9 to 10.0 kg (mean = 9.1 kg) for males and from 6.9 to 9.0 kg (mean = 7.9 kg) for females at randomization.

### 2.6. Administration

Dogs were offered diets containing 0 (basal diet control), 5000, 20,000, or 35,000 ppm *R,R*-monatin (groups 1–4; Table 1) for 2–4 h *per* day for up to 92 days based on the results of previous 90-day studies in rats and mice (Hlywka et al., 2011, 2013) and a 7-day palatability study in dogs (data not shown). The mean amount of *R,R*-monatin consumed (mg/kg bw/day) by each sex *per* dose group was calculated from the mean feed consumed (g/kg bw/day) and the appropriate target concentration of test article in the feed (Table 1).

### 2.7. Parameters evaluated

#### 2.7.1. Clinical observations and survival

All animals were observed twice daily, once in the morning and once in the afternoon, for mortality and moribundity. Clinical examinations were performed once daily and detailed physical examinations were conducted on all animals approximately weekly. A moribund female was euthanized by an intravenous injection of sodium pentobarbital followed by exsanguination, and a necropsy was performed as described in Section 2.7.7.

**Table 1**
Group assignments and mean *R,R*-monatin consumption in a 90-day study with Beagle dogs.

| Group number[a] | Treatment | Dietary concentration (ppm) | Mean *R,R*-monatin consumption[b] (mg/kg bw/day ± SD) | |
|---|---|---|---|---|
| | | | Males | Females |
| 1 | Basal Diet | 0 | 0 | 0 |
| 2 | *R,R*-Monatin | 5000 | 151 ± 7.5 | 168 ± 16.7 |
| 3 | *R,R*-Monatin | 20,000 | 625 ± 40.3 | 637 ± 101.7 |
| 4 | *R,R*-Monatin | 35,000 | 1088 ± 31.3 | 1101 ± 73.8 |

SD = standard deviation.
[a] 4 animals/sex/group.
[b] Calculated from weekly individual feed consumptions and nominal dietary concentrations. Actual dietary concentrations were analyzed and found to contain 97.2 to 114% of target concentrations.

*2.7.2. Body weights*

Body weights were recorded at least once weekly, beginning one week prior to randomization, through 13 weeks of dosing. Final fasted body weights were recorded prior to scheduled necropsy.

*2.7.3. Feed consumption*

Individual feed consumption was recorded daily, beginning approximately two weeks prior to test diet administration, through 13 weeks of dosing. Feed intake was calculated weekly as g/animal/day and g/kg bw/day.

*2.7.4. Functional observational battery (FOB)*

Motor, sensory, and autonomic pathways were examined using home cage observations (*i.e.*, general posture, general demeanor, head posture, tremors/convulsions, salivation, lacrimation, palpebral closure, body weight, excreta, and emesis), open field observations (*i.e.*, time to the first step, gait, and behavior), and table-top functional observations and measurements (*i.e.*, respiration rate, respiration pattern, perineal reflex, rectal temperature, blood pressure and heart rate, cliff avoidance, pinna reflex, pupillary reflex, pupillary size, nystagmus, ocular position, palpebral reflex, righting reflex, patellar reflex, pinch reflex, tricep reflex, proprioceptive positioning, posterior extension thrust, wheel barrowing, hemistanding/hemiwalking, capillary refill time, jaw and tongue examination, gag reflex, auditory response, and menace reaction) to assess neurological function of control and test article-treated animals. All animals were evaluated once during study week -1 and again during study week 12.

*2.7.5. Clinical pathology*

Standard hematology, serum chemistry and urinalysis parameters, consistent with OECD and FDA Redbook guidelines, were evaluated during study weeks -1, 6 and 12. Blood samples were collected from the jugular vein of all animals into tubes containing $K_3$EDTA (hematology), sodium citrate (coagulation), or no anticoagulant (serum chemistry). Non-fasted samples were also collected for hematology and serum chemistry evaluations from one high-dose female euthanized *in extremis* on study day 17. During scheduled intervals, the animals were fasted overnight prior to blood collection while using cage pans for urine collection.

*2.7.6. Ophthalmic examinations*

Ocular examinations were conducted on all animals prior to randomization and near the end of the treatment period. All ocular examinations were conducted using an indirect ophthalmoscope and slit lamp biomicroscope preceded by pupillary dilation with an appropriate mydriatic agent.

*2.7.7. Electrocardiographic data*

Multilead (I, II, III, aVR, aVL, aVF, and V2) electrocardiograms (ECGs) were recorded for all animals prior to the initiation of test diet administration (study week -2) and during study week 12 (approximately 1–2 h following completion of the daily feeding period). Qualitative interpretation and measurements of ECG tracing (heart rate and PR, RR, QRS, QT, and QTcV intervals) were performed. Van de Water's correction was used to calculate QTcV = QT-0.087*(RR-1) (Spence et al., 1998; Van de Water et al., 1989).

*2.7.8. Macroscopic examination and organ weights*

Surviving animals were sedated with a subcutaneous injection of acepromazine, euthanized by an intravenous injection of sodium pentobarbital, and exsanguinated. A complete necropsy was conducted on all animals. The necropsies included, but were not limited to, examination of the external surface, including palpable masses, all orifices, and the cranial, thoracic, abdominal and pelvic cavities including viscera. A comprehensive list of tissues and organs (including all gross lesions), consistent with OECD and FDA Redbook guidelines, were collected and placed 10% neutral-buffered formalin fixative for further histological processing. Additionally, epididymides and testes were fixed in Bouin's solution while eyes were fixed in Davidson's solution.

The following organs were weighed at the scheduled necropsy: adrenals, brain, epididymides, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes, thymus, thyroids with parathyroids, and uterus with cervix. Organ-to-final body weight and organ-to-brain weight ratios were calculated.

*2.7.9. Microscopic examination*

After fixation, tissues were trimmed, processed into paraffin blocks, sectioned at 4 to 8 microns, mounted on glass microscope slides, and stained with hematoxylin and eosin. Microscopic examination was performed on all tissues from all animals at the scheduled necropsy, as well as the one female dog that was euthanized *in extremis*.

*2.8. Statistical analyses*

All statistical tests were performed using an in-house validated system, WIL Toxicology Data Management System (WTDMS™). Analyses were conducted using two-tailed tests (except as noted otherwise) for minimum significance levels of 1 and 5%, comparing each test article-treated group to the control group by sex. Each mean was presented with the standard deviation (SD), standard error (SE), and the number of animals (n) used to calculate the mean.

Body weight, body weight change, food consumption, quantitative FOB, ECG measurements, clinical pathology, and organ weight data were subjected to a parametric one-way analysis of variance (ANOVA) (Snedecor and Cochran, 1980) to determine intergroup differences. If the ANOVA revealed statistically significant ($p < 0.05$) intergroup variance, Dunnett's test (Dunnett, 1964) was used to compare the test article-treated groups to the control

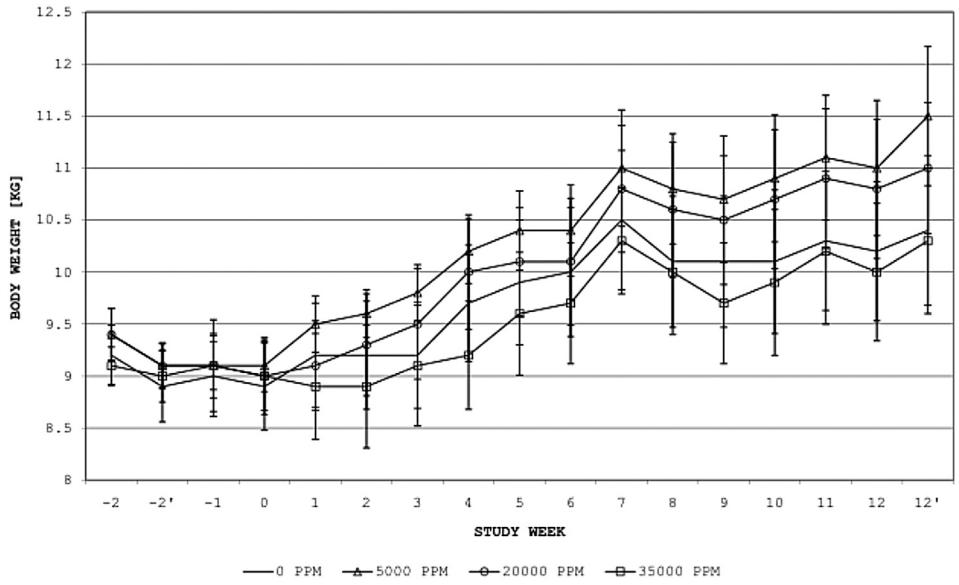


**Fig. 2.** Weekly mean body weights (±standard error) of male Beagle dogs fed *R,R*-monatin for up to 92 days.

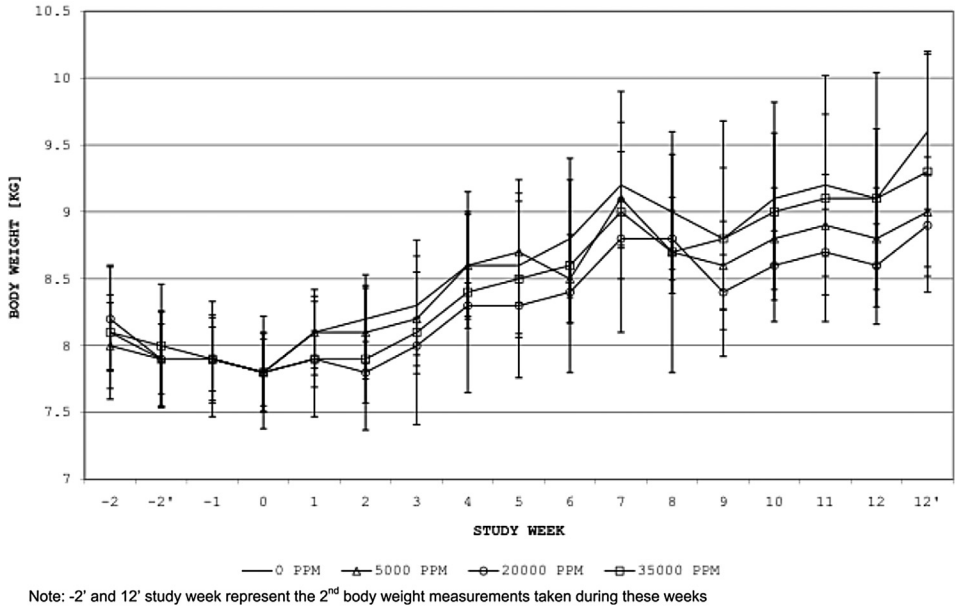


**Fig. 3.** Weekly mean body weights (±standard error) of female Beagle dogs fed *R,R*-monatin for up to 92 days.

group. FOB parameters that yielded scalar or descriptive data were analyzed using Fisher's Exact Test (Steel and Torrie, 1980).

## 3. Results

The use of the term "significant" indicates a statistically significant change from control values and does not describe severity of the change.

### 3.1. Confirmation of test article concentration in diet

The analyzed dietary admixes were found to contain 97.2% to 114% of the target test article concentration, which was within the acceptable range defined by analytical standard operating procedures (SOPs), with the exception that on study day 55, the high-dose group diet measured at 119% of the target (exceeding the SOPs) and a new formulation was prepared for use on day 57. The test diets were homogeneous and were stable at room temperature for up to 10 days. The test article was not detected in the analyzed basal diet offered to the control group.

### 3.2. Survival and clinical observations

All animals survived to scheduled necropsy with the exception of one high-dose female euthanized *in extremis* on study day 17. This animal was prostrate, had pale gums, ear(s) cool to the touch, increased respiration rate, clear material on mouth, forelimb(s), and ventral neck, emesis, and red and mucoid feces prior to death on study day 17. These clinical signs were consistent with the moribund state of this animal and were not considered test article-related.

**Table 2**
Statistically significant changes (±SD) in hematology and coagulation parameters of Beagle dogs fed *R,R*-monatin for up to 92 days.

| | 0 ppm | 5000 ppm | 20,000 ppm | 35,000 ppm | HC mean (±SD) | HC Min/Max |
|---|---|---|---|---|---|---|
| **Males (n = 4)** | | | | | | |
| *MCHC* (g/dL) | | | | | 33.9 ± 1.04 | 31.6/35.9 |
| Week -1 | 32.6 ± 0.36 | 32.6 ± 0.60 | 32.4 ± 0.49 | 32.3 ± 0.37 | | |
| Week 6 | 34.6 ± 0.64 | 33.9 ± 0.13 | 33.3 ± 0.40** | 33.2 ± 0.17** | | |
| Week 12 | 33.3 ± 0.21 | 33.0 ± 0.17 | 32.2 ± 0.26** | 31.8 ± 0.31** | | |
| *APTT* (s) | | | | | 10.2 ± 0.62 | 9.7/10.7 |
| Week -1 | 10.7 ± 0.14 | 11.4 ± 1.21 | 10.4 ± 0.24 | 11.1 ± 0.68 | | |
| Week 6 | 10.5 ± 0.30 | 11.6 ± 1.71 | 11.8 ± 0.56 | 14.4 ± 2.06** | | |
| Week 12 | 11.0 ± 0.39 | 12.5 ± 2.08 | 12.4 ± 0.92 | 14.9 ± 1.78** | | |
| *Basophil, absolute* ($\times 10^3$/μL)) | | | | | 0.05 ± 0.022 | 0.02/0.10 |
| Week -1 | 0.07 ± 0.005 | 0.06 ± 0.013 | 0.06 ± 0.019 | 0.05 ± 0.019 | | |
| Week 6 | 0.09 ± 0.033 | 0.06 ± 0.010 | 0.04 ± 0.017* | 0.04 ± 0.021* | | |
| Week 12 | 0.10 ± 0.034 | 0.07 ± 0.031 | 0.07 ± 0.019 | 0.04 ± 0.016 | | |
| *Eosinophils* (%) | | | | | 2.5 ± 1.22 | 0.7/6.3 |
| Week -1 | 1.7 ± 0.50 | 2.9 ± 1.12 | 3.1 ± 2.04 | 1.6 ± 0.48 | | |
| Week 6 | 1.2 ± 0.62 | 1.8 ± 0.68 | 3.2 ± 3.01 | 2.6 ± 0.23 | | |
| Week 12 | 1.5 ± 1.23 | 2.5 ± 0.49 | 2.0 ± 0.93 | 3.7 ± 0.81* | | |
| *LUC, absolute* ($\times 10^3$/μL) | | | | | 0.05 ± 0.023 | 0.02/0.17 |
| Week -1 | 0.05 ± 0.014 | 0.05 ± 0.015 | 0.05 ± 0.013 | 0.05 ± 0.013 | | |
| Week 6 | 0.08 ± 0.034 | 0.07 ± 0.024 | 0.04 ± 0.010* | 0.04 ± 0.013* | | |
| Week 12 | 0.12 ± 0.033 | 0.09 ± 0.034 | 0.12 ± 0.019 | 0.08 ± 0.024 | | |
| *RDW* (%) | | | | | 12.8 ± 0.61 | 12.0/14.3 |
| Week -1 | 13.2 ± 0.46 | 12.8 ± 0.66 | 13.1 ± 0.57 | 13.5 ± 0.68 | | |
| Week 6 | 13.4 ± 0.54 | 12.9 ± 0.46 | 13.9 ± 0.81 | 14.6 ± 0.34* | | |
| Week 12 | 12.7 ± 0.25 | 13.0 ± 0.68 | 13.6 ± 1.36 | 14.2 ± 0.70 | | |
| **Females (n = 4)** | | | | | | |
| *RBC* ($\times 10^6$/μL) | | | | | 6.97 ± 0.488 | 5.93/7.92 |
| Week -1 | 7.59 ± 0.030 | 7.23 ± 0.354 | 7.20 ± 0.229 | 6.82 ± 0.357** | | |
| Week 6 | 6.84 ± 0.235 | 6.45 ± 0.279 | 6.40 ± 0.270 | 6.63 ± 0.856 | | |
| Week 12 | 6.95 ± 0.259 | 6.52 ± 0.485 | 6.04 ± 0.194** | 5.81 ± 0.150** | | |
| *Hemoglobin* (g/dL) | | | | | 15.7 ± 1.05 | 13.2/17.1 |
| Week -1 | 16.4 ± 0.38 | 15.7 ± 0.76 | 16.0 ± 0.31 | 15.0 ± 0.83* | | |
| Week 6 | 15.7 ± 0.53 | 14.9 ± 0.54 | 14.9 ± 0.43 | 15.2 ± 2.15 | | |
| Week 12 | 15.4 ± 0.78 | 14.7 ± 0.83 | 13.8 ± 0.54* | 13.1 ± 0.64** | | |
| *Hematocrit* (%) | | | | | 46.4 ± 3.35 | 38.6/52.8 |
| Week -1 | 50.9 ± 1.28 | 48.7 ± 2.45 | 49.5 ± 0.92 | 46.5 ± 2.87 | | |
| Week 6 | 45.9 ± 1.36 | 43.9 ± 1.49 | 44.7 ± 0.99 | 45.9 ± 6.16 | | |
| Week 12 | 46.4 ± 1.72 | 44.5 ± 2.77 | 42.9 ± 1.62 | 40.6 ± 1.61** | | |
| *MCV* (fL) | | | | | 66.6 ± 1.95 | 62.8/72.0 |
| Week -1 | 67.1 ± 1.89 | 67.3 ± 2.30 | 68.8 ± 2.86 | 68.1 ± 0.80 | | |
| Week 6 | 67.2 ± 1.84 | 68.0 ± 1.73 | 69.9 ± 2.71 | 69.3 ± 1.11 | | |
| Week 12 | 66.8 ± 1.47 | 68.3 ± 1.48 | 71.1 ± 2.84* | 69.8 ± 1.36 | | |
| *MCHC* (g/dL) | | | | | 33.8 ± 1.06 | 31.7/36.2 |
| Week -1 | 32.3 ± 0.44 | 32.3 ± 0.21 | 32.3 ± 0.06 | 32.3 ± 0.48 | | |
| Week 6 | 34.1 ± 0.39 | 33.9 ± 0.10 | 33.5 ± 0.26* | 33.0 ± 0.17** | | |
| Week 12 | 33.2 ± 0.50 | 33.0 ± 0.19 | 32.3 ± 0.21** | 32.2 ± 0.32** | | |
| *Prothrombin time* (s) | | | | | 7.2 ± 0.23 | 7.1/7.3 |
| Week -1 | 8.9 ± 0.39 | 9.0 ± 0.17 | 9.0 ± 0.38 | 9.1 ± 0.18 | | |
| Week 6 | 9.5 ± 0.10 | 9.5 ± 0.43 | 9.8 ± 0.56 | 10.3 ± 0.21* | | |
| Week 12 | 9.2 ± 0.37 | 9.3 ± 0.19 | 9.5 ± 0.62 | 10.0 ± 0.31 | | |
| *HDW* (g/dL) | | | | | 1.6 ± 0.11 | 1.3/1.9 |
| Week -1 | 1.44 ± 0.090 | 1.46 ± 0.079 | 1.45 ± 0.095 | 1.40 ± 0.022 | | |
| Week 6 | 1.75 ± 0.030 | 1.65 ± 0.053 | 1.59 ± 0.059** | 1.68 ± 0.070 | | |
| Week 12 | 1.66 ± 0.044 | 1.57 ± 0.048 | 1.52 ± 0.086 | 1.61 ± 0.100 | | |

SD, standard deviation; MCHC, mean corpuscular hemoglobin concentration; APTT, activated partial thromboplastin time; LUC, large unstained cells; RDW, red cell distribution width; MCV, mean corpuscular volume; HDW, hemoglobin distribution width; HC, Historical Control; Min/Max, minimum and maximum of study means.
*Significantly different from control group at 0.05 using Dunnett's test.
**Significantly different from control group at 0.01 using Dunnett's test.

There were no test article-related clinical observations in animals that survived to the scheduled necropsy. A higher incidence of clear and/or green discharge from the eye(s) was noted primarily in the high-dose females. As this is a commonly reported finding for laboratory dogs of this age and breed, and not consistently observed in males and females, it is unlikely to be related to test article administration.

All other observations in the test article-treated groups (*e.g.*, soft feces, mucoid feces, diarrhea, and emesis) were noted with similar incidence in the control group, were limited to single animals, were not noted in a dose-related manner, and/or were common findings for laboratory dogs of this age and breed.

### 3.3. Body weights

Body weights were unaffected by test article administration (Figs. 2 and 3). A transient, significant ($p < 0.05$) mean body weight loss was noted in high-dose males during study week 8 to 9, but was likely due to individual animal variability and was not considered related to test article administration. The mean body weight of high-dose males at week 9 (9.7 kg) was within the laboratory's historical control value (10.5 ± 1.43 kg) for dogs of this age and breed.

### 3.4. Feed consumption

Feed consumption (g/animal/day or g/kg bw/day) was unaffected by test article administration (data not shown). A transient, significant ($p < 0.05$) lower feed consumption (g/kg bw/day) was noted in high-dose males during study week 1 to 2, but was likely due to individual variability in food consumption and not related to test article administration. Normal feed efficiency fluctuations occurred throughout the study, which were unrelated to test article administration. There were no other significant differences when the control and test article-treated groups were compared.

### 3.5. Function observational battery

Home cage observations, functional observations and measurements, and open field observations were unaffected by test article administration (data not shown).

### 3.6. Clinical pathology

#### 3.6.1. Hematology and coagulation

Non-adverse, but significant, test article-related effects on hematology and coagulation parameters included lower red blood cells (RBC), hemoglobin, and hematocrit values in mid- and high-dose females at study week 12, and higher red cell distribution width (RDW) in high-dose males at study week 6, higher activated partial thromboplastin times (APTT) in high-dose males at weeks 6 and 12, and higher prothrombin (PT) times in high-dose females at study week 6 (Table 2).

RBC, hemoglobin, and hematocrit changes in females were minimal and were considered non-adverse. Minimally higher RDW values noted in males were indicative of erythrocyte size variation

**Table 3**
Statistically significant changes (±SD) in serum chemistry parameters of Beagle dogs fed *R,R*-monatin for up to 92 days.

| | 0 ppm | 5000 ppm | 20,000 ppm | 35,000 ppm | HC mean (±SD) | HC Min/Max |
|---|---|---|---|---|---|---|
| **Males (n = 4)** | | | | | | |
| *Alkaline phosphatase* (IU/L) | | | | | 73 ± 22.3 | 36/156 |
| Week -1 | 83 ± 22.7 | 91 ± 14.4 | 100 ± 26.9 | 84 ± 16.5 | | |
| Week 6 | 79 ± 24.0 | 101 ± 20.3 | 144 ± 40.4* | 163 ± 45.0* | | |
| Week 12 | 68 ± 23.3 | 83 ± 19.9 | 113 ± 24.6 | 126 ± 28.7* | | |
| *Alanine aminotransferase* (IU/L) | | | | | 41 ± 8.5 | 30/60 |
| Week -1 | 31 ± 10.2 | 31 ± 9.5 | 31 ± 2.1 | 28 ± 1.7 | | |
| Week 6 | 35 ± 8.5 | 33 ± 8.7 | 48 ± 6.7 | 89 ± 16.2** | | |
| Week 12 | 36 ± 9.8 | 41 ± 10.5 | 46 ± 3.9 | 69 ± 14.5** | | |
| *Calcium* (mg/dL) | | | | | 11.1 ± 0.47 | 10.4/12.2 |
| Week -1 | 11.1 ± 0.25 | 11.1 ± 0.15 | 11.0 ± 0.28 | 11.1 ± 0.26 | | |
| Week 6 | 12.0 ± 0.30 | 12.1 ± 0.32 | 11.6 ± 0.39 | 11.7 ± 0.10 | | |
| Week 12 | 12.3 ± 0.32 | 12.0 ± 0.17 | 11.8 ± 0.35 | 11.5 ± 0.53* | | |
| *Chloride* (mEq/L) | | | | | 110 ± 1.6 | 106/113 |
| Week -1 | 108 ± 1.9 | 109 ± 1.7 | 109 ± 2.2 | 110 ± 1.0 | | |
| Week 6 | 111 ± 1.9 | 113 ± 1.0 | 115 ± 1.0** | 115 ± 1.0** | | |
| Week 12 | 111 ± 1.7 | 114 ± 2.8 | 117 ± 1.8** | 119 ± 0.6** | | |
| *Phosphorus* (mg/dL) | | | | | 6.1 ± 0.79 | 4.7/8.2 |
| Week -1 | 7.0 ± 0.45 | 6.7 ± 0.48 | 6.8 ± 1.02 | 6.7 ± 0.55 | | |
| Week 6 | 6.2 ± 0.29 | 6.4 ± 0.44 | 6.8 ± 1.94 | 7.5 ± 0.57 | | |
| Week 12 | 5.4 ± 0.13 | 5.5 ± 0.25 | 6.1 ± 1.26 | 6.8 ± 0.36* | | |
| *Potassium* (mEq/L) | | | | | 4.54 ± 0.262 | 4.18/4.96 |
| Week -1 | 4.81 ± 0.311 | 4.58 ± 0.073 | 4.62 ± 0.397 | 4.65 ± 0.368 | | |
| Week 6 | 4.39 ± 0.527 | 3.98 ± 0.208 | 3.65 ± 0.448 | 3.53 ± 0.344* | | |
| Week 12 | 4.45 ± 0.338 | 4.01 ± 0.136 | 3.95 ± 0.435 | 3.50 ± 0.150** | | |
| *Sorbitol dehydrogenase* (IU/L) | | | | | 4.6 ± 1.38 | 2.6/7.5 |
| Week -1 | 4 ± 1.2 | 5 ± 1.0 | 4 ± 0.5 | 5 ± 0.5 | | |
| Week 6 | 3 ± 0.8 | 5 ± 1.3 | 6 ± 1.5** | 6 ± 1.0* | | |
| Week 12 | 3 ± 1.3 | 5 ± 0.0* | 7 ± 1.7** | 6 ± 1.0** | | |
| Females (n = 4) | | | | | | |
| *Total protein* (g/dL) | | | | | 5.6 ± 0.30 | 5.0/6.2 |
| Week -1 | 5.5 ± 0.19 | 5.6 ± 0.17 | 5.3 ± 0.17 | 5.4 ± 0.06 | | |
| Week 6 | 5.6 ± 0.13 | 5.6 ± 0.17 | 5.3 ± 0.38 | 5.6 ± 0.25 | | |
| Week 12 | 5.6 ± 0.15 | 5.6 ± 0.06 | 5.2 ± 0.24* | 5.2 ± 0.23 | | |
| *Alkaline phosphatase* (IU/L) | | | | | 73 ± 20.6 | 40/135 |
| Week -1 | 90 ± 4.8 | 76 ± 24.1 | 103 ± 17.1 | 88 ± 17.7 | | |
| Week 6 | 80 ± 9.0 | 91 ± 31.2 | 145 ± 32.4* | 148 ± 24.3* | | |
| Week 12 | 67 ± 9.0 | 76 ± 31.0 | 120 ± 23.2* | 121 ± 13.1* | | |
| *Chloride* (mEq/L) | | | | | 111 ± 1.5 | 108/114 |
| Week -1 | 109 ± 1.3 | 109 ± 0.8 | 109 ± 1.0 | 110 ± 1.3 | | |
| Week 6 | 112 ± 1.0 | 112 ± 0.8 | 115 ± 1.0** | 115 ± 0.6** | | |
| Week 12 | 112 ± 1.0 | 114 ± 1.0 | 118 ± 1.5** | 117 ± 0.6** | | |
| *Phosphorus* (mg/dL) | | | | | 6.0 ± 0.75 | 4.8/7.8 |
| Week -1 | 6.9 ± 0.28 | 6.9 ± 0.93 | 6.8 ± 0.80 | 6.7 ± 0.88 | | |
| Week 6 | 5.9 ± 0.13 | 6.3 ± 1.02 | 7.4 ± 0.71* | 7.6 ± 0.45* | | |
| Week 12 | 4.9 ± 0.69 | 5.5 ± 0.65 | 6.6 ± 0.36** | 7.3 ± 0.35** | | |
| *Potassium* (mEq/L) | | | | | 4.53 ± 0.255 | 4.11/4.97 |
| Week -1 | 4.71 ± 0.314 | 4.71 ± 0.280 | 4.65 ± 0.386 | 4.69 ± 0.283 | | |
| Week 6 | 4.42 ± 0.303 | 3.79 ± 0.124* | 3.61 ± 0.264** | 3.95 ± 0.443 | | |
| Week 12 | 4.31 ± 0.253 | 4.02 ± 0.184 | 3.39 ± 0.112** | 3.68 ± 0.215** | | |

SD, standard deviation; HC, Historical Control; Min/Max, minimum and maximum of study means.
*Significantly different from control group at 0.05 using Dunnett's test.
**Significantly different from control group at 0.01 using Dunnett's test.

(Brockus and Andreason, 2003). The RDW changes were not associated with other erythrocyte findings in males and were not considered adverse. The higher APTT and PT (females only) values noted at study weeks 6 and 12 were minimal, were not associated with clinical observations of coagulation or bleeding abnormalities, and were not considered adverse. With few exceptions, the observed changes in hematology and coagulation parameters were within the laboratory's historical control range for Ridglan Beagle dogs aged 28 to 50 weeks (see Table 2).

Some additional significant differences were reported (Table 2) when the control and test article-treated groups were compared and included lower mean corpuscular hemoglobin concentration (MCHC) values in mid- and high-dose males and females at study weeks 6 and 12, lower absolute basophil and large unstained cell (LUC) counts in mid- and high-dose males at study week 6, higher eosinophil count in high-dose males at week 12, higher mean corpuscular volume (MCV) in mid-dose females at week 12, and lower hemoglobin distribution width in mid-dose females at week 6. These changes were small, similar to pre-test values (week -1), within historical control ranges, not dose-dependent, not significantly different from corresponding controls, not seen in both sexes and/or not associated with any gross or histopathological findings at necropsy, and therefore not considered test article-related.

### 3.6.2. Serum chemistry

Non-adverse but significant, test article-related effects on serum chemistry parameters included higher alkaline phosphatase (ALP) and chloride values in mid- and high-dose males and females at weeks 6 and 12, higher alanine aminotransferase (ALT) values in high-dose males at week 12, and higher sorbitol dehydrogenase (SDH) values in mid- and high-dose males at weeks 6 and 12 (Table 3).

The higher mean ALP, ALT, and SDH values were not associated with liver weight or microscopic liver changes. ALP and ALT values at study week 12 were generally decreased from study week 6 values in males and females (ALP only) and were similar to or within historical control ranges for Ridglan Beagle dogs aged 28 to 50 weeks (Table 3). Minimal changes in liver-related serum enzyme values have been associated with hepatocellular metabolic enzyme induction (Amacher et al., 2001).

Significantly higher mean chloride values were noted in the mid- and high-dose males and females at study weeks 6 and 12, while lower (inconsistently significant) mean potassium values were noted in mid- and/or high-dose males and females at study weeks 6 and/or 12 (Table 3). Mean serum chloride values at study week 12 increased from study week 6 values in the mid- and high-dose males and females. The study week 12 mean potassium value in the low-dose females was increased from the study week 6 value, while mean potassium values in the mid- and high-dose females had decreased at study week 12. A similar trend for mean potassium values was not noted in the males. Chloride changes were observed without corresponding changes in sodium values.

Some additional significant differences, which were not considered test article-related, were reported when the control and test article treated groups were compared and included lower mean calcium values in high-dose males at study week 12, higher mean phosphorus values in high-dose males at study week 12 and in mid- and high-dose females at study weeks 6 and 12, and higher mean SDH value in low-dose males at study week 12 (Table 3). These differences were not considered test article-related as the values were similar or slightly higher than the pretest values and/or the concurrent control group values were lower than pretest values. Additionally, the values at weeks 6 and 12 were within the laboratory's historical control ranges for Ridglan Beagle dogs aged 28 to 50 weeks (see Table 3).

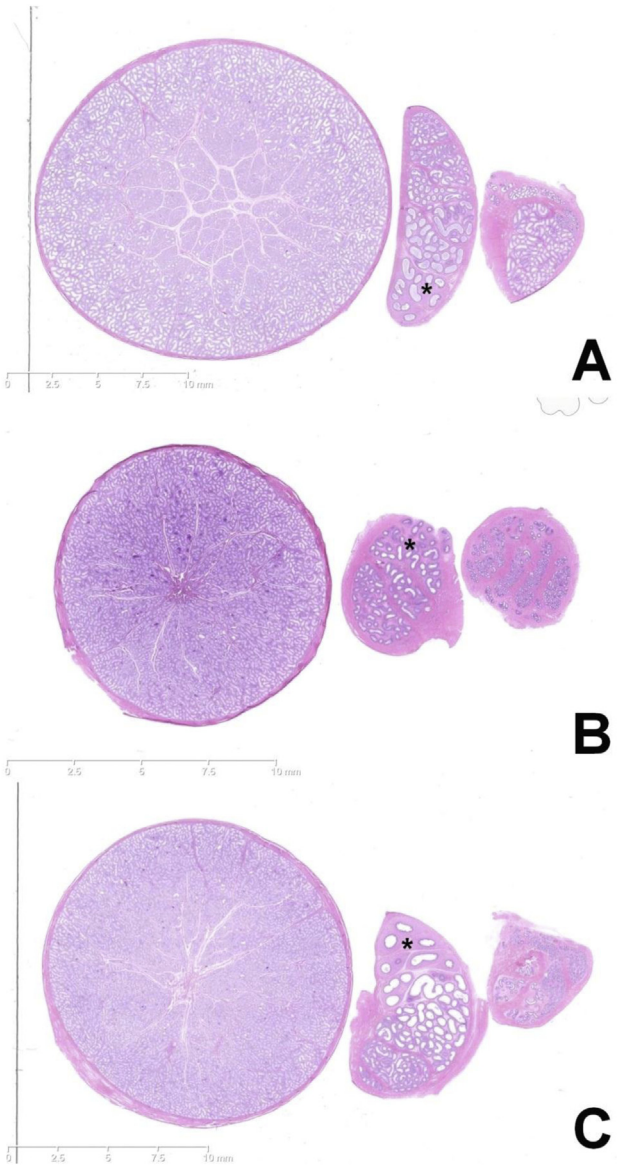


**Fig. 4.** Testes and epididymides from dogs that received 0 (panel A) or 35,000 (panels B and C) ppm *R,R*-monatin. The testes in dogs that received 35,000 ppm *R,R*-monatin were smaller and the epididymides (*) did not contain sperm.

### 3.6.3. Urinalysis

With the exception of significantly higher pH values in mid- and high-dose females at week 12 (pH of 6.3 ± 0.29, 6.6 ± 0.25, 7.0 ± 0.41 and 7.0 ± 0.00 for control, low-, mid-, and high-dose groups, respectively), the urinalysis parameters examined were similar to control values. The higher mean urine pH changes were not associated with microscopic kidney findings.

### 3.7. Ophthalmic examinations

No ophthalmic lesions indicative of toxicity were observed in any of the test article-treated groups. All other findings observed throughout the study were typical in prevalence and appearance for laboratory dogs of this age and strain.

### 3.8. Electrocardiographic evaluations

All the electrocardiograms were qualitatively and quantitatively interpreted and within normal limits. No test article-related effects

**Table 4**
Absolute and relative testes and epididymides weights (±SD) in male Beagle dogs (n = 4) fed *R,R*-monatin for up to 92 days.

| | 0 ppm | 5000 ppm | 20,000 ppm | 35,000 ppm |
|---|---|---|---|---|
| Testes | | | | |
| *g* | 16.34 ± 3.964 | 13.91 ± 3.719 | 10.27 ± 2.285 | 7.11 ± 3.559** |
| *g/100 g final bw* | 0.158 ± 0.0316 | 0.122 ± 0.0252 | 0.095 ± 0.0251* | 0.067 ± 0.0284** |
| *g/100 g brain* | 21.159 ± 5.2963 | 17.839 ± 5.3941 | 12.618 ± 2.3648 | 9.240 ± 4.5454** |
| Epididymides | | | | |
| *g* | 2.31 ± 0.587 | 2.65 ± 0.380 | 2.30 ± 0.577 | 1.49 ± 0.530 |
| *g/100 g final bw* | 0.022 ± 0.0057 | 0.024 ± 0.0047 | 0.021 ± 0.0031 | 0.014 ± 0.0035 |
| *g/100 g brain* | 2.982 ± 0.7900 | 3.386 ± 0.6147 | 2.827 ± 0.6191 | 1.934 ± 0.6454 |

SD = standard deviation, bw = body weight.
*Significantly different from control group at 0.05 using Dunnett's test.
**Significantly different from control group at 0.01 using Dunnett's test.

**Table 5**
Incidence of selected histopathological findings of male Beagle dogs (n = 4) fed *R,R*-monatin for up to 92 days.

| | 0 ppm | 5000 ppm | 20,000 ppm | 35,000 ppm |
|---|---|---|---|---|
| | Number of animals | | | |
| **Epididymides** | | | | |
| Reduced sperm, luminal | 0 | 1 | 1 | 4 |
| Mild | – | 1 | 0 | 0 |
| Moderate | – | 0 | 1 | 0 |
| Severe | – | 0 | 0 | 4 |
| **Testes** | | | | |
| Germ cell hypocellularity | 0 | 1 | 1 | 4 |
| Minimal | – | 0 | 1 | 0 |
| Mild | – | 1 | 0 | 1 |
| Moderate | – | 0 | 0 | 2 |
| Severe | – | 0 | 0 | 1 |

on heart rate, RR, PR, QRS, QT or QTcV intervals were found at any dose level based on comparison of pre-dose and post-dose group mean values and control group values. No arrhythmias were found.

### 3.9. Macroscopic examination and organ weights

Smaller testes were observed in mid- and high-dose males and were associated with test article-related microscopic findings (Fig. 4, see Section 3.10).

Necropsy of the high-dose female euthanized *in extremis* on study day 17, revealed red fluid in the thoracic cavity, dark red areas and discoloration of firm left lung lobes, dark red thymus, and dark red or reddened areas at the ileo-colic junction and the large intestine. The lung lesions resulted from bacterial infection. The remaining inflammation and hemorrhage of organs in the thoracic cavity (aorta, thymus, and heart) extended from the primary lung lesion. The cause of death was necrotizing inflammation in the lungs and subacute inflammation of the heart, which were not attributed to treatment with the test article. These findings likely represented a pre-existing condition as they were limited to this single animal and not reported in any other animal surviving to the scheduled necropsy.

Organ weights and organ weight ratios were similar between test article-treated and control animals with the exception of significantly lower mean absolute testes weights and mean testes weights relative-to-body and brain weights in high-dose males (Table 4). Mean testes weights relative-to-body weights were also significantly lower in mid-dose males. These lower mean testes weights were associated with test article-related changes, which were observed microscopically in the 5,000, 20,000 and 35,000 ppm group males (see Section 3.10). Lower, but not statistically significant, epididymides weights (Table 4) were noted in high-dose males and associated with test article-related microscopic changes (see Section 3.10).

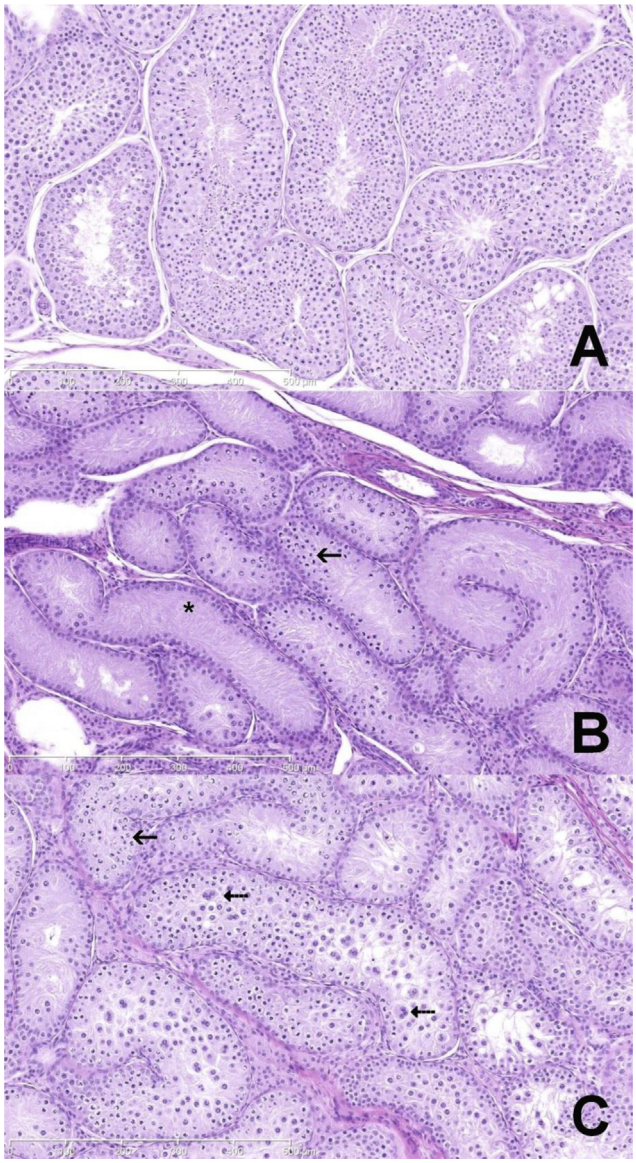


**Fig. 5.** Higher magnification (10×) of seminiferous tubules in testes of dogs that received 0 (panel A) or 35,000 (panels B and C) ppm *R,R*-monatin. Seminiferous tubules were fully populated with spermatocytes and elongated spermatids. Dogs that received 35,000 ppm *R,R*-monatin had germ cell hypocellularity (more severe in panel B) with many tubules only lined by Sertoli cells (*). There was cellular debris and degeneration (solid arrows) and/or syncytial cells (dotted arrows) in the majority of seminiferous tubules.

### 3.10. Microscopic examination

Testicular and epididymal changes reported in males are shown in Table 5. One low-dose male, one mid-dose male, and all high-dose males had mild to severe reduced luminal sperm in the epididymides characterized by fewer mature spermatozoa and cell debris (Fig. 5). There was minimal to severe germ cell hypocellularity in the testes in the low- (1 male), mid- (1 male), and high-dose (all males) groups. Germ cell hypocellularity was characterized by fewer germ cells in the seminiferous tubules with germ cell sloughing, syncytial giant cells, and/or sperm stasis (stasis only observed in the low-dose male). Germ cell hypocellularity was noted in all stages of seminiferous tubules.

The degree of germ cell hypocellularity with associated epididymal changes was more pronounced at higher dietary concentrations of the test article. While some of the testicular and epididymal changes can be observed in sexually immature dogs (see Discussion below), the observation of germ cell sloughing and the dose-related occurrence of germ cell hypocellularity, coupled with the macroscopic and organ weight findings was considered consistent with a test article-related effect. Further, testicular and epididymal findings were considered adverse in the 5,000, 20,000, and 35,000 ppm groups.

In one high-dose female, there was mild, focally extensive subcapsular atrophy of hepatocytes with bile ducts remaining. This correlated with a grossly visible small left liver lobe. This was not considered test article-related given the isolated nature of the finding.

All other histological changes were considered incidental findings or related to some aspect of experimental manipulation, rather than administration of the test article. There was no test article-related alteration in the prevalence, severity, or histological character of those incidental tissue alterations.

## 4. Discussion and conclusions

The objective of this study was to evaluate the potential systemic toxicity of *R,R*-monatin when administered *via* the diet for 90 days to male and female Beagle dogs at concentrations of 5000, 20,000, and 35,000 ppm, respectively. With the exception of one female at 35,000 ppm (see Section 3.9), all dogs survived to the scheduled necropsy. There were no effects on body weights, food consumption, FOB observations, ECG readings, or ophthalmic examinations. No adverse hematology, coagulation, serum chemistry, or urinalysis changes were reported. Conversely, lower (as compared to control) mean absolute and relative (to brain and body weight) testes weights at 35,000 ppm (statistically significant at $p < 0.01$) and lower mean relative (to body weight) testes weight at 20,000 ppm (statistically significant at $p < 0.05$) associated with immature testes (germ cell hypocellularity and hypospermatogenesis) in four males at 35,000 ppm and one male at 20,000 ppm were reported. These observations (germ cell hypocellularity and hypospermatogenesis of the testes) were not noted for control group males. One male at 5000 ppm was also noted with immature testes; however, mean absolute and relative (to brain and body weight) testes weights were similar to the controls. Overall, the exposure to R,R-monatin was well tolerated at all dose levels and did not result in overt systemic toxicity; however, dose-related adverse effects were observed for the testes in the form of lower mean absolute and/or relative organ weights at necropsy and histopathological findings.

Spontaneous and age-related testicular findings such as hypospermatogenesis, atrophy/hypoplasia of seminiferous tubules, cellular debris, retained spermatids, syncytial cells and/or apoptotic cells are normally seen in young Beagle dogs and can complicate the interpretation of studies (Creasy, 2012; Foley, 2001; Goedken et al., 2008; James and Heywood, 1979; Rehm, 2000). These age-related findings are typically segmental; often occurring in low numbers of randomly scattered groups of tubules and are usually of low severity (Rehm, 2000). Thus, familiarity with testicular maturation, normal and abnormal stages of spermatogenesis, and recognition of cellular degeneration during testes development are important criteria for the interpretation of toxicity studies utilizing dogs as the test species (Creasy, 1997; Foley, 2001; Goedken et al., 2008; James and Heywood, 1979).

Although the FDA Redbook states that “Testing should begin when dogs are no older than 4 to 6 months of age” (FDA, 2000) and OECD testing guideline similarly states “in the case of the dog, dosing should begin preferably at 4 to 6 months and not later than 9 months of age” (OECD, 1998), it is acknowledged that sexual maturity in male Beagle dogs occurs at 9 to 10 months of age, while dogs 4 to 6 months of age are sexually immature. Therefore, adhering to FDA Redbook and OECD testing guidelines for sub-chronic oral toxicity evaluation of non-rodents means *de facto* that the males are likely not fully sexually mature at study initiation and may be at differing stages of testes development within and between the small group sizes employed. In addition, to be reasonably assured that dog testes will be fully developed at the time of termination in a 90-day exposure study, it is advised that dogs be 10 to 12 months of age at termination (Lanning et al., 2002). In this study, the dogs were approximately 6 to 7 months of age at initiation of dose administration and about 9 to 10 months of age at termination. Consequently, it is very possible that some dogs in this study were still either immature or peripubertal at termination. If so, then the histological appearance of the immature testes and epididymides may be indistinguishable from test article-induced toxicity, and assessing the relationship of such changes to *R,R*-monatin may have been impacted by this confounding factor, particularly with a total study population of 16 animals and a small group size of four animals/sex/dose. While control group males did not exhibit the germ cell hypocellularity and hypospermatogenesis that was observed in the treated groups, it is possible that the male dogs in the control group were more sexually mature than test article-treated males due to the small study population and high degree of individual animal variability in development of the testes. At the age the dogs were terminated, some presented incomplete spermatogenesis and no epididymal sperm, whereas others just started producing sperm with some sperm present in the epididymis; a few others produced substantial amounts of sperm. This confounding factor for data interpretation may be circumvented by ensuring that dogs are at least 10 months of age, preferably 12 months of age at termination for this type of study, rather than 9 to 10 months of age (Goedken et al., 2008).

Investigation of possible mechanisms for the testicular findings seen in this study would include determination of whether the hypothalamic-pituitary-gonadal axis or the testes were involved by establishing whether testosterone was decreased or blocked relative to Sertoli cell histopathological changes (Boekelheide, 2011; Creasy, 1997, 2001). However, these endpoints are not routinely investigated under the current dog (non-rodent) 90-day sub-chronic toxicity study design per the FDA Redbook or OECD. Both of these mechanisms typically result in an effect on certain stages of development for seminiferous tubules, which was not observed in the present study.

In conclusion, immature testes were observed at termination in all *R,R*-monatin-treated groups, especially at 35,000 ppm. Due to the young age of the dogs at study initiation and termination, and the small group sizes recommended under FDA and OECD testing guidelines, it is difficult to definitively conclude whether the higher frequency of immature testes observed in the high-dose group

compared to the control group is strictly related to exposure to the test article. Alternatively or additionally, the findings may be possibly a function of the use of young dogs, the accompanying variability in individual male sexual development and/or statistical probability. This study illustrates the possibility that the use of younger, still sexually maturing dogs in repeat-dose oral toxicity studies, and a limited number of dogs *per* group, has the potential to confound the interpretation of the study results due to the high likelihood of spontaneous and age-related testicular findings such as hypospermatogenesis, which has a background frequency of about 33% observed in normal young dogs (Creasy, 2011).

Nevertheless, the current study data support a conclusion, based on adverse testicular and epididymal findings for treated males, of a no-observed-adverse-effect level (NOAEL) of 35,000 ppm for females (equivalent to an exposure level of 1101 mg/kg bw/day) and <5000 ppm for males (equivalent to an exposure level of <151 mg/kg bw/day).

## Declaration of conflicting interests

Authors Crincoli and Brathwaite are employees of Cargill, the funding sponsor of this study. Author Eapen is currently an employee of Cargill, but was an employee of WIL Research at the time of study conduct. Authors Rihner and Nikiforov are consultants to Cargill, the study sponsor. Authors Greeley and Harris have no competing interests to declare.

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## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2015.08.020.

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