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Fertility suppression effects of a new plant extract shikonin-quinestrol (ND-1) on mid-day gerbil (*Meriones meridianus*) populations

Heping Fu[a,b,c,1], Shuai Yuan[a,b,c,1], Darhan Bao[a,b,1], Man Duhu[a,b,1], An Xing[a,b,1], Suwen Yang[a,b], Xiaodong Wu[a,b,c,*]

[a] Inner Mongolia Agricultural University, 29, Ordos East Street, Saihan District, Hohhot, Inner Mongolia, 010011, China
[b] Key Lab. of Grassland Resources, Ministry of Education, 29, Ordos East Street, Saihan District, Hohhot, Inner Mongolia, 010011, China
[c] Key Laboratory of Forage Cultivation, Processing and High Efficient Utilization of the Ministry of Agriculture, 29, Ordos East Street, Saihan District, Hohhot, Inner Mongolia, 010011, China

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ABSTRACT

Controlling fertility of rodent pests has become a widely used means of controlling populations of grassland rodents in China. Research has recently focused on environmentally-friendly fertility inhibitors that lead to a more sustainable control of pest rodent populations. Using plant-derived fertility inhibitors may be an appropriate method. In this study, we used experimental populations of wild mid-day gerbils (*Meriones meridianus* Pallas) – the dominant rodents in typical Chinese desert regions – to test the effects of shikonin-quinestrol (ND-1) (also known as Nong Da-1), a new plant-derived fertility inhibitor. Mid-day gerbils were divided into control, female treatment and male treatment groups, and the effects of the fertility inhibitor ND-1 were studied. We found that there was a significant decrease in average litter size in the male treatment group ($P < 0.01$) during the first breeding period of the gerbil's annual breeding season. The average litter size of the male treatment group was 1.42 ± 0.12 individuals and the fertility suppression rate was 42.86%. The second litter showed a significant decrease ($P < 0.05$) in the average litter size in both female and male treatment groups (1.42 ± 0.13 and 0.14 ± 0.04 individuals, respectively) and fertility suppression rates were 57.14% and 85.78%, respectively. The control group bred two to three times during the breeding season, while the two treatment groups bred zero to two times, which was significantly lower than the control group ($P < 0.01$). Reproductive start-up periods (RSP) of the two treatment groups were delayed. The female treatment group showed a 15-22d delay in reproductive period, and the male treatment group showed a 32-157d delay. The fertility inhibitor ND-1 induced infertility in both males and females. As a plant-based agent, ND-1 is an effective and non-polluting agent for inhibiting fertility in mid-day gerbils.

1. Introduction

Mid-day gerbils (*Meriones meridianus* Pallas) are a common rodent distributed in China's semi-desert steppe and desert areas. The gerbils do not hibernate, and mainly appear in the evening. Their peak activities are around midnight from April to October each year, and at dusk from November to March of the following year (Zhao, 1981, 1985; Ma et al., 1987; Wu et al., 2009). The gerbils breed two to three times a year, rearing four to nine pups per litter. Each hectare of grassland has 32–38 burrows, and each adult consumes 32.4–34.6 g of herbage each day (Song and Liu, 1984; Zhou et al., 1999). With regular breeding seasons and population outbreaks, the gerbils cause damage to grassland vegetation, especially in autumn. Therefore, there is demand for effective management strategies to control growth of gerbil populations. Sustainable and effective population control methods have attracted a lot of attention in recent years (Zhang, 1995; Zhang et al., 2004; Han et al., 2013), in particular with fertility control as a focus. Screening of chemical fertility inhibitors, laboratory experiments and fertility control experiments on partially wild populations have led to some progress (Zhang, 2015). In addition, theoretical studies have used ecological models to examine the effects of fertility control (Zhang, 1995, 2000; Shi et al., 2002; Liu et al., 2008, 2013). In recent years, one focus of research has been on selection of environmentally friendly fertility inhibitors for the sustainable control of rodent populations in China. Tran and Hinds (2013) summarized more than 40 plant extracts with infertility effects, and selected 13 plant extracts with effects on

* Corresponding author. Inner Mongolia Agricultural University, 29, Ordos East Street, Saihan District, Hohhot, Inner Mongolia, 010011, China.
E-mail address: wuxiaodong_hgb@163.com (X. Wu).
[1] These authors contributed equally to this work.

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follicle formation in female rats and mice. They further proposed application routes and identified several extracts with potential. In China, plant sourced inhibitors that have been applied in experiments have included *Tripterygium wilfordii* F., Gossypol, *Ruta graveolens* L., *Camellia oleifera* Abel., *Trichosanthes kirilowi* Maxim., colchicine, *Ricinus communis* L., *Curcuma aeruginosae* Val. and *Melia azedarach* L., all of which have been shown to have some effect, although studies on practical applications have been unsatisfactory (Liu et al., 2011). Issues such as bait palatability, food intake amounts, duration of drug action, and sterility effects on different sexes and species need to be further explored.

Arnebia euchroma (Royle) Johnst. is a plant belonging to the perennial Boraginaceae family that is widely distributed in arid and semi-arid regions of Inner Mongolia, Xinjiang and Gansu Province in China. The plant contains shikonin (Chen and Lin, 2006; Wu and Liu, 2008). Several studies have shown that shikonin has anti-inflammatory, anti-tumor, anti-HIV and sterility effects (Yin and Guo, 1994). However, direct experimental evidence of its fertility suppression effects applied to wild animals is still sparse. Specifically, an extract from *Onosma armeniacum* K., which is similar to shikonin, has been analyzed for its contraceptive effects on embryos implanted in rats, and further clinical studies were recommended by researchers in Turkey (Salman et al., 2009). Further studies are needed to test different shikonin application methods.

In our previous studies, we tested the effects of shikonin on fertility in mice, and found that it atrophied the uteri of female mice, causing a significant decrease in the juvenile birth rate of the female treatment group, but shikonin had no significant effects on the male treatment group (Fu et al., 2016). Studies have shown that shikonin lacked estrogen-like activity (Findley and Jacobs, 1980; Findley, 1981), which implies a need to add xenoestrogen into shikonin to compensate for this shortcoming. Quinestrol had been found to be an effective fertility inhibitor when applied to male Mongolian gerbils (*Meriones unguiculatus* Milme-Edwauds) (Shen et al., 2011; Zhang, 2015). Therefore, we reasoned that mixing shikonin with quinestrol might improve its fertility suppression effects.

In this study, we tested whether a combined inhibitor, shikonin-quinestrol (ND-1) (also known as Nong Da-1), has fertility-reducing effects on mid-day gerbil populations in captivity. As shikonin is derived from plants, we hypothesized that ND-1 may be an effective and environmental-friendly fertility inhibitor for population control among mid-day gerbils.

2. Materials and methods

2.1. Materials

Shikonin (molecular formula $C_{16}H_{16}O_5$, analytical standard ≥97%, molecular weight 288.30) is extracted from *Arnebia euchroma*, and its main components are naphthoquinone compounds. The shikonin used in this experiment was provided by Shanghai Jingchun Biochemical Technology Co. Ltd. The quinestrol (assay 99.27%, using a product that complies with specification USP22) was provided by Beijing Zizhu Tiangong Science and Technology Co. Ltd. Mid-day gerbils (*Meriones meridianus*) were captured from typical desert habitats located in southern Alashan Desert in Inner Mongolia in October 2014. They were kept alone in mice cages (40 cm × 40 cm × 60 cm) with a wooden box (20 cm × 8 cm × 8 cm) in the Research Laboratory Base of Desert Ecology and Pest Rodent Control of Inner Mongolia Agricultural University (located in the southern Alashan). The wooden box was placed in the mice cages in order to offer a shelter and maintain rodent body temperature. Animals were supplied with ventilation and natural light as well as plentiful feed, and the mid-day gerbils were allowed to feed freely. The animal testing laboratory met the required standards for cleanliness and sanitation. The gerbils were kept on a 14 h: 10 h daylight-dark cycle. After feeding for five months and after safe overwintering, 45 sexually mature and healthy female and male gerbils were selected in April 2015. A total of 90 individuals were used for the experiments.

In all experiments, animal care and treatment were carried out in accordance with the guidelines issued by the Ethical Committee of Inner Mongolia Agricultural University. The committee required that all researchers and students related to experimental animals were certified in accordance with the requirements of the Institutional Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences (Permit Number: IOZ11012).

2.2. Overview of study design

Shikonin (concentration 100 mg/kg) was mixed with appropriate quinestrol to prepare a shikonin-quinestrol edible oil solution (15 ml/kg) as a solvent. Experimental gerbils were randomly divided into control, female treatment and male treatment groups. Each group consisted of 15 females and 15 males, which were kept separately. All of them were adults, and average body weight was 62.78 g ± 10.08 g (SD). Female gerbils were given the fertility inhibitor solution by gavage twice a week at three day intervals, and, one week after the last gavage, the female gerbils copulated with untreated male gerbils. Male gerbils were given the fertility inhibitor solution using the same method as for females, and then they copulated with normal female gerbils. Gerbils in the control group were given the same volume of edible oil by gavage twice a week with a three-day interval, and one week after the last gavage males and females were mated. We defined the reproductive start-up period (RSP) as the days from gerbils copulating to when the first litter was born. The experimental observation period totaled 183d (April 5th- October 5th). If the animal did not reproduce during the observation period, the observation day number was categorized as within the RSP. Birth was defined as the litter we observed during experimental period. Reproduction cycles were defined as days from the end of first breeding to the start of the next breeding. Experimental gerbils were observed between April and October, and breeding date, litters, litter size, RSP and reproduction cycles were observed daily and recorded.

2.3. Data analysis

Litter size, birth and their interaction were analyzed by generalized linear mixed-effects model (Proc Glimmix, SAS9.0) with a Poisson error distribution. A random statement with the first order autoregressive structure was included in the model to specify the birth effect. Because the birth × treatment interaction was significant ($F_{4,\ 83} = 5.21$, $P < 0.001$), glimmix models were used for each birth with treatment as the fixed effect. Glimmix models were also conducted on the number of litters and RSP of each group. Means were separated by the Least Squares Means (LSMs). Significance level of all analysis were set at $P < 0.05$. The fertility suppression ratio of ND-1 on mid-day gerbils was calculated using the following formula:

$$\textit{Fertility ratio} = \frac{\textit{Number of fertile female gerbils}}{\textit{Number of all female gerbils}}$$

$$\textit{Fertility suppression ratio} = \textit{Fertility ratio in control} - \textit{Fertility ratio in treatment}$$

3. Results

3.1. Comparison of fertility ratio and litter size

During the experimental feeding process, one accidental death occurred due to fighting between gerbils during the initial matching period. Fourteen pairs of gerbils were therefore available in each

Table 1
Fertility rate of gerbils.

	Control	Female treatment	Male treatment
First birth	14/14	14/14	8/14
Second birth	14/14	6/14	2/14
Third birth	4/14	0/14	0/14

Note:Numerator was number of fertile female in each group. Denominator was all of female numbers in each group.

treatment or control group. In the first birth, the female treatment group and control group had the same fertility ratio (i.e. 14/14), while the fertility ratio of the male treatment group was lower at 8/14 (Table 1). In the second birth, the fertility ratio of the female and male treatments showed a decrease in the two treatment groups (Table 1). The fertility suppression ratios of the female and male treatment groups in the second birth were 8/14 and 12/14, respectively, which were both higher than the first litter (Table 1). In the third birth, four pairs in the control group bred, but the treatment groups did not breed. Six pairs gerbils in the male treatment group never bred during the experimental observation period (Table 1).

In the first birth, litter sizes differed between the three groups ($F_{2,\ 22.76} = 8.82$, $P = 0.002$), and average litter size in the control, female treatment and male treatment groups were 4.28 ± 0.72, 3.71 ± 0.28 and 1.42 ± 0.12 individuals, respectively. Litter size of the male treatment group was significantly lower than both female treatment and control groups in the first birth (Fig. 1). The female treatment showed a trend for being lower than the control (Fig. 1). In the second birth, ND-1 had a significant effect on litter size in treatment groups ($F_{2,\ 17.90} = 13.43$, $P = 0.0003$). The litter sizes for the two treatment groups were significantly lower than the control group (Fig. 1), and the male treatment was significantly lower than the female treatment (Fig. 1). In the third birth, all values were further reduced, and both male and female treatments had no litter. So the convergence was not met for litter size in the third birth. Both male and female treatments had no litter, while the control was significantly higher than zero (mean = 1.29, 95%CI 2.52–0.06, Fig. 1). Therefore, ND-1 had a significant fertility suppression effect on females and males in the third birth. The last litter of gerbils in the control group occurred on September 20th, which is close to the reproduction dormancy period, but it cannot be completely ruled out that termination of reproduction in the treatment groups was due to the effects of ND-1.

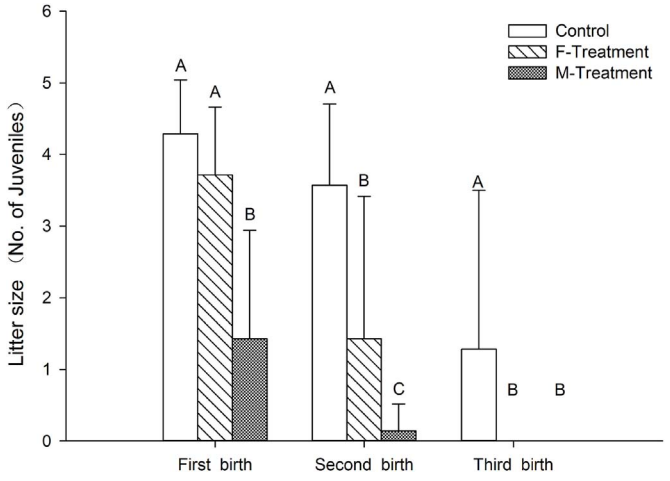

Fig. 1. Comparison of gerbil litter size (± SD) under different treatments. Note: The F-Treatment represents females treated with ND-1; M-Treatment represents males treated with ND-1. Bars with different letters indicate significant differences between groups with LSMs ($P < 0.05$). In the third birth, all values were further reduced and both male and female treatments had no litter, so bars for male and female treatment groups were zero.

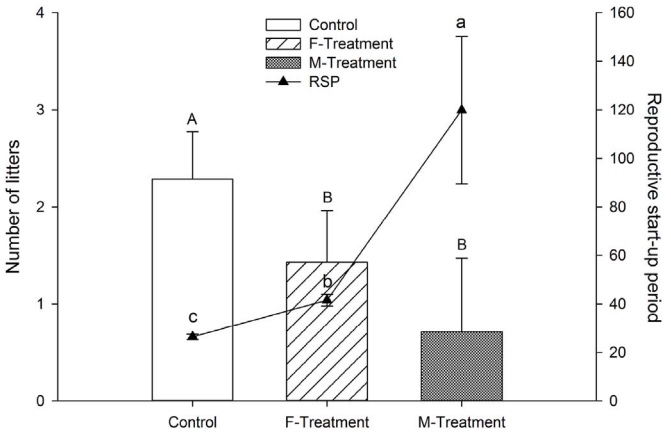

Fig. 2. The number of (± SD) and the reproductive start-up period (± SD) of gerbils in different treatment groups. Note: The F-Treatment and M-Treatment represent the female and male treatment groups, respectively. Bars with different letters indicate significant differences between treatments with LSMs ($P < 0.05$).

3.2. Comparison of reproductive measures on mid-day gerbils

The control group had two to three litters, the female treatment group had one to two litters, and the male treatment group had zero to two litters (Fig. 2). We observed significant differences among the three groups ($F_{2,\ 9.35} = 7.00$,$P < 0.001$; Fig. 2). ND-1 significantly decreased the litters of the treatment groups.

ND-1 had a significant effect on RSP among treatments ($F_{2,\ 9.69} = 150.81$, $P < 0.001$, Fig. 2). Both female treatment and male treatment had significant differences with the control group (Fig. 2). RSP in the male treatment was significantly higher than that in female treatment (Fig. 2). The RSP of the female treatment group was delayed on average by 15.2 ± 1.3 days more than the control group, and the longest delay was 22 days. The RSP of the male treatment group delayed on average by 93.4 ± 3.4 days, and the longest delay was 157 days (Fig. 3). ND-1 delayed the RSP of the female treatment group by 15–22 days, and the male treatment group by 32–157 days (Figs. 2 and 3), and ND-1 significantly extended the RSP, especially in males.

4. Discussion

The ideal fertility inhibitor to control pest rodents should be environmentally friendly, palatable for target rodents, and have

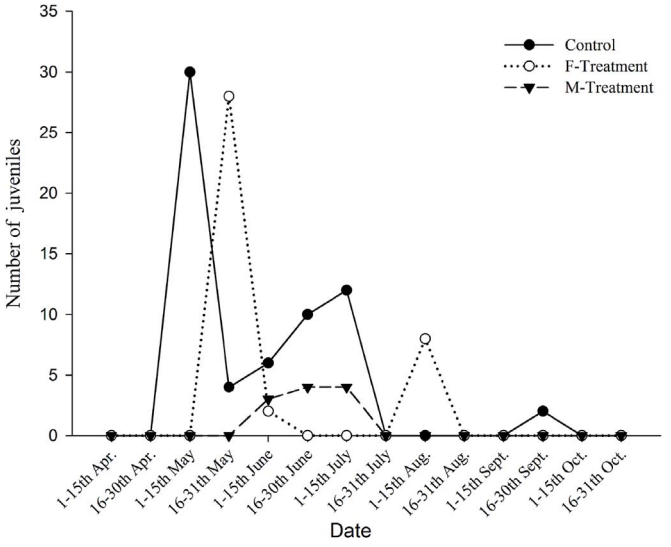

Fig. 3. Dynamics of gerbil juvenile numbers during the experimental period.

sustainable control effects on both sexes. Inhibitors should cause no harm or cause non-negative effects on non-target animals inhabiting the same environment, such as humans, livestock, birds or beneficial insects, and should also naturally decompose in a short period (Zhang, 2015), and have a significant effect on the reproduction of gerbils. Plant extracts have become major targets for researchers. The Chinese classic herbal book, Shennong's *Materia Medica*, states that shikonin has anti-inflammatory and fertility inhibition effects (CPC, 2010). Moreover, shikonin is relatively easily obtainable due to its simple extraction process (Wu and Liu, 2008). Shikonin is composed of pure natural compounds that should not pollute the environment. Shikonin is biodegradable under conditions of natural sunlight in excess of 12 h and under alkaline conditions (pH > 8) (Qiao et al., 2004). On this basis, we selected shikonin extract from *Arnebia euchroma*, a plant that is extensively distributed in China. Because shikonin has no estrogen-like activity (Findley and Jacobs, 1980; Findley, 1981), we added the xenoestrogen, quinestrol, to enhance effects on infertility.

Quinestrol is an estrogen that has no obvious effects on non-target animals (e.g. birds), while influencing the fertility of some small rodents (Zhang, 2015). It also has a relatively short half-life period and can naturally degrade in the soil (5.5–15.6d) and water (< 3d) (Zhang, 2015). Therefore, we used an adequate amount of quinestrol to improve the fertility inhibition effects of shikonin on both sexes. We applied ND-1 to experimental mid-day gerbil populations in captivity, and observed effects on gerbil reproduction.

ND-1 reduced the fertility of both female and male mid-day gerbils, and also changed the reproductive start-up period (RSP). Our results showed that the effect of ND-1 was stronger in males than in females. The reasons for these effects might include the following. Quinestrol in ND-1 had a significant fertility inhibition effect on males. Previous studies have shown that quinestrol has fertility suppression effects on both females and males in some small rodents (Zhang, 2015), but its fertility suppression effects were stronger in males than in females, and fertility could recover from a one-time administration of quinestrol (Huo et al., 2007; Zhao et al., 2007; Shen et al., 2011; Zhang, 2015). Other research on male mice reproductive mechanisms suggest that the mice estrogen was mediated by ERα (estrogen receptor-α, which plays an important role in spermatogenesis). Shikonin can inhibit the activation of ERα (Yao et al., 2010; Xu and Wu, 2015) and the ERα gene of mice fertility could be suppressed by shikonin with damaged spermatogenic epitheliums, reduced sperm counts, increased abnormal sperm morphology and reduced reproductive ability (Hess et al., 2001; Zhou et al., 2001). Therefore, the effects of ND-1 on males could be due to the dual function of shikonin and quinestrol.

The appropriate ratio of shikonin and quinestrol in ND-1 could play an important role in controlling fertility. We observed significant effects on fertility using the current proportion of shikonin and quinestrol in ND-1. In this study, we did not find synchronous control of fertility in both sexes when applying ND-1, which may be related to the combined effect of naphthoquinone compounds and their derivatives in shikonin. Further studies should be conducted to better understand the mechanisms through which ND-1 influences reproduction of mid-day gerbils.

Screening methods and infertility agents for fertility control in pest rodents have achieved significant results that may be useful for controlling rodent populations in the wild. For example, the fertility inhibitor EP-1 or its components reduce the fertility of small rodents, resulting in infertility in both sexes with relatively little effect on the environment (Zhang, 2015). Cabergoline inhibits female reproduction in mice, but its effects on early and late pregnancy were dose-dependent (Su et al., 2014). Jacob et al. (2004), Jacob and Rahmini (2006) used tubal ligation to control the fertility of female rice-field rats (*Rattus argentiventer* Robinson & Kloss) in a closed population, which resulted in sterility in more than 50% of female individuals, and they suggested that their results could control population growth and reduce the harm caused by these animals to paddy fields. However, they suggested that migrating animals could influence the reproduction of animals in the study area, so further field experiments would be required to assess the effects on target populations at a large scale. Tran and Hinds (2013) summarized the infertility effects of a variety of plant extracts, and postulated that plant extracts could directly or indirectly affect the normal development of follicles in female rodents and interrupt estrus, but cautioned that the effects of one-time administration of plant extracts could be reversible. Therefore, the ideal plant extract would cause primordial follicles in the female animal to become non-renewable. The ND-1 used in this study not only had significant effects in controlling the number of offspring produced by mid-day gerbils, thus decreasing the fertility rate, but also effectively delayed the RSP. However, its effects on formation of female follicles or interruption of estrus need to be further explored.

The annual number of juveniles in the treatment groups showed a decreasing trend, and there were no indications of reversal of the effects of ND-1 on fertility (Shen et al., 2011; Fu et al., 2011, 2013). The most important effects of ND-1 are the decrease in the number of juveniles, and the increase in the fertility suppression rate throughout the treatment period. These results suggest that ND-1 can control reproduction of mid-day gerbils in laboratory conditions. ND-1 could potentially be used for effective control of wild populations of mid-day gerbil. Delay in RSP clearly affects the number and the structure of rodent populations, and it would most likely affect overwintering survival and the number of offspring in a wild population in subsequent periods. However, evidence from field tests on wild populations is still required.

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