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ORIGINAL ARTICLE

Anti-fertility effect of levonorgestrel and quinestrol in Brandt's voles (*Lasiopodomys brandtii*)

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Abstract

The combination of levonorgestrel and quinestrol (EP-1) has been shown to have anti-fertility effects on several wild rodents, but the mechanism underlying these effects is poorly understood. We investigated the effects of EP-1 and each of its components, levonorgestrel (P) and quinestrol (E), on the fertility of Brandt's voles (Lasiopodomys brandtii) by using a gastric gavage method. The doses for EP-1, E and P were 1, 0.34 and 0.66 mg/kg body weight, respectively. Male voles (n = 98) were treated daily for 5 or 14 days, then the testes and epididymides were collected, weighed and examined histologically at 30 (D30), 60 (D60) or 90 (D90) days after the end of treatment. Four males were allowed to mate with normal females at D90. Female voles (n = 75) were treated for 3 days and a further 3 days after a 7-day interval. The uteri and ovaries were weighed and examined histologically at 15 (D15), 30 (D30) or 75 (D75) days after the end of treatment. Each of three females were mated with fertile males at D30 and D75, respectively. Our results indicated that quinestrol (E) significantly decreased the sperm numbers in the testes as well as the weight of the testes and epididymides, with both of these tissues showing obvious structural abnormalities, and significantly reduced the litter size and the pup weight for females mated with males of the E treatment group. For female voles, treatment with E, P or EP-1 resulted in no marked influence on the fertility status. These data indicate that quinestrol (E) alone has a significant anti-fertility effect on male Brandt's voles, but is ineffective in combination with levonorgestrel (P).

Key words: Brandt's voles (*Lasiopodomys brandtii*), fertility control, levonorgestrel, quinestrol.

INTRODUCTION

Brandt's vole (*Lasiopodomys brandtii*) (Scientific Database of Animals in China) is a major rodent pest in the grasslands of Inner Mongolia, China. The breeding period of this species is from March to September. Over-wintering voles usually reproduce 3–4 times each year, and the average litter size is eight with a maximum of 14. The voles live in groups of 2–3 voles in spring and 6–8 voles in early summer, and approximately 22 or even 30 voles live together in a nest system in later summer or autumn (Zhang *et al.* 2003). The population sizes of the voles oscillate greatly within and between years, with

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major outbreaks occurring irregularly; these changes are often driven by climatic factors (Zhang *et al.* 2003). In autumn the density ranges from 2.4 to 528.8 voles per ha. Large populations of Brandt's voles have contributed to serious degradation of the grasslands, and have a negative impact on livestock production and the environment. Overgrazing may facilitate increases in Brandt's vole population sizes, because they prefer short and sparse grass vegetation (Zhong *et al.* 1999). During outbreak years, it is imperative that Brandt's voles be controlled. Poison baiting is widely used for vole control in the grasslands of Inner Mongolia, but this often brings serious pollution problems and poses a risk to non-target species (Zhong *et al.* 1999; Shi *et al*. 2002). Fertility control has been proposed as a potentially effective control method via simulation modeling (Zhang 2000a,b; Shi *et al.* 2002), but no fertility control technique currently exists for this vole species.

The combination of levonorgestrel and quinestrol (EP-1; at a ratio of 6:3) has been commercially used as a contraceptive for women. Zhang *et al*. (2004) reported that EP-1 reduces the fertility of Brandt's voles (*Lasiopodomys brandtii*), gray hamsters (*Cricetulus migratorius*) and mid-day gerbils (*Meriones meridianus*) on the basis of laboratory data. Subsequently, both laboratory and field experiments also showed that EP-1 and its individual components, levonorgestrel (synthetic progesterone, P) and quinestrol (synthetic estradiol, E), affected the structure of the reproductive organs, and exerted anti-fertility effects in many wild rodent species, for example the greater long-tailed hamster (*Tscherskia triton*; Zhang *et al.* 2005, 2006), the Djungarian hamster (*Phodopus campbelli*; Wan *et al.* 2006) and Mongolian gerbils (*Meriones unguiculatus*; Huo *et al.* 2006; Liang *et al.* 2006).

With respect to the contraceptive effects of quinestrol and levonorgestrel in women, levonorgestrel mainly inhibits the release of follicle stimulating hormone from the pituitary to block ovulation, and disrupts the proliferation and differentiation of endometrial cells, preventing embryonic implantation. Quinestrol, a stable estradiol homologue, can be stored in adipose tissue and released slowly. It mainly inhibits the release of gonadotropin releasing hormone (GnRH) from the hypothalamus and thus inhibits follicle growth. Although EP-1 has been shown to be effective in reducing the fertility of female rodents, it remains unclear how each hormone (levonorgestrel and quinestrol) functions individually.

To optimize this contraceptive for rodents, it is necessary to determine the respective anti-fertility effects of EP-1 and its components. The purpose of this study was to investigate the effects of EP-1, P and E on the fertility of both male and female Brandt's voles in the laboratory.

MATERIALS AND METHODS

Reagents

EP-1 is a mixture of levonorgestrel and quinestrol at a ratio of 6:3. Eighteen milligrams of EP-1, 12 mg of levonorgestrel and 6 mg of quinestrol were each ground and mixed with 18 mL distilled water. The suspension was delivered by oral gavage (approximately 50 μL per day) to conscious animals at a dose of 1 mg kg^{-1} body weight for EP-1, 0.67 mg kg^{-1} body weight for P and 0.33 mg kg^{-1} body weight for E.

Animals

The Brandt's voles were part of a captive colony maintained at the Institute of Zoology, Beijing. The colony was derived from wild-caught stock from the Xilinhot Field Research Station, the Chinese Academy of Sciences, Inner Mongolia. The animals were housed in individual cages with a 12 h light : 12 h dark light cycle, and food was available *ad libitum*. The room was kept at 20–21ºC and 35–50% humidity. Ninety-eight males (body weight 54.4 ± 8.3 g) and 75 females (body weight 48 ± 7.9 g) were randomly assigned to the control and treatment groups. All animal experiments were conducted using protocols approved by the Institute of Zoology Research Animal Resources Center (Beijing, China).

Experimental groups

During the course of the experiments, some of the animals died of natural causes. Therefore, the total number of animals listed below is larger than that presented in the Results section, but there were at least three animals per group at each time point.

Ninety-eight male voles were divided into seven groups. Groups P5 and P14 were treated daily with levonorgestrel for 5 or 14 days, respectively; groups E5 and E14 were treated daily with quinestrol for 5 or 14 days, respectively; groups EP5 and EP14 were treated daily with EP-1 for 5 or 14 days, respectively; group C received an equal volume of distilled water daily for 14 days. Testes and epididymides of three animals per group were collected at 30 (D30), 60 (D60) and 90 (D90) days after the end of treatment. Tissues were weighed, measured and flash frozen in liquid nitrogen. At D90, 3–4

animals in each group were randomly selected to mate with 3–6 fertile females for 2 weeks. The time to birth of the pups, pup weight and litter size for each female were recorded. The whole experiment lasted approximately 130 days.

Seventy-five female voles were divided into four groups: levonorgestrel (group P), quinestrol (group E), EP-1 (group EP) and distilled water (group C). Each group received its assigned treatment for 3 days, and then after a 7-day interval, treatment for a further 3 days. Uteri and ovaries of three female Brandt's voles per group were collected at D15, D30 and D75 after the end of treatment. All tissues were weighed and frozen in liquid nitrogen. At D30 and D75 after the end of treatment, 3–4 animals per group were randomly selected to mate with three fertile males for 2 weeks, and the time to birth, pup weight and litter size were recorded. The experiment lasted approximately 110 days.

Tissue preparation and staining

All tissues were fixed in 4% paraformaldehyde at 4ºC for 10 h, then gradually dehydrated in ethanol and embedded in paraffin. Sections of 6 μm thickness were collected on glass slides. Routine hematoxylin and eosin (HE) staining was performed and the sections were observed using a light microscope (Leica, Germany).

Statistics

Specimens from at least three animals per group at each time point were harvested and analyzed. Statistical analysis was performed using ANOVA and $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of E, P and EP-1 on fertility of male Brandt's voles

At D30, D60 and D90, no significant differences in the size and weight of testes and epididymides were observed between the control group and groups EP5, EP14, P5 or P14. However, for groups E5 and E14, at each time point, the volume and weight of testes and epididymides were significantly less than those of the control group (Tables 1, 2).

Treatment with EP-1 and P did not appear to affect either the structure of the seminiferous tubules in the testes, or the epididymides, or the quantity of sperm present in these organs. However, treatment with E significantly altered the structure of the testis and epididymis. The diameter of the seminiferous tubules and the quantity of sperm were markedly reduced compared with control animals (Fig. 1). The number of Sertoli and Leydig cells in the testes of the E-treated animals also decreased compared with control animals. These effects were evident at D30, D60 and D90.

The fertility of males treated with EP-1 or P was unaffected: pregnancy rates (proportion of pregnant females), time to birth of the females giving birth, litter size and pup weight were similar to control animals. However, the fertility of males treated with E was reduced (Table 3): pregnancy rates, litter sizes and pup weights in this group were significantly lower and the time to delivery of pups was delayed for 3–4 days compared with the control group. One pup in group E5 died a few hours after birth.

Effect of E, P and EP-1 on fertility of female Brandt's voles

The weights of the uteri and ovaries of females treated with EP-1, P or E were not significantly different from those of control females at D15, D30 and D75 (Table 4). There was no obvious structural change in the uterine luminal and glandular epithelia and stroma, or the follicles in the ovaries for any of the groups at the different time points (Fig. 2).

The fertility of these animals as assessed by mating them with fertile males also revealed no significant difference in pregnancy rates, days to delivery, litter sizes or pup weights between any treatment group and the control group at either D30 or D75 after the end of treatment (Table 5).

DISCUSSION

The results of this study show that none of EP-1, levonorgestrel and quinestrol alone had any effect on the fertility of female Brandt's voles. This data is in contrast with the results of an earlier study (Zhang *et al.* 2004) in which treatment of female Brandt's voles with EP-1 led to a 3-day delay in the time to birth and a reduced number of litters. However, the two studies used different experimental designs. Zhang *et al*. (2004) performed an assessment of the effects on fertility by mating females with males within 1–2 days after the end of treatment. In our study, assessments were made considerably later: observations of female reproductive organs began on D15 after the end of treatment, and mating experiments were performed on D30 and D75 after the end of treatment. The results of the two studies suggest that the contraceptive effect of the hormones in female Brandt's

Table 1 Effect of levonorgestrel plus quinestrol (EP) and levonorgestrel (P) and quinestrol (E) alone on the weight of testes and epididymides in male Brandt's voles at different times after the end of treatment

Organs	Treatment group	No. days between end of treatment and autopsy		
		30	60	90
Testis (g per 10g body weight)	Control	0.53 ± 0.12 (n = 4)	0.57 ± 0.15 (n = 4)	0.64 ± 0.2 (n = 4)
	P5	0.49 ± 0.093 (n = 4)	0.52 ± 0.134 (n = 6)	0.62 ± 0.187 (n = 4)
	P14	0.52 ± 0.201 (n = 4)	0.53 ± 0.097 (n = 6)	0.62 ± 0.086 (n = 6)
	E5	0.33 ± 0.065 (n = 6)**	0.34 ± 0.102 (n = 6)**	0.37 ± 0.076 (n = 6)**
	E14	0.36 ± 0.083 (n = 4)**	0.36 ± 0.091 (n = 6)**	0.36 ± 0.058 (n = 4)**
	EP5	0.49 ± 0.111 (n = 6)	0.52 ± 0.165 (n = 6)	0.55 ± 0.096 (n = 6)
	EP15	0.52 ± 0.130 (n = 4)	0.51 ± 0.0167 (n = 6)	0.54 ± 0.173 (n = 4)
Epididymis (g per 10g body weight)	Control	0.58 ± 0.145 (n = 4)	0.53 ± 0.094 (n = 4)	0.65 ± 0.023 (n = 4)
	P5	0.59 ± 0.077 (n = 4)	0.57 ± 0.089 (n = 6)	0.62 ± 0.165 (n = 4)
	P15	0.63 ± 0.176 (n = 4)	0.61 ± 0.089 (n = 6)	0.61 ± 0.143 (n = 6)
	E5	0.38 ± 0.085 (n = 6)**	0.36 ± 0.071 (n = 6)**	0.37 ± 0.065 (n = 6)**
	E15	0.39 ± 0.11 (n = 4)**	0.39 ± 0.091 (n = 6)**	0.37 ± 0.088 (n = 4)**
	EP5	0.59 ± 0.203 (n = 6)	0.59 ± 0.157 (n = 6)	0.49 ± 0.12 (n = 6)
	EP15	0.62 ± 0.231 (n = 4)	0.56 ± 0.099 (n = 6)	0.56 ± 0.146 (n = 4)

n, number of tissues measured. Compared with the control: *P < 0.05; **P < 0.01.

Table 2 Effect of levonorgestrel plus quinestrol (EP) and levonorgestrel (P) and quinestrol (E) alone on the size of testes in male Brandt's voles at different times after the end of treatment

	Treatment group	No. days between end of treatment and autopsy		
		30	60	90
Testis length (cm)	Control	0.49 ± 0.08 (n = 4)	0.63 ± 0.11 (n = 4)	0.64 ± 0.16 (n = 4)
	P5	0.51 ± 0.1 (n = 4)	0.54 ± 0.14 (n = 6)	0.60 ± 0.17 (n = 4)
	P14	0.52 ± 0.15 (n = 4)	0.59 ± 0.13 (n = 6)	0.59 ± 0.09 (n = 6)
	E5	0.36 ± 0.08 (n = 6)**	0.33 ± 0.104 (n = 6)**	0.38 ± 0.13 (n = 6)**
	E14	0.37 ± 0.09 (n = 4)**	0.38 ± 0.14 (n = 6)**	0.36 ± 0.12 (n = 4)**
	EP5	0.49 ± 0.11 (n = 6)	0.54 ± 0.12 (n = 6)	0.61 ± 0.09 (n = 6)
	EP15	0.52 ± 0.12 (n = 4)	0.55 ± 0.14 (n = 6)	0.63 ± 0.18 (n = 4)

n, number of tissues measured. Compared with the control: *P < 0.05; **P < 0.0.

Table 3 Effect of levonorgestrel plus quinestrol (EP) and levonorgestrel (P) and quinestrol (E) alone on the fertility of male Brandt's voles 90 days after the end of treatment

Treatment group	No. males	No. females	No. littering females (%)	Total litters	Litter size	Time to birth	Pup weight (g)
P	4	4	4 (100)	20	5 ± 2	29 ± 2.6	2.97 ± 0.28
E	4	6	3 (50)	5	0.83 ± 0.11*	32 ± 1.8	2.54 ± 0.32
EP	4	4	4 (100)	20	5 ± 2	28 ± 2.3	2.87 ± 0.37
Control	3	3	3 (100)	15	5 ± 1	29 ± 2	3.01 ± 0.41

Compared with control: *$P < 0.05$.

Table 4 Effect of levonorgestrel plus quinestrol (EP) and levonorgestrel (P) and quinestrol (E) alone on the weight of uteri (including ovaries) in female Brandt's voles at different times after the end of treatment

Treatment group	No. days between end of treatment and autopsy		
	15	30	75
Control	0.28 ± 0.014 (n = 3)	0.29 ± 0.02 (n = 3)	0.3 ± 0.026 (n = 3)
P	0.28 ± 0.032 (n = 3)	0.31 ± 0.013 (n = 3)	0.3 ± 0.017 (n = 3)
E	0.25 ± 0.01 (n = 3)	0.27 ± 0.026 (n = 3)	0.29 ± 0.02 (n = 3)
EP	0.27 ± 0.048 (n = 3)	0.28 ± 0.023 (n = 3)	0.28 ± 0.036 (n = 3)

Table 5 Effect of levonorgestrel plus quinestrol (EP) and levonorgestrel (P) and quinestrol (E) alone on the fertility of female Brandt's voles at 30 and 75 days after the end of treatment

Group	No. females	No. males	Total litters	Litter size	Time to birth	Littering females (%)	Pup weight (g)
D30							
P	3	4	17	5.4 ± 0.21	28 ± 1.5	75	2.91 ± 0.19
E	3	3	18	6 ± 0.17	30 ± 2.2	100	2.84 ± 0.21
EP	3	4	15	5 ± 0.11	32 ± 2.3	75	2.99 ± 0.16
Control	3	3	20	5 ± 0.27	28 ± 1.6	100	3.1 ± 0.25
D75							
P	3	3	17	5.6 ± 0.11	28 ± 0.75	100	2.96 ± 0.37
E	3	4	17	5.6 ± 0.27	29 ± 1.17	75	3.04 ± 0.18
EP	3	4	15	5 ± 0.11	31 ± 1.36	75	2.93 ± 0.21
Control	3	3	19	5 ± 0.17	28 ± 1.6	100	3.0 ± 0.15

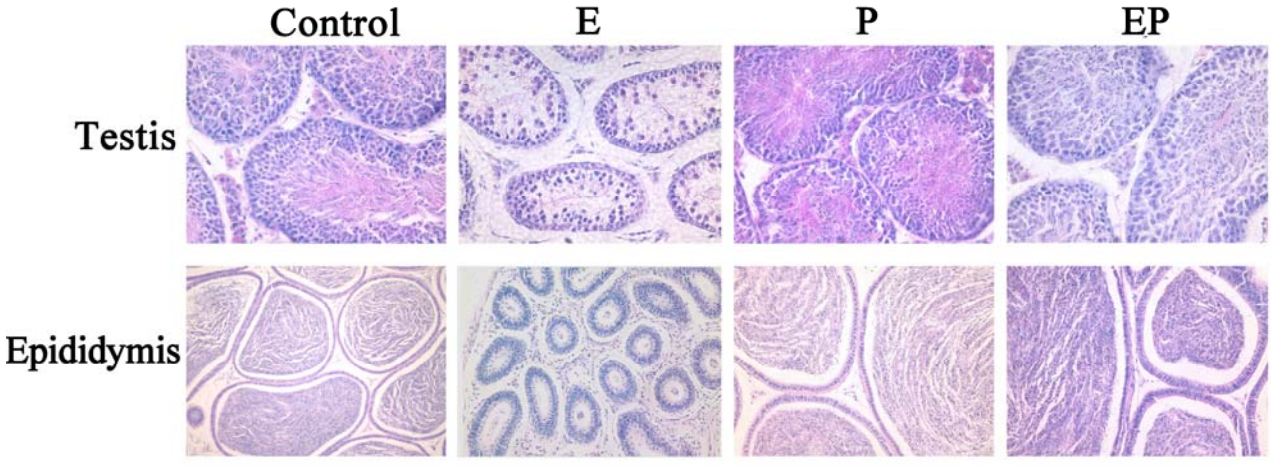

Figure 1 Structure of the testis and epididymis at 60 days after the end of treatment with levonorgestrel plus quinestrol (EP-1) and levonorgestrel (P) and quinestrol (E) alone. Hematoxylin and eosin stain; original magnification: ×100.

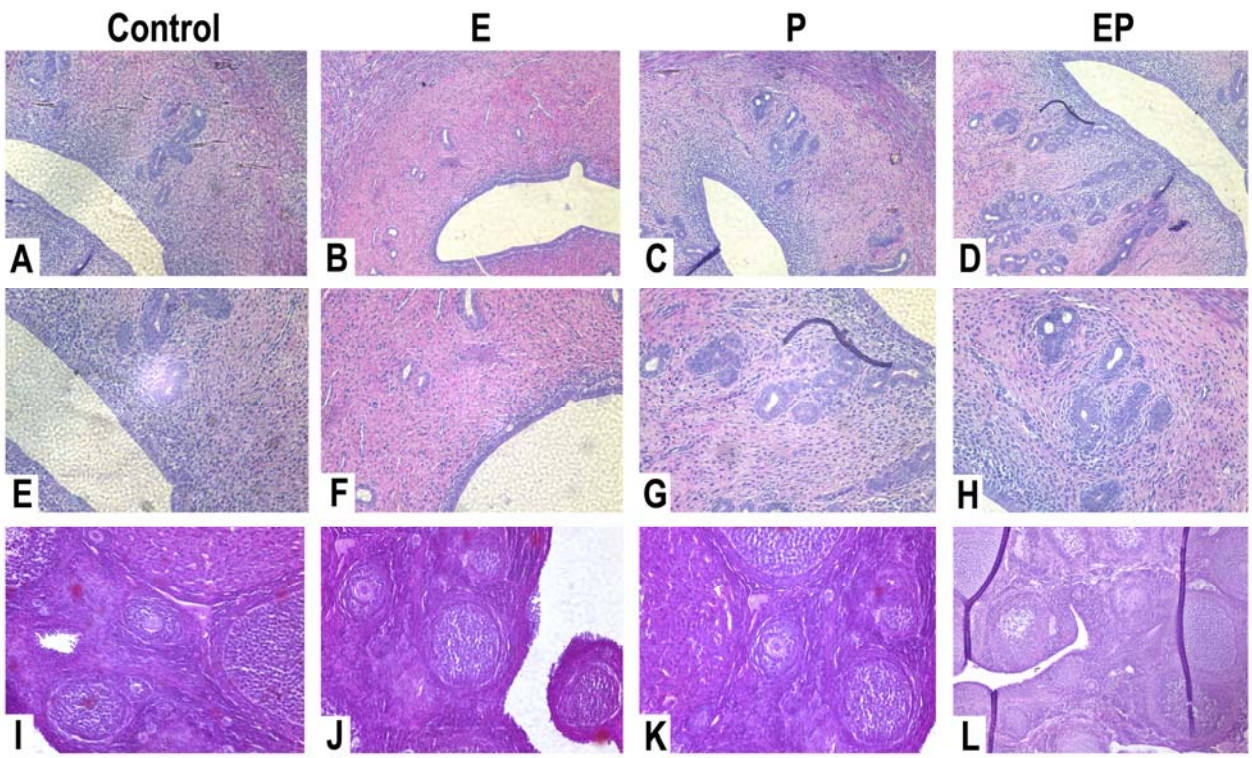

Figure 2 Histological features of the ovary (A-H) and uterus (I-L) at day 75 after the end of treatment with levonorgestrel plus quinestrol (EP) and levonorgestrel (P) and quinestrol (E) alone. Hematoxylin and eosin stain; original maginifcation: x100 for A-D and I-L; x200 for E-H.

voles may be short-lived and more continuous exposure may be required if this treatment is to be effective in the field. This response of female Brandt's vole differs from the responses of two other rodent species, which seem to be more sensitive to EP-1: female gray hamsters and mid-day gerbils had reduced litter sizes and pregnancy rates for up to 3 months after the end of treatment (Zhang *et al.* 2004).

However, male Brandt's voles were sensitive to treatment with quinestrol, but not levonorgestrel or EP-1. Treatment of male Brandt's voles with E for 5 or 14 consecutive days caused a significant decrease in the size of the testis and epididymis, damage to testicular histology and a decrease in sperm number. Mating with fertile females resulted in a lower pregnancy rate, litter size and pup weight. The contraceptive effect lasted at least 90 days after the end of treatment. The effect of quinestrol on male Brandt's voles seems to be similar to that of environmental estrogens on male infertility. There is a large body of data showing that male animals that are exposed *in utero* or perinatally to exogenous estrogens (e.g. diethylstilboestrol, ethinyl estradiol, bisphenol A) or anti-androgens (flutamide, vinclozlin, 1,1-trichloro-2,2-bis (p-chlorophenyl) ethylene) may develop hypospadias, undescended testis and/or low sperm counts. Mounting evidence from clinical observations and large epidemiological studies also indicates that male infertility may be related to environmental estrogens or estrogen mimics (Carlsen 1992).

It is also interesting that in this study we did not find a significant difference between treatment for 5 and 14 days in male voles. This indicates that a quinestrol dosage of 0.35 mg kg^{-1} body weight for male voles may be high enough for practical control in the field. This dosage is actually lower than that being tested in hamsters and gerbils, further indicating that male voles are very sensitive to quinestrol.

Our results for male and female Brandt's voles, plus the various results from other studies indicate a wide range of responses to EP-1 and/or its individual components. Some of the variations in responses appear to be related to dose and duration of exposure and whether the studies are undertaken in laboratory or field conditions. Zhang *et al*. (2006) reported that EP-1 affected the reproductive organs of male greater long-tailed hamsters (*Tscherskia triton*) in a laboratory experiment. One week and 3 weeks after the hamsters were fed with wheat bait containing 0.001% EP-1 for 6 consecutive days, the size and weight of the testis, seminal vesicle and epididymis were significantly reduced compared with those of the control animals. The data indicate that EP-1 is able to effectively reduce the fertility of both male and female hamsters.

Recently, Huo *et al.* (2006) reported that 1 mg kg^{-1} EP-1 administered by oral gavage for 3 consecutive days to mature female Mongolian gerbils (*Meriones unguiculatus*) affected uterine structure in more than 50% of animals. Uterine structure was severely disrupted by a dose of 5–10 mg kg^{-1}, and death occurred at a dose of 10–60 mg kg^{-1}. Field and laboratory tests (Liang *et al.* 2006) further confirmed the efficacy of EP-1 in reducing fertility in female and male Mongolian gerbils. In studies using semi-natural experimental enclosures, male and female animals were treated with EP-1, and the results revealed that one-pulse baits containing 0.001–0.003% EP-1 could effectively reduce the reproductive success of the greater long-tailed hamster (Zhang *et al.* 2005).

Wan *et al*. (2006) assessed the effect of EP-1 on recruitment of the Djungarian hamster (*Phodopus campbelli*) in a pasture of 800 ha in Abagaqi, Inner Mongolia. Wheat baits containing 0.01% EP-1 were delivered at a rate of 1.25 kg ha^{-1} in May 2004 at the beginning of the hamster breeding season. Wan *et al*. found that the uterine morphology of about 80% of mature females in the two baited areas was affected by EP-1 for more than 4 months, and the pregnancy rate and the litter size were reduced to 20% and 66% of the controls, respectively. It seems that EP-1 is more effective in hamsters in semi-natural or natural conditions compared with laboratory conditions. This is most likely to be due to the food-caching behavior of hamsters. This behavior leads to a more prolonged intake of EP-1 and therefore a more prolonged effect on reproduction (Zhang *et al.* 2005; Wan *et al.* 2006).

In a contraceptive model, Zhang (2000b) showed that sterile males indirectly contributed to a reduction in the pregnancy rate of females due to competitive interference with fertile males. This anti-fertility effect of sterile males mostly depends on the mating system of the focal species. For species with a strictly monogamous system or a polygynous system, the anti-fertility effect will be largest, but for species with a polyandrous or polygamous system, the anti-fertility effect of sterile males will become smaller. Thus, the anti-fertility effect of quinestrol on males may be related to the mating system of rodents. EP-1 has an anti-fertility effect on both males and females of some species (e.g. hamsters and gerbils). It is expected that EP-1 will yield better results than traditional culling.

Both levonorgestrel and quinestrol are synthetic ster-

oid hormone contraceptives. Levonorgestrel is a progesterone analog with a methyl introduced on C18. It has been widely used for emergency contraception in women. Human studies have demonstrated that levonorgestrel can cause pronounced alkalization of the intrauterine fluid and increased viscosity of the cervical mucus, leading to immobilization of sperm. The effect involves blockade or delay of follicular development and ovulation, and its "window of effect" seems to be rather narrow, beginning after selection of the dominant follicle, but before the luteinizing hormone surge. Levonorgestrel does not affect endometrial development or steroid receptor expression in the Fallopian tubes (Gemzell-Danielsson & Marions 2004). Treatment of rats and monkeys with levonorgestrel does not affect fertilization or implantation (Muller *et al*. 2003). In the present study, levonorgestrel alone did not cause a significant anti-fertility effect for either female or male Brandt's voles. However, its short-term effects need to be further elucidated considering its narrow "window of effect" in humans.

Quinestrol, a synthetic estrogen homolog, is the major component of long-term oral contraceptives for women. After ingestion, it can be stored in adipose tissue as proto type and then released slowly into the circulation. It is believed that quinestrol can inhibit the release of hypothalamus GnRH, causing disturbance of the hypothalamus–pituitary–ovary axis and inhibition of follicle development and ovulation. Little is known regarding the effect of quinestrol on male fertility. At present, it is unclear whether quinestrol affects androgen release via feedback through the hypothalamus and pituitary. In animals, a decrease in androgen may lead to testicular atrophy. On the other hand, it is well known that progesterone can antagonize the effect of estrogen. In the present study, the effect of quinestrol in male Brandt's voles was blocked by levonorgestrel, suggesting that P and E may work in opposing ways in the endocrine regulation of male reproduction.

In summary, the results of the present study have indicated that quinestrol alone has a significant anti-fertility effect in male Brandt's voles, and that levonorgestrel may act antagonistically against E. Our study suggests that quinestrol may be a good candidate for fertility control in Brandt's voles.

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