2011

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Integrative Zoology 2011; 6: 409-419 doi: 10.1111/j.1749-4877.2011.00247.x

Feeding of grey squirrels (*Sciurus carolinensis*) with the contraceptive agent DiazaCon™: effect on cholesterol, hematology, and blood chemistry

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Abstract

Grey squirrels (*Sciurus carolinensis*) are an invasive species in Britain and Italy. They have replaced native red squirrels (*Sciurus vulgaris*) throughout most of Britain, and cause damage to trees. Currently, lethal control is used to manage grey squirrel populations in Britain, but nonlethal methods might be more acceptable to the public. One such method is contraception with 20,25-diazacholesterol dihydrochloride (DiazaCon™). DiazaCon™ inhibits the conversion of desmosterol to cholesterol, resulting in increasing desmosterol concentrations and decreasing cholesterol concentrations. Because cholesterol is needed for the synthesis of steroid reproductive hormones, such as progesterone and testosterone, inhibition of cholesterol synthesis indirectly inhibits reproduction. Desmosterol is used as a marker of efficacy in laboratory studies with species that do not reproduce readily in captivity. Grey squirrels were gavaged with a DiazaCon™ solution for 2 days, and then fed DiazaCon™-coated peanuts for an additional 8 days at target doses of 50 and 100 mg DiazaCon™ per kg body weight. There was a significant difference in cholesterol concentrations in the treatment groups compared to the control group. Cholesterol was reduced by ≥40% for 2 months in both treatment groups. There were no differences among groups with respect to blood chemistry and hematology parameters, and mean values are reported. The mean overall dose of DiazaCon™ received was 29.0 ± 1.6 and 55.3 ± 4.3 mg/kg in the low (50 mg/kg) and high dose (100 mg/kg) groups, respectively. DiazaCon™ might provide an effective, acceptable alternative to lethal control.

Key words: 20,25-diazacholesterol dihydrochloride, cholesterol, contraception, DiazaCon™, grey squirrel.

INTRODUCTION

The grey squirrel (*Sciurus carolinensis*) was introduced to Britain and Ireland from North America in the late 1800s (Middleton 1932) and to northern Italy during the mid- to late-1900s. Grey squirrels compete with native red squirrels (*Sciurus vulgaris*) and have replaced them throughout much of Britain and Ireland (Lloyd 1983; Gurnell 1987; Gurnell & Pepper 1993, O'Teangana *et al.* 2000). Without effective control, they will potentially do the same in Italy (Currado 1998; Bertolino *et al.* 2008).

Grey squirrels evolved in the mixed oak forest of North America and are physiologically more adapted to neutralizing phytotoxins in acorns, allowing them to use

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these more efficiently (Kenward & Holm 1989, 1993). In areas where both species are present, grey squirrels raid red squirrel caches of seed, leading to reduced reproductive and juvenile recruitment rates for red squirrels (Wauters *et al.* 2002; Gurnell *et al.* 2004). Grey squirrels also carry Squirrelpox virus, which is lethal to red squirrels but has little effect on grey squirrels (Rushton *et al.* 2000; Sainsbury *et al.* 2000; Tompkins *et al.* 2002; Thomas *et al.* 2003; McInnes *et al.* 2006). Grey squirrels also cause bark stripping damage to trees (Kenward 1983; Rowe & Gill 1985; Dagnall *et al.* 1998), and this can have serious economic impacts as well as influence woodland composition (Mayle *et al.* 2009).

Despite their impact on red squirrel populations, grey squirrels are well liked by the public where there are no red squirrels. This might be because grey squirrels are often the only wild mammal seen by the public. Although lethal control methods are currently used, the public is more amenable to nonlethal control measures, such as live-trapping and contraception (Barr *et al.* 2002). Translocation is not an option in Britain because, as an introduced species, grey squirrels may not be released once caught. Contraception offers a potential nonlethal option for reducing the rate of spread of the grey squirrel, limiting the risk of Squirrelpox virus disease transmission and reducing tree damage.

Immunocontraception based upon a sperm-antigen approach has been investigated for the grey squirrel (Moore *et al.* 1997), but an effective single dose agent with a long-term effect through oral delivery is not yet available. Potential oral contraceptives with a short-term effect exist, but have not been tested on squirrels. One such contraceptive is 20,25-diazacholesterol dihydrochloride (DiazaCon™).

DiazaCon™ inhibits the conversion of desmosterol to cholesterol, increasing desmosterol concentrations and decreasing cholesterol concentrations (Yoder *et al.* 2004). Because cholesterol is needed for the synthesis of steroid reproductive hormones, such as progesterone and testosterone, inhibition of cholesterol synthesis indirectly inhibits reproduction. Many wild species for which fertility control could provide a practical management tool do not reproduce readily in captivity; therefore, desmosterol is used as a marker of efficacy in laboratory studies with these species (Johnston *et al.* 2003). DiazaCon™ has been used successfully to inhibit reproduction in mice, rats and prairie dogs (Hikim & Chakraborty 1986; Singh & Chakravarty 2003; Nash *et al.* 2007).

The objectives of the present study were to determine the minimum dose of DiazaCon™ in grey squirrels that would sufficiently reduce plasma cholesterol with no adverse health effects. Body weight, complete blood counts and blood chemistry were used to monitor general health. We also wanted to establish baseline data on hematology and blood chemistry of grey squirrels as very little information exists in the published literature.

MATERIALS AND METHODS

The study protocol was reviewed and approved by the National Wildlife Research Center's Animal Care and Use Committee. DiazaCon™ was provided by the Avitrol Corporation (Tulsa, OK). Grey squirrels (48) were imported from Oklahoma. Squirrels were individually housed indoors in cages equipped with a 45 cm length of PVC pipe capped on one end for use as a nest cavity. The light cycle throughout the study was 14 h of daylight and 10 h of darkness. Squirrels were maintained on a maintenance diet of nuts (peanuts, almonds, pecans and hazelnuts), cracked corn and fruit throughout the study, except where otherwise noted, with water freely available.

Squirrels were ranked by weight and randomly assigned to treatment groups such that each treatment group consisted of 16 animals. Each squirrel was fitted with a fingerling ear tag with a unique identifying code. There were 3 treatment groups as follows: (1) control; (2) 50 mg DiazaCon™ per kg body weight; and (3) 100 mg DiazaCon™ per kg body weight. Squirrels were handled for all procedures by placing them in DecapiCones (Braintree Scientific).

DiazaCon™ gavage solutions were prepared such that they contained either 50 mg DiazaCon™ or 100 mg DiazaCon™ per 1 mL of water. Squirrels were then gavaged according to each animal's individual body weight such that each animal received either 50 mg/kg or 100 mg/kg DiazaCon™. Squirrels were gavaged using a straight 20 gauge 7.6 cm stainless steel feeding needle with a 2.25 mm diameter ball on the tip. All animals were gavaged on days 1 and 2 of the study. On the second day of gavaging, it became apparent that the handling of the animals was causing them significant stress. On the second day, 2 squirrels died after being handled. Therefore, peanuts coated with DiazaCon™ were used for the remaining 8 days of treatment. The 2 squirrels that died during gavaging were not replaced in the study. To allow for recovery, there were 35 days between the last day of gavaging and the first day of feed-

ing treated peanuts. It was determined that DiazaCon™ should have been mostly eliminated from the squirrels' systems by 35 days after gavage. This was based on the squirrels having only received 2 doses of DiazaCon™, cholesterol and desmosterol results from 5 and 19 days after gavaging, and prior data for mice and rats (P Nash, unpubl. data).

Peanut baits were formulated using an average squirrel weight of 470 g. Based on this, the average squirrel needs 23.5 or 47 mg DiazaCon™ daily to receive a dose of 50 or 100 mg/kg, respectively. Grey squirrels will consume 34–40 g of feed in captivity (Short & Duke 1971). Therefore, 5 g was chosen as the amount of peanuts to coat with DiazaCon™ to ensure consumption of the entire DiazaCon™ dose. Peanuts were formulated such that 5 g of peanuts contained either 23.5 or 47 mg DiazaCon™. In addition to DiazaCon™, each batch also contained 2% table sugar and 1% sticker. The sticker was prepared by mixing Alcolec-S and corn oil in a 3:1 ratio (75% Alcolec-S, 25% corn oil). The appropriate amounts of DiazaCon™ and table sugar were dissolved in water. Raw shelled peanuts were placed in a Hobart mixer, and with the mixer running, half the sticker solution was slowly poured over the peanuts and mixed for 5 min. Next, the DiazaCon™ and table sugar mixture was slowly poured over the peanuts with the mixer still running, and mixed for a further 5 min. Finally, the remainder of the sticker was slowly poured over the peanuts and mixed for 5 min. Control peanuts were coated with the sticker and sugar water only.

Each squirrel was offered treated peanuts for 8 consecutive days. With the exception of the third day, all squirrels received 5 g of peanuts daily. On the third treatment day, the squirrels in the 100 mg/kg group inadvertently received 10 g of peanuts. Cages and nest cavities were checked for cached food, and all food was removed from each cage prior to offering the treated peanuts. Treated peanuts were offered for 8 h, and no other food was available during this time. At the end of 8 h, any remaining peanuts were removed from the cage and the maintenance diet was offered. Any peanuts remaining were weighed to determine food consumption for each squirrel.

Blood samples (3 mL) were taken from either the femoral or saphenous vein once prior to treatment, and 5 and 19 days after the last gavage. In addition, blood samples were taken 5, 11, 18, 25, 42, 62, 83 and 95 days after the last day of feeding treated peanuts. It was important to follow desmosterol and cholesterol concentrations weekly for the first month post-treatment. One month is the minimum time desired for DiazaCon™ to be effective in the field. Blood samples collected after the first month were more spread out because all that was needed was to determine how many months DiazaCon™ could still be detected in the blood. Cholesterol concentrations were monitored for 3 months post-treatment because 3 months is the desired length of treatment in the field as this will provide contraception for 1 breeding season. Blood was collected into heparanized tubes for blood chemistry and EDTA tubes for hematology, cholesterol and desmosterol analysis. Hematology was immediately performed using the Abaxis HMT hematology analyzer. Only blood samples from 5 days post-gavage were analyzed for hematology because the machine was being repaired the day that the pretreatment samples were taken. The remaining samples were centrifuged at 13 000 rpm for 3 min. The plasma was removed and stored at –70 °C until analysis. Plasma cholesterol (non-esterified) and desmosterol concentrations were determined using high performance liquid chromatography (Johnston *et al.* 2003). Blood chemistry was performed using the Abaxis VetScan blood chemistry analyzer. Only blood samples from the pretreatment day and 5 days post-gavage were analyzed for blood chemistry. This is because acute effects from DiazaCon™ should have been most apparent immediately following treatment. Squirrels were weighed at each blood collection.

We compared plasma cholesterol and desmosterol concentrations, blood chemistry, body weights and peanut bait consumption among treatment groups using mixed model analysis (PROC MIXED; SAS Institute). Squirrels were treated as random effects, and treatments as fixed effects. We performed mean separations with PDMIX800 (Saxton 1998). Hematology parameters were analyzed using a general linear model with fixed effects (PROC GLM; SAS Institute), and means were separated using the least significant difference. The mean DiazaCon™ dose during the peanut phase was calculated by determining the exact dose for each animal each day, and averaging the daily dose across each treatment group (PROC MEANS; SAS Institute).

RESULTS

There were significant group, treatment day and interaction effects for both cholesterol and desmosterol ($P < 0.0001$ for all). However, mean cholesterol and desmosterol did not differ between the 50 mg/kg and 100 mg/kg groups (Figs 1 and 2). Mean cholesterol concentrations on day

11 after feeding peanuts were 35.2 ± 10.3 and 25.7 ± 10.9 μg/mL (mean ± standard mean error [SEM]) in the 50 mg/kg (n = 15) and 100 mg/kg (n = 13) groups, respectively, compared to 176.9 ± 10.2 μg/mL in the control group (n = 15). Mean desmosterol concentrations on day 11 after feeding peanuts were 204.1 ± 9.7 and 202.1 ± 10.3 μg/mL in the 50 mg/kg (n = 15) and 100 mg/kg (n = 13) groups, respectively, compared to 11.8 ± 9.7 μg/mL in the control group (n = 15). Mean cholesterol concentrations on day 19 after gavaging were 181.3 ± 10.0, 96.7 ± 10.3 and 85.1 ± 10.6 μg/mL in the control (n = 16), 50 (n = 15) and 100 mg/kg (n = 14) groups, respectively. Mean desmosterol concentrations on day 19 after gavaging were 5.9 ± 9.5, 155.2 ± 9.7 and 136.6 ± 10.0 μg/mL in the control (n = 16), 50 mg/kg (n = 15) and 100 mg/kg (n = 14) groups, respectively. The method limit of detection (mean ± SEM) was 3.7 ± 0.2 μg/mL (n = 11; range 2.5 to 4.9) and 2.1 ± 0.1 μg/mL (n = 11; range 1.3 to 2.8) for cholesterol and desmosterol, respectively. Percent recovery (mean ± SEM) was 94.6 ± 1.2% (n = 44; range 73 to 115) and 91.6 ± 0.7% (n = 44; range 82.4 to 104) for cholesterol and desmosterol, respectively.

The highest dose received by an individual squirrel on any one particular day was 180 mg/kg. The actual doses received by feeding peanuts were 30 and 55 mg/kg, approximately half the intended target dose. Even when just the data from 11 days post-peanut feeding and onwards were analyzed for group differences, there was no significant difference between the treatment groups in plasma cholesterol and desmosterol concentrations.

There were no significant differences among groups in any of the blood chemistry parameters (Tables 1 and 2), nor were there differences between treatment days in hematology (Table 3). Body weights did not differ among groups (P = 0.8720), but did differ among treatment days (P < 0.0001; Table 4). Overall, body weights tended to increase during the course of the study.

Peanut consumption varied among groups and treatment days, and there was a significant interaction effect (P < 0.0001 for all; Fig. 3). However, mean peanut consumption did not differ between the 50 mg/kg and 100 mg/kg groups. Peanut consumption was 3.2 ± 0.19 g (mean ± SEM) and 3.04 ± 0.20 g in the 50 mg/kg (n = 120) and 100 mg/kg (n = 108) groups, respectively, compared to 4.3 ± 0.19 g in the control group (n = 120). There was a significant decrease in intake on day 4 in the high dose group (Fig. 3). Of 14 animals, 6 ate ≤ 0.6 g, 1 ate 1.9 g, and 7 ate ≥ 4.2 g of peanuts that day. Consumption increased gradually after this to levels similar to the control group by the end of the study. Consumption on day 8 of feeding was 4.9 ± 0.3, 3.9 ± 0.3 and 4.6 ± 0.3 in the control, 50 mg/kg, and 100 mg/kg groups, respectively. The mean overall dose of DiazaCon™ received on treated peanuts was 29.0 ± 1.6 and 55.3 ± 4.3 mg/kg (mean ± SEM) in the 50 mg/kg (n = 120) and 100 mg/kg (n = 108) groups, respectively.

DISCUSSION

To reduce reproduction, plasma cholesterol concentrations must be lowered by approximately 40% (Yoder *et al.* 2005). DiazaCon™ reduced cholesterol by greater than 40% for 2 months in both treatment groups. Results were confounded by the need to use 2 different delivery methods. However, it is apparent that there is a large margin of safety in grey squirrels.

Squirrels in the high dose group significantly decreased their intake of peanuts on day 4 (Fig. 3). Squirrels in the high dose group had inadvertently been fed 10 g of treated peanuts instead of 5 g on day 3, but ate only half of this. There are 2 possible explanations for the decreased consumption observed in the high dose group on day 4. One is that the larger amount of peanuts eaten on day 3 provided squirrels with a higher dose of DiazaCon™ and they might have felt ill as a result, causing them to decrease food intake the following day. On day 3, squirrels in the high dose group consumed an average of 5.1 ± 0.7 g of peanuts. However, on days 7 and 8 of feeding, squirrels in the high dose group consumed 4.6 ± 0.1 g of peanuts on each day. The difference in the amount of DiazaCon™ between 4.6 and 5.1 g of peanuts is 5 mg. This does not seem enough to cause the squirrels to feel ill. An alternate explanation might be that the squirrels were sated on day 3 due to the additional consumption and, therefore, did not need to consume as much on day 4. Neither explanation is completely satisfactory, and although the results might be statistically significant, they are likely not biologically significant. Compared to an average total daily intake of 34–40 g of food, a decrease of approximately 1.5 g is likely to be within normal variation.

Although reproductive studies could not be conducted with grey squirrels in this setting, it is predicted that DiazaCon™ will impair reproduction when fed at rates similar to those in the present study. It might not be necessary to feed DiazaCon™ for 10 days. Adequate results have been obtained in birds with as few as 5 feedings (Yoder *et al.* 2005). DiazaCon™ does not need to be fed on consecutive days to be effective as it accumulates in the liver (Yoder *et al.* 2005; Nash *et al.* 2007). This is advantageous for field applications as the same squirrel might not eat peanut bait on consecutive days.

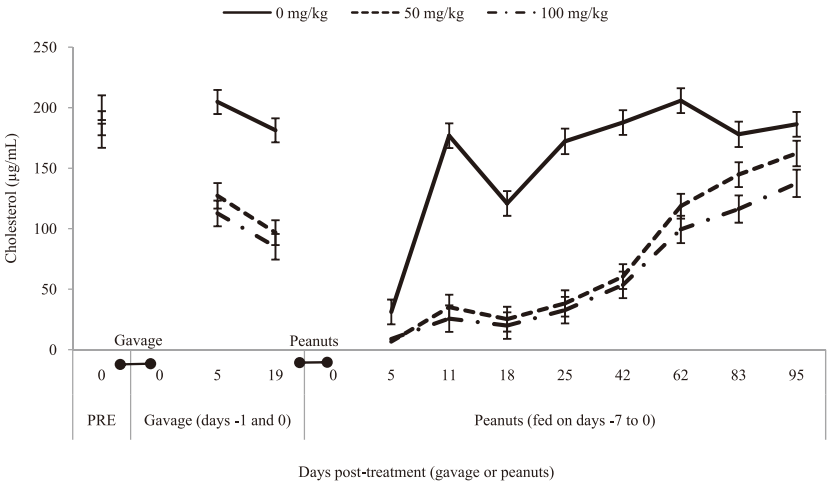

Figure 1 Non-esterified cholesterol concentrations (µg/mL ± SEM) for grey squirrels (*Sciurus carolinensis*) treated with 0 mg/kg, 50 mg/kg, or 100 mg/kg DiazaCon™.

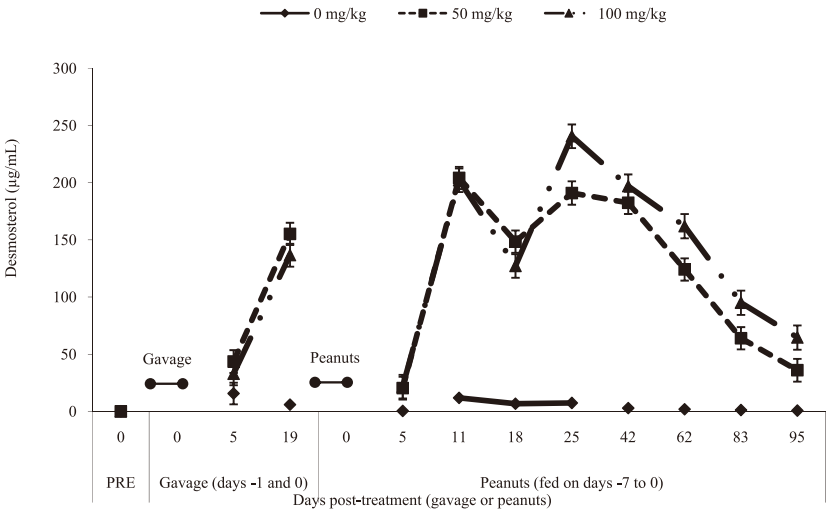

Figure 2 Desmosterol concentrations (µg/mL ± SEM) for grey squirrels (*Sciurus carolinensis*) treated with 0 mg/kg, 50 mg/kg, or 100 mg/kg DiazaCon™.

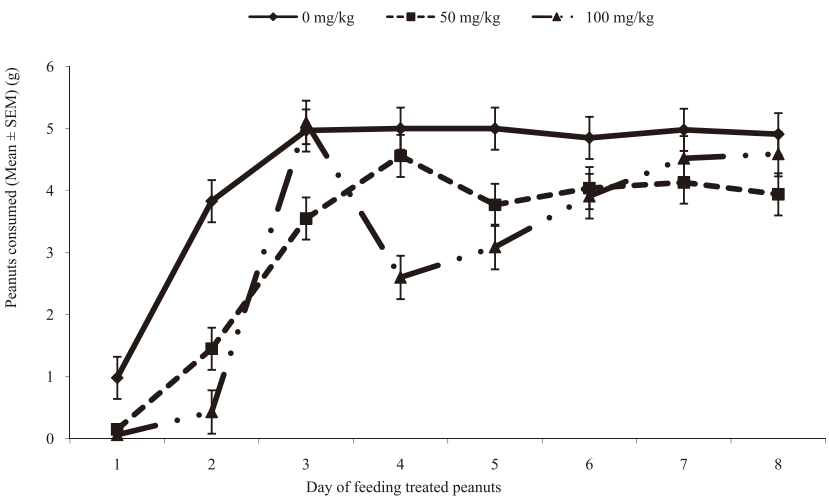

Figure 3 Peanut consumption (g) for grey squirrels (*Sciurus carolinensis*) treated fed DiazaCon™-coated peanuts (0 mg/kg, 50 mg/kg, or 100 mg/kg DiazaCon™).

Table 1 Blood chemistry parameters (mean ± SEM) for grey squirrels (*Sciurus carolinensis*) treated with 0 mg/kg, 50 mg/kg or 100 mg/kg DiazaCon™

	0 mg/kg (control)		50 mg/kg		100 mg/kg		
Parameter (units)	PRE	PG5d	PRE	PG5d	PRE	PG5d	*P*
Albumin (g/dL)	2.6 ± 0.1	2.7 ± 0.1	2.8	2.6 ± 0.1	2.6 ± 0.1	2.5 ± 0.1	0.7582
Alkaline phosphatase (U/L)†	330.3 ± 83.7	284.0 ± 42.9	300.0	295.5 ± 29.3	337.7 ± 86.2	275.6 ± 49.1	0.2542
Alanine aminotransferase (U/L)	7.3 ± 1.9	7.4 ± 0.8	4.5	6.4 ± 0.7	6.0 ± 1.5	5.5 ± 0.7	0.7893
Amylase (U/L)	2070 ± 255	2900 ± 274	2553	2503 ± 181	2549 ± 328	2495 ± 110	0.9344
Total bilirubin (mg/dL)	0.4 ± 0.0	0.4 ± 0.0	0.3	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.7754
Blood urea nitrogen (mg/dL)	12.7 ± 2.7	17.4 ± 2.0	6.0	18.5 ± 1.6	16.3 ± 0.7	19.6 ± 1.6	0.1142
Calcium (mg/dL)	9.2 ± 0.1	9.8 ± 0.2	9.9	9.6 ± 0.3	10.1 ± 0.3	9.7 ± 0.3	0.1634
Phosphorus (mg/dL)	7.5 ± 0.1	6.1 ± 0.3	6.9	5.8 ± 0.2	7.4 ± 0.4	6.8 ± 0.4	0.1892
Creatinine (mg/dL)	0.6 ± 0.1	0.6 ± 0.0	0.6	0.6 ± 0.0	0.7 ± 0.1	0.6 ± 0.0	0.1427
Glucose (mg/dL)	130.7 ± 26.9	159.7 ± 6.1	131.0	144.2 ± 10.2	121.0 ± 4.0	133.0 ± 10.0	0.5778
Sodium (mmol/L)	148.7 ± 1.9	154.7 ± 2.0	151.0	151.7 ± 1.6	154.3 ± 3.5	152.1 ± 1.4	0.1130
Potassium (mmol/L)	7.3 ± 0.8	6.9 ± 0.3	9.0	6.7 ± 0.3	7.4 ± 0.9	6.9 ± 0.5	0.6237
Total protein (g/dL)	7.2 ± 0.4	8.1 ± 0.1	8.7	7.8 ± 0.2	7.7 ± 0.2	7.8 ± 0.2	0.1215
Globulin (g/dL)	4.7 ± 0.4	5.4 ± 0.2	5.9	5.2 ± 0.2	5.0 ± 0.2	5.2 ± 0.2	0.1618

Sample sizes were 3 squirrels pretreatment and 7 squirrels 5 days post-gavage in the 0 mg/kg and the 100 mg/kg groups. In the 50 mg/kg group, sample sizes were 1 squirrel pretreatment and 11 squirrels 5 days post-gavage. †The data for alkaline phosphatase contained outliers; therefore, these values are for the data with the outliers removed. The data containing the outliers had a mean of 380.8 ± 127.0 U/L during pretreatment and 398.4 ± 66.1 U/L 5 days post-gavage in the 0 mg/kg group. In the 50 mg/kg group, the pretreatment value was 300.0 U/L (n = 1), and the mean 5 days post-gavage was 295.5 ± 29.3 U/L. Data containing outliers in the 100 mg/kg group had a mean of 274.5 ± 87.8 U/L during pretreatment and 296.1 ± 59.6 U/L 5 days post-gavage. PRE, pretreatment; PG5d = sample taken 5 days post-gavage.

Table 2 Ranges of blood chemistry parameters (mean ± SEM) for grey squirrels (*Sciurus carolinensis*) treated with 0 mg/kg, 50 mg/kg or 100 mg/kg DiazaCon™

	0 mg/kg (control)		50 mg/kg		100 mg/kg	
Parameter (units)	PRE	PG5d	PRE	PG5d	PRE	PG5d
Albumin (g/dL)	2.4–2.7	2.4–3.1	NA †	2.0–3.0	2.5–2.8	2.1–2.9
Alkaline phosphatase (U/L)†	192.0–481.0	159.0–509.0	NA	185.0–502.0	245.0–510.0	130.0–481.0
Alanine aminotransferase (U/L)	5.0–11.0	4.5–9.0	NA	4.5–10.0	4.5–9.0	4.5–9.0
Amylase (U/L)	1616–2498	1933–4000	NA	1355–3303	1915–3012	2105–2906
Total bilirubin (mg/dL)	0.3–0.4	0.3–0.4	NA	0.3–0.4	0.3–.04	0.3–0.4
Blood urea nitrogen (mg/dL)	9.0–18.0	12.0–28.0	NA	8.0–26.0	15.0–17.0	13.0–25.0
Calcium (mg/dL)	9.1–9.4	9.0–10.4	NA	8.5–11.5	9.5–10.5	8.7–10.9
Phosphorus (mg/dL)	7.3–7.8	4.9–7.1	NA	4.6–7.3	6.8–8.3	5.6–8.8
Creatinine (mg/dL)	0.5–0.7	0.5–0.7	NA	0.5–0.8	0.5–0.8	0.5–0.8
Glucose (mg/dL)	94.0–183.0	141.0–181.0	NA	101.0–198.0	113.0–126.0	90.0–161.0
Sodium (mmol/L)	145.0–151.0	145.0–159.0	NA	143.0–159.0	148.0–160.0	148.0–158.0
Potassium (mmol/L)	6.4–9.0	5.4–8.3	NA	5.7–8.4	5.9–9.0	5.3–9.0
Total protein (g/dL)	6.7–8.1	7.6–8.7	NA	6.7–8.6	7.3–8.1	7.1–8.6
Globulin (g/dL)	4.3–5.4	4.9–6.3	NA	3.9–6.3	4.7–5.5	4.5–6.0

Sample sizes were 3 squirrels pretreatment and 7 squirrels 5 days post-gavage in the 0 mg/kg and the 100 mg/kg groups. In the 50 mg/kg group, sample sizes were 1 squirrel pretreatment and 11 squirrels 5 days post-gavage. †The data for alkaline phosphatase contained outliers, therefore these values are for the data with the outliers removed. The data containing the outliers had a mean of 380.8 ± 127.0 U/L during pretreatment and 398.4 ± 66.1 U/L 5 days post-gavage in the 0 mg/kg group. In the 50 mg/kg group, the pretreatment value was 300.0 U/L (n = 1), and the mean 5 days post-gavage was 295.5 ± 29.3 U/L. Data containing outliers in the 100 mg/kg group had a mean of 274.5 ± 87.8 U/L during pretreatment and 296.1 ± 59.6 U/L 5 days post-gavage. PRE, pretreatment; PG5d, sample taken 5 days post-gavage; NA, not applicable because the sample size was 1 animal during pretreatment. This was due to not having a large enough plasma sample to run all tests.

Table 3 Hematology parameters 5 days post-gavage for grey squirrels (*Sciurus carolinensis*) treated with 0 mg/kg, 50 mg/kg or 100 mg/kg DiazaCon™

	0 mg/kg (control)		50 mg/kg		100 mg/kg		
Parameter (units)	Mean ± SEM	Range	Mean ± SEM	Range	Mean ± SEM	Range	*P*
White blood cells (m/mm^3)	7.20 ± 0.61	3.47–9.61	8.13 ± 0.81	3.13–14.91	9.32 ± 0.97	5.22–15.09	0.0691
Lymphocytes (%)	24.8 ± 3.2	11.7–43.5	25.7 ± 4.1	9.5–66.7	25.1 ± 3.3	9.5–51.0	0.8525
Monocytes (%)	2.1 ± 0.2	1.2–3.0	1.9 ± 0.2	0.7–3.7	2.9 ± 0.5	1.2–7.5	0.1676
Granulocytes (%)	73.1 ± 3.3	54.3–85.3	72.4 ± 4.1	31.3–89.1	72.0 ± 3.3	47.0–88.5	0.9096
Red blood cells (m/mm^3)	7.59 ± 0.26	6.27–9.06	7.56 ± 0.20	5.97–8.70	7.31 ± 0.15	6.54–8.31	0.5716
Hematocrit (%)	52.8 ± 1.6	43.1–61.4	51.4 ± 1.7	35.6–57.8	50.0 ± 1.4	42.1–57.4	0.4010
Hemoglobin (g/dL)	15.3 ± 0.5	13.0–18.4	15.4 ± 0.3	13.8–17.8	14.7 ± 0.3	12.9–16.6	0.3390
Platelets (m/mm^3)†	260 ± 31	124–398	265 ± 15	179–366	218 ± 14	161–312	0.4041

Sample sizes were 15, 15, and 14 squirrels for the 0 mg/kg, 50 mg/kg and 100 mg/kg groups, respectively. †The data for platelet count contained outliers; therefore, these values are for the data with the outliers removed. The data containing the outliers had a mean of 303 ± 57 m/mm^3 and a range of 54–854 m/mm^3 for the 0 mg/kg group. Data containing outliers had a mean of 289 ± 28 m/mm^3 and a range of 179–630 m/mm^3 for the 50 mg/kg group. For the 100 mg/kg group, data containing outliers had a mean of 230 ± 26 m/mm^3 and a range of 99–503 m/mm^3.

Table 4 Body weights (g; mean ± SEM) for grey squirrels (*Sciurus carolinensis*) treated with 0 mg/kg, 50 mg/kg or 100 mg/kg DiazaCon™

Treatment day	0 mg/kg	50 mg/kg	100 mg/kg
Pretreatment	461.9 ± 13.6	467.5 ± 13.6	468.8 ± 13.6
5 days post-gavage	472.8 ± 13.6	473.3 ± 13.7	467.4 ± 13.9
19 days post-gavage	487.3 ± 13.6	496.8 ± 13.7	500.6 ± 13.9
5 days post-peanuts	521.1 ± 13.7	523.3 ± 13.7	520.9 ± 13.9
11 days post-peanuts	512.4 ± 13.7	524.0 ± 13.7	517.1 ± 13.9
18 days post-peanuts	510.4 ± 13.7	521.0 ± 13.7	519.4 ± 13.9
25 days post-peanuts	501.9 ± 13.7	509.0 ± 13.7	511.1 ± 13.9
42 days post-peanuts	501.3 ± 13.7	512.8 ± 13.7	511.1 ± 13.9
62 days post-peanuts	520.1 ± 13.7	526.3 ± 13.7	548.9 ± 13.9
83 days post-peanuts	530.3 ± 13.7	535.6 ± 13.7	545.9 ± 14.0
95 days post-peanuts	525.5 ± 13.7	523.2 ± 13.8	537.4 ± 14.0

With the exception of the fifth day after feeding peanuts, the control cholesterol concentrations observed in the present study were consistent with ranges reported previously in the literature (Guthrie & Mosby 1966; Guthrie *et al.* 1967; Hoff *et al.* 1976). All groups experienced a drastic decrease in plasma cholesterol concentrations on the fifth day after feeding peanuts (Fig. 1). Plasma samples from these days were reanalyzed to confirm the values with no significant changes, indicating that this was a real physiological phenomenon. Confinement stress is associated with decreased plasma cholesterol concentrations (Guthrie *et al.* 1967). Guthrie *et al.* (1967) only observed an 8–20% decrease, which is not adequate to fully explain the decrease in our study. However, Guthrie *et al.* (1967) only exposed squirrels to acute stress rather than chronic stress, as was the case in the present study. Another possible explanation for the decrease is that caloric restriction can affect serum cholesterol concentrations and the activity of key enzymes in cholesterol synthesis. Serum free cholesterol decreased in the low density lipoprotein fraction of mice fasted for 24 h (van Ginneken *et al.* 2007). The activity of hydroxymethyl glutaryl CoA reductase (HMG-CoA reductase), a key enzyme in cholesterol synthesis, is reduced in fasting animals (Mayes 1993). Because both cholesterol and desmosterol concentrations were reduced in the squirrels, it is plausible that an enzyme in the cholesterol synthetic pathway could have been affected. At this time, there is no good explanation for these results.

DiazaCon™ treatment was not associated with any ill health effects during this study. Although 2 squirrels died on the second day of gavaging, this was due to stress rather than DiazaCon™. Overall, body weights tended to increase during the course of the study. Blood chemistry and hematology were only investigated during the initial phase of the study post-gavage; therefore, more data should be obtained in the future. In particular, it would be of value to determine these parameters during the phase when cholesterol concentrations are lowest.

Glucose, blood urea nitrogen, calcium and phosphorus values observed in this study are within the previously reported ranges for grey squirrels (Guthrie *et al.* 1967; Hoff *et al.* 1976). Total protein was slightly higher in the present study than the values observed by Hoff *et al.* (1976), but this might be attributed to different assays being used to determine total protein. Hematocrit and hemoglobin values were greater than previously reported mean values, but were within the reported ranges (Guthrie *et al.* 1967; Barker & Boonstra 2005). Percentages of lymphocytes and monocytes were close to reported values (Guthrie *et al.* 1967; Barker & Boonstra 2005). White and red blood cell counts were elevated compared to mean values reported by Barker and Boonstra (2005), but this might be due to different techniques.

Peanut consumption was low for the first 2 days in all groups, and the treated peanuts appeared to be slightly unpalatable to squirrels. This might be resolved by the use of a higher sugar concentration on the peanuts, a different masking agent, or microencapsulation of DiazaCon™. Squirrels in the 100 mg/kg group ate more on the day that they received twice as many peanuts. This indicates that the concentration of DiazaCon™ on the peanuts could be reduced, and more peanuts offered to achieve the same target dose. Grey squirrels regularly cache large food items such as peanuts and acorns in the wild (Steele & Koprowski 2001). No peanuts were cached by squirrels during the course of the study, possibly because all peanuts were shelled. However, other researchers have found caches of shelled peanuts (B Mayle, pers. comm.). We might not have observed caching in our study due to the small amount of peanuts being offered. Because these studies were conducted in the laboratory, it is unclear how big an impact caching behavior might have on treated food consumption in the field.

In Great Britain, juvenile recruitment levels are believed to be a major factor influencing the risk of bark-stripping damage. Kenward and Parish (1986) demonstrate that damage occurs when juvenile density is high (0.25 per ha). Contraception offers a potential nonlethal option for reducing the rate of spread of the grey squirrel, limiting the risk of Squirrelpox virus disease transmission, and reducing damage to woodlands. DiazaCon™ might be particularly useful for field delivery using oral baits, as animals do not need to feed on consecutive days because DiazaCon™ accumulates in the liver. However, minimum effective dose rates, nontarget risks and suitable field delivery methods will need to be investigated before DiazaCon™ can be approved for field use. A feeder is currently in use in Britain for delivery of warfarin-poisoned bait to grey squirrels. This excludes larger mammals while allowing grey, but not red squirrels or smaller mammals, to access bait (Mayle *et al.* 2007). This feeder could be used to deliver DiazaCon™ to grey squirrels and to minimize nontarget hazards. Therefore, DiazaCon™ might provide an effective alternative to lethal control where such methods are not acceptable.

ACKNOWLEDGMENTS

Thanks to K Crane, R Mauldin, J Kemp and J O'Hare for providing technical assistance. LA Miller provided comments on an earlier version of the manuscript. This study was conducted under QA-1365 study protocol.

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