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Sprague Dawley Female Rat Consumption of a Liquid Bait Containing Vinylcyclohexene Diepoxide and Triptolide Leads to Subfertility

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ABSTRACT: Worldwide, Norway rats cause significant infrastructure damage, agricultural losses, and carry zoonotic diseases. Due to rat fecundity, killing them by mechanical and/or poison does not lead to sustainable management of their populations. Our biotechnology company, SenesTech, Inc, has developed an environmentally safe oral bait taken by rats, causing them to produce fewer rat pups and take longer to deliver, without any observable adverse effects. The fertility control bait is an emulsion and contains two chemicals that cause ovarian follicle elimination; vinylcyclohexene diepoxide (VCD) targets the finite pool of primordial/primary follicles, and triptolide (T) targets growing follicles. Female Sprague Dawley rats were 33 days old when provided bait with 0.1% VCD and increasing amounts of T with unlimited chow and water for 15 days (n = 8 rats per group). The rats consumed >5% of their body weight of bait and thrived during their rapid growth phase. The day after the end of baiting, female rats were bred with untreated, proven male breeders for 21 days, then litter size and time to delivery was tracked over the next 25 days. The control group that consumed bait without active ingredients had an average litter size of 11.5 pups, treatment groups that consumed bait with T at 400, 800, or 1200 µg/kg body weight had average litter sizes respectively of 9.6, 8.3, and 3.6, and pup production per treatment group compared to control group was 83.5%, 72.2%, and 29.6% respectively with increasing T doses. Time to delivery increased significantly as T dose increased: control rats took 26.0 days versus 31.5, 38.9, and 38.2 days to delivery. We conclude our fertility control bait is a palatable liquid readily consumed by growing female rats that causes subfertility, with significantly fewer pups/litter taking 1.5 times longer to deliver. Fertility control bait effects on rat population dynamics are currently being tested in field locations.

KEY WORDS: fertility control, liquid bait, Norway rat, *Rattus norvegicus*, rodent control, Sprague-Dawley rats, triptolide, vinylcyclohexene diepoxide

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INTRODUCTION

Norway rats (*Rattus norvegicus*) are carriers of diseases, cause agricultural crop losses, and through burrowing and gnawing cause infrastructure damage. The primary means to control rat populations is the use of lethal methods, mostly poisons (Capizzi et al. 2014). However, due to their rapid breeding, methods for controlling rat populations through lethal methods are often ineffective in the long term. More importantly, some poisons are very potent and non-specific. They can kill non-target animals, pets, and wildlife, and some toxicants persist in the environment. Since 1993, as many as 15,000 children under age 6 have been exposed to rodenticides annually in the United States, and in recent years an average of 3,617 children were treated in health care facilities for such exposure, with an averages of 115 cases per year resulting in symptoms of rodenticide toxicity (USEPA 2013). Due to the numerous limitations in sustainably managing rat populations through lethal means, fertility control has been proposed as a means to manage rodent pest populations.

To cause infertility in a free living, wild Norway rat population the bait active ingredients must be orally active. In addition, the oral bait must be palatable and attractive to rats so they will choose to eat it in the presence of abundant familiar food sources. Rats that consume the fertility control bait cannot become sick as they will avoid more takes and their cohorts will learn from the sick rat to not consume the bait (Meehan 1984). The fertility control bait must rapidly reduce the rat's

fertility. This is necessary because of the life cycle of the Norway rat: Female and male rats are sexually mature at 40-60 days and thereafter can produce litters of up to a dozen pups every 3 weeks until death, which is typically less than a year (Meehan 1984). Thus, for a fertility control bait to significantly impact a rat population, the onset of infertility must be rapid.

We have developed a proprietary fertility control bait that is an emulsion with two active ingredients. One is triptolide (T), a plant-derived herbal supplement used in traditional Chinese medicine for centuries (Graziose et al. 2010); the other is the industrial ovotoxic chemical 4-vinylcyclohexene diepoxide (VCD) (Hoyer et al. 2001). We have demonstrated that these active ingredients, when eaten in bait by Sprague Dawley female rats, lead to significantly reduced ovarian follicle counts (Dyer et al. 2013). Here, we describe the effect of our fertility control bait on female rat fertility.

METHODS AND MATERIALS

Chemicals/Reagents

The active ingredients are triptolide (CAS No. 38748-32-2) that was >98% pure (Stanford Materials Corp. Irvine, CA), and VCD (CAS No. 106-87-6) that was >98% pure (Sigma-Aldrich, St. Louis, MO). The proprietary bait emulsion contained generally regarded as safe (GRAS) food-grade emulsifier, oil, sweetener, and laboratory-grade water, with increasing concentrations of T while the VCD concentration was held constant at 0.1%.

Animals

Female 22-day-old Sprague Dawley rats purchased from Charles River Laboratories (Chicago, IL) were housed individually in a room at 22-23°C and a 12-h light/12-h dark schedule. They were acclimated to the environment for 6 days, and the study was begun when they were 28 days old. Proven breeder male Sprague Dawley rats were purchased from Harlan (Houston, TX) and housed in individual cages until the start of breeding. Laboratory rodent chow (Purina LabDiet Laboratory Rodent Diet #5001, Purina, St. Louis, MO) and water were provided *ad libitum*.

Experimental Design

Twenty-eight day-old female rats were randomly grouped into one of 4 groups referred to as Control, Low, Medium, and High, with 8 rats per group. Individually housed rats in each group were pre-baited with 25 ml of control emulsion (no active ingredients), provided in glass feeding tubes present in their cages from 4 PM to 9 AM. After 5 days, the control bait was switched to bait with active ingredients for 15 days. The baits with active ingredients had calculated T dose of Low (400 µg/kg body weight), Medium (800 µg/kg body weight), or High (1200 µg/kg body weight) and all had a constant concentration of 0.1% VCD. On Treatment Day 6, emulsion volume was raised to 35 ml/day to compensate for increases in body weight and consumption among all groups. Unlimited rat chow and water were provided through the entire baiting period. Emulsion consumption, body weights, and rat condition were recorded in the morning when feeding tubes were removed from the cages and refilled for the next night of baiting.

On the first day after the last dosing, two females were housed with an untreated, proven male breeder for 21 days, after which females were housed separately for 25 days, followed by a repeat of the next breeding round. Litter sizes and days to parturition, from Day 0 of breeding, when female and male rats were co housed, were recorded. Rats in the Low and Medium groups were euthanized after the second breeding round, while rats in the Control and High groups were bred a third time with 1:1 pairing with an untreated, proven male breeder.

Statistical Analysis

Data are represented as the mean ±SEM. Significance of difference was evaluated using linear regression, one-way ANOVA, and student's t-test. Statistical differences were considered significant at $P < 0.05$.

RESULTS

Bait Consumption

During the baiting period, emulsion consumption was recorded daily and normalized to body weight (Figure 1); results from a rat from the Medium dose group along with a rat from the High dose group were removed from the data sets, as they failed to consume at least 5% of their body weight on average. Over the 20-day feeding period, 5 days of pre-baiting plus 15 days of treatment baiting, neither the Control nor the Low group showed significant changes in consumption, while the Medium and High groups' consumption was significantly decreased. The

Medium group consistently ate more than both the High and Low groups until Treatment Day 9, when consumption dropped from approximately 16% body weight to 11% body weight for the rest of the treatment period. After the introduction of the High bait, that group's overall consumption decreased but did not fall below 6% body weight.

Active Ingredient Dosing

Due to the differences in consumption between the Medium and High groups, both received equal amounts of T but different amounts of VCD in cumulative dosing (Figure 2). The Low group consumed 0.41 ± 0.01 mg/kg/day T, compared to 1.07 ± 0.03 mg/kg/day T in the Medium group, or 1.11 ± 0.05 mg/kg/day T consumed by rats in the High group. By the end of the treatment period, the Low group consumed a total of 6.22 ± 0.27 mg/kg T, significantly less than the 16.06 ± 1.26 or 16.62 ± 1.19 mg/kg T that the Medium and High groups consumed, respectively. As for VCD, both the High and Low groups consumed significantly less than the Medium group. The Medium group consumed a total of $2,172.44 \pm 170.86$ mg/kg body weight VCD, while the Low group consumed $1,681.59 \pm 74.04$ mg/kg body weight, and the High group consumed $1,498.56 \pm 107.13$ mg/kg body weight.

All Rats Gained Weight

The rats were immature at the start of the experiment, thus in a rapid growth phase. There were no signs of systemic toxicity or behavioral changes in rats as they were well-groomed, inquisitive, and active. All rats consistently gained weight (Figure 3). However, the Medium and High groups gained weight at a significantly slower rate than did the Control and Low groups, resulting in 16-33% difference in total weight gain among all groups.

Effect on Litter Sizes and Days to Parturition

All pups survived to post-natal Day 4 and were healthy without apparent defects. In the first breeding round, there was an inverse dose-response relationship; as T dose increased, there were fewer pups per litter (Figure 4a), but only in the High group were there significantly fewer pups/litter. The High group exhibited the most profound effects on litter size, with 2 treated females not giving birth, 2 treated females giving birth to one pup each, and the rest of the females having significantly smaller litters than all other groups. The Control group had an average litter size of 11.5 ± 1.4 , while the High group had an average litter size of 3.6 ± 1.5 pups. As for the Medium and Low groups, the first breeding round showed no significant decrease in litter sizes, as these groups had 8.3 ± 1.3 and 9.6 ± 1.4 pups, respectively.

There was a direct T dose-response relationship effect on days to parturition (Figure 4b). All treatment groups showed a significant increase in the days to parturition, with the Low group taking 31.5 ± 2.3 days, the Medium group taking 38.9 ± 0.7 days, and the High group taking 38.2 ± 0.7 days to give birth. The Control group averaged giving birth at 26.0 ± 1.6 days. There were no significant differences in days to parturition among the groups in the second and third breeding rounds.

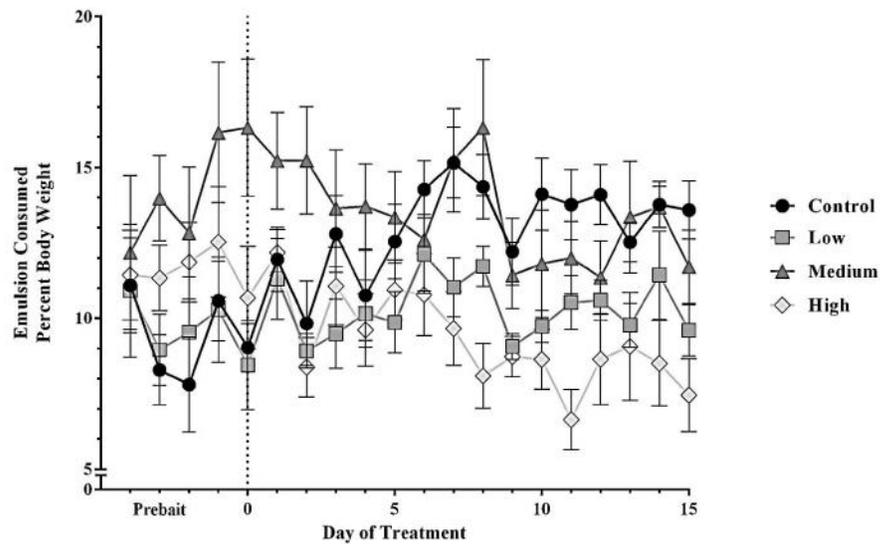


Figure 1. The amount of emulsion consumed by each group as percent body weight. Each group was pre baited with control emulsion for 5 days, followed by the introduction of emulsion containing 0.109 % VCD and either T at 400, 800, or 1,200 $\mu\text{g}/\text{kg}$ body weight (Low, Medium, and High groups, respectively). The Control group was provided bait without active ingredients. Values are mean \pm SEM. Control and Low n = 8, Medium and High n = 7.

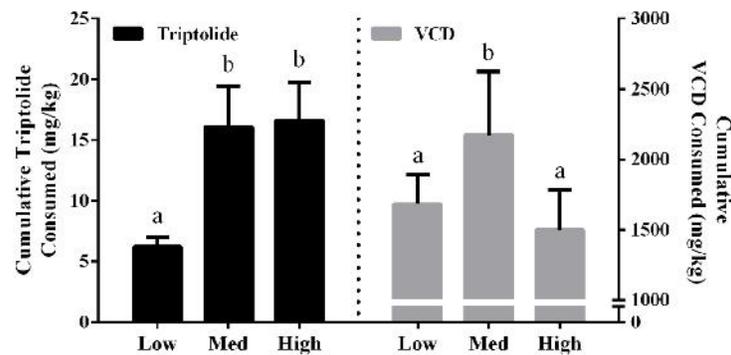


Figure 2. Cumulative T (mg/kg) and VCD (mg/kg) consumed by rats provided emulsion for 15 days containing 400 $\mu\text{g}/\text{kg}$, 800 $\mu\text{g}/\text{kg}$, or 1,200 $\mu\text{g}/\text{kg}$ T and 0.109 % of VCD. Values represent mean \pm SEM. Control and Low n = 8, Medium and High n = 7. Unpaired letters show significant differences.

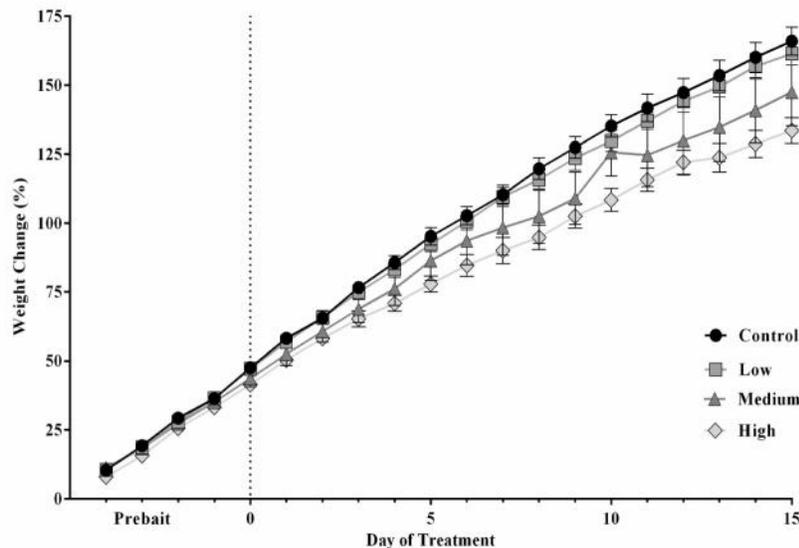


Figure 3. Females were weighed daily throughout the feeding period. The graph represents the cumulative percent weight change calculated from Day 0 of pre baiting. Values are the mean \pm SEM. Control and Low n = 8, Medium and High n = 7.

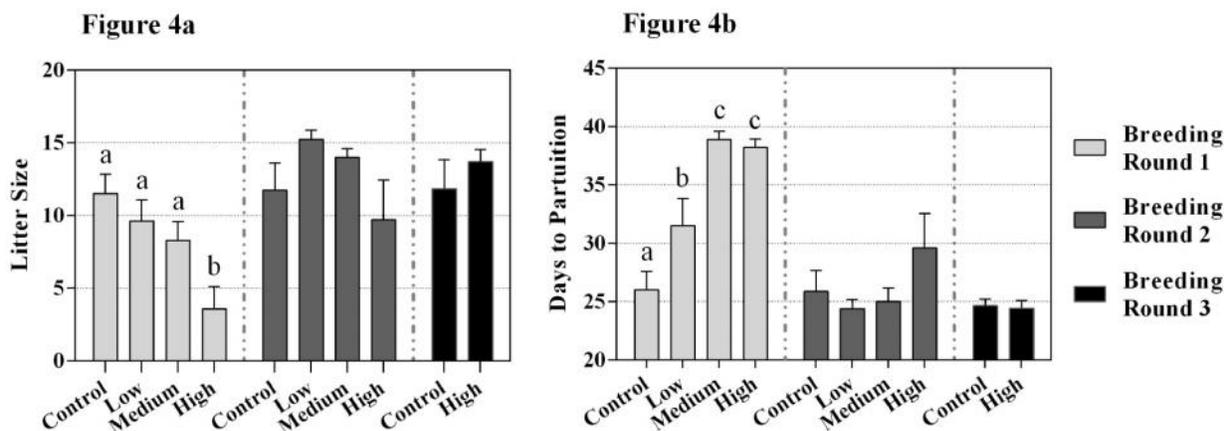


Figure 4. To assess fertility, two females were housed with one proven male breeder for 21 days, then housed individually for a 25-day observation period. Litter size (4a) and days to parturition (4b) from Day 0 of breeding were recorded for each round. Values are represented as mean ± SEM. Breeding rounds 1, 2 Control and Low n = 8, Medium and High n = 7; breeding round 3 Control n = 8, High n = 7. Unpaired letters show significant difference.

DISCUSSION

The essential requirement of fertility control bait is that rats eat enough to be exposed to concentrations of active ingredients that cause subfertility. Of the 24 rats divided among the Low, Medium, and High groups, 22 of 24 (87.5%) ate more than 5% of their body weight over the 15 days of dosing. The rats in the High group had significantly reduced litter sizes and significantly increased days to parturition; whereas the rats in the Low, Medium, and High groups all took significantly longer to give birth, suggesting that frequency of ovulations in these animals was less. But all rats that demonstrated either significantly reduced litter size or significantly longer time to parturition recovered their fertility by the second breeding round. The effect of active ingredients was reversible, as there was full recovery of fertility 86 days after the start of dosing.

The combination of VCD and T significantly reduces the number of all stage ovarian follicles, and most importantly, there are no corpora lutea, indicating an absence of ovulation (Dyer et al. 2013). The two active ingredients accelerate apoptotic-mediated elimination of all stages of ovarian follicles. VCD causes primordial/primary follicle depletion, ultimately leading to premature ovarian failure (Hoyer et al. 2001). However, since subfertility was reversed, it appears that primordial follicles were not depleted in the 15 days of VCD exposure, since premature ovarian failure did not occur (Mayer et al. 2002). T causes growing follicle depletion, leading to reduced or no ovulations (Xu and Zhao 2010, Liu et al. 2011). T targets granulosa cells of growing follicles causing reduced production of progesterone and estradiol, leading to increases in plasma LH and FSH and disrupting hormonal regulation of ovulation (Zhang et al. 2012a). But our results show that their impact on follicle growth and maturation is reversible, because treated female rats that were subfertile in the first breeding round, recovered full fertility within 3 months. These results indicate that to sustain subfertility, female rats have to be re-baited with the active ingredients.

The active ingredients are formulated into an emulsion that is highly palatable since the rats ate >5% of their body weight with unlimited chow and water available for 15 days. The most effective T dose was 1,200 µg/kg body weight and 0.1% VCD. Although the rats in the High group ate significantly less bait with active ingredients, suggesting they could sense the presence of these chemicals, nonetheless they continued to eat the bait and there was no indication of toxicity or abnormal behavior. Another sign the rats were healthy was that they all consistently gained weight throughout dosing.

Bolus gavage of female rats with 400 µg/kg body weight T causes acute toxicity leading to anorexia or death (Liu et al. 2010, Liu et al. 2011). But there were no signs of toxicity from any amount of T consumed by the rats in this study. For the High group, the amount of T consumed averaged 1.11 mg/kg/day, or more than twice the concentration of a bolus dose reported to cause acute toxicity and possibly death. Chemicals administered by gavage rapidly reach a high concentration in the blood stream, unlike the slow absorption seen in rats eating the same chemicals in bait overnight in their cages (Nebendahl 2000). Due to the slow absorption, the rats were able to consume significantly higher concentrations of T without experiencing the toxicity reported in other studies. While the daily doses and cumulative dose of T were similar for the Medium and High groups, only consumption of the High dose caused reduced litter size. Perhaps the High bait was more effective, as rats consume more T per lick than either Medium or Low baits: thus, more T was introduced with each lick and this amount was able to reach an effective dose in the ovary.

For our fertility control bait to be safer than rat poison, it must not pose a high risk for human exposure, not accumulate in rat tissues, and not be excreted in active form or persist in the environment. Our fertility control bait is 99.8% inert ingredients with less than 0.2% active ingredients. The low concentrations of active ingredients reduce risk that could occur with human exposure. Another reason our fertility control bait has low risk is

that the active ingredients have blood half-lives of less than 15 minutes. Each active ingredient is inactivated by liver enzymes: VCD by microsomal epoxide hydrolase (Flaws et al. 1994) and T by P450 CYP3A (Tai et al. 2014). Over 90% of the inactive metabolites from each active ingredient are excreted in urine and there is less than 1% of each active ingredient excreted in the urine. There is no accumulation of VCD or T in rat tissues, making the risk of non-target animal exposure (e.g., via carcass consumption) very low. Finally, our fertility control bait will be put out in Tier 1 bait boxes, which are tamper proof, weather proof, pet proof, and child proof to reduce risk of human exposure.

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The experiment was performed according to the protocol approved by SenesTech's Institutional Animal Care and Use Committee (IACUC).

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