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# Ratio-dependent effects of quinestrol and levonorgestrel compounds (EP-1) on reproductive parameters of adult male Swiss mice

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ABSTRACT

Fertility control is considered as the second-generation pest rodent management strategy. Most previous studies have focused on the dosage-dependent effects of quinestrol and levonorgestrel compounds (EP-1) at a ratio of 1:2, but the ratio-dependent effects of EP-1 have not been fully investigated, especially in male rodents. To test the ratio-dependent antifertility effects of EP-1 with different ratios (1:2, 1:1, and 2:1) on male Swiss outbred strain of laboratory mice, forty male mice were randomly assigned into four groups ($n$ = 10). Mice in the three treatment groups were provided one of the three EP-1 mixture compounds for 3 successive days via gavage at a dosage of 50 mg/kg$_{(body\ weight)}$, and then all mice were sacrificed 15 days after the gavage treatment. Reproductive organ weights, sperm density and motility, levels of testosterone (T), estradiol (E2), luteinizing hormone (LH), and follicle stimulating hormone (FSH) in serum and/or testis, and androgen receptor (AR), estrogen receptor α (ERα), estrogen receptor β (ERβ), luteinizing hormone receptor (LHR), and aromatase in testis were determined. Each of the ratios of quinestrol and levonorgestrel significantly decreased the density and motility of sperm and induced atrophy of the epididymis and seminal vesicle. The combination of compounds also significantly reduced serum T and LH levels, increased testicular T levels and decreased testicular estradiol ERβ and aromatase levels. EP-1 delivered at a ratio of 1:1 induced the most significant effects on the reproductive parameters assessed and shows the potential for use in fertility control of male rodents.

## 1. Introduction

Fertility control is considered as a non-lethal and sustainable way to manage pest species populations (Knipling, 1959; Singleton et al., 1999; Zhang, 2000), and is referred to as the second generation of pest management strategies (Krebs, 2014). One common approach to control pest rodent fertility has been through the use of steroid hormones, particularly natural and synthetic oestrogens, progestins and androgens (e.g., Su et al., 2015, 2016, 2017; Chen et al., 2017; Massawe et al., 2018), which may disturb the normal endocrine function of the hypothalamic-pituitary-gonadal axis. Nevertheless, not all endocrine disrupting steroid hormones meet the requirement for effective fertility control of pest rodents, since many of these compounds have poor palatability, are short-acting, have many side effects, and are expensive. Notably, compounding several drugs could alleviate some problems which occur when compounds are used individually.

The combination of quinestrol and levonorgestrel is widely used in contraception for woman. In the once-a-month contraceptive pill (10 mg) for women, the ratio of quinestrol and levonorgestrel is 1:2, and this exerts good contraceptive efficacy with few side effects, such as nausea, vomiting, dizziness, and headaches (Fang et al., 2007). Using a higher dosage, Zhang et al. (2004) reported the antifertility effects of quinestrol and levonorgestrel compounds (EP-1, 1:2) on three wild rodent species in laboratory tests. Later on, Zhang et al. (2005, 2006) showed that a single baiting (EP-1, 1:2) can inhibit reproduction in male and female greater long-tailed hamster (*Tscherskia triton*) in semi-natural enclosure tests. Wan et al. (2006) reported that EP-1(1:2) showed significant antifertility effects on desert hamsters (*Phodopus campbelli*) in the grasslands of Inner Mongolia. By now, many laboratory and field tests have confirmed that EP-1 is effective in reducing the fertility of many wild rodent species (e.g. Zhao et al., 2007; Liu et al., 2012a, 2012b; Fu et al., 2013; for review, see: Zhang, 2015).

Most previous studies have focused on the dose-dependent effects of EP-1 at a ratio of 1:2 of quinestrol and levonorgestrel, but the ratio-

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dependent effects and the endocrinale mechanism of EP-1 have not been fully investigated. The effects of the three ratio groups (1:2, 1:0 and 0:1) of quinestrol and levonorgestrel on the fertility of Brandt's voles (*Lasiopodomys brandtii*) and Plateau pika (*Ochotona curzonia*) have been assessed (Zhao et al., 2007; Liu et al., 2012b). Massawe et al. (2018) evaluated the effects of EP-1 of the three ratio groups (1:0, 1:1, and 0:1) on the reproduction of *Mastomys natalensis* in Africa. However, the results of these studies are not consistent, and the ratio-dependent antifertility effects of EP-1 remains equivocal especially in male rodents.

In this study, we compared the effects of different ratios (1:2, 1:1, and 2:1) of quinestrol and levonorgestrel (EP-1 at 50 mg/kg body weight) on reproductive organ weights, sperm density and motility, levels of testosterone (T), estradiol (E2), luteinizing hormone (LH), and follicle stimulating hormone (FSH) in serum and/or testis, and androgen receptor (AR), estrogen receptor α (ERα), estrogen receptor β (ERβ), luteinizing hormone receptor (LHR), and aromatase in testis of male Swiss mice. The objective is to determine an optimal EP-1 formulation for rodent fertility control.

## 2. Materials and methods

### 2.1. Animals and drugs

Adult Swiss mice were purchased from the Guangdong Medical Laboratory Animal Center, and then bred in the laboratory of the Guangdong Institute of Applied Biological Resources. When the male offspring were sexually mature, they were individually housed in plastic cages (30 cm × 20 cm × 16 cm) under controlled conditions of temperature (25 °C ± 1 °C), humidity (60%–70%) and light (from 08:00 AM to 20:00 PM). Mice were fed ad libitum with tap water and commercial mouse pellets (Guangdong Medical Laboratory Animal Center). All procedures involving animals were carried out in accordance with the Policy on the Care and Use of Animals, approved by the Ethics Committee of Guangdong Institute of Applied Biological Resources.

Quinestrol and levonorgestrel (purity 99.9%, Beijing Zizhu Medicine Co., Ltd., Beijing, China) were firstly dissolved in sunflower seed oil, and put it in an ultrasonic cleaner (working frequency 40 kHz) for 15 min and let it dissolve completely. Finally, the ratio of quinestrol and levonorgestrel mixture used was 1: 2 (c1 group), 1:1(c2 group), and 2:1(c3 group), and the concentration of mixture in three solutions was set at 2.94 mg/mL according to administration dosage, volume and body weight of mice.

### 2.2. Experiments

After 1 week of acclimatization, male mice (average weight, 47.0 g ± 0.5) were randomly assigned into four groups ($n = 10$ per each group). Mice in the three treatment groups were provided one of the three EP-1 mixture compounds for 3 successive days via oral gavage at a dosage of 50 mg/kg $_{(\text{body weight})}$. The dosage was selected on the basis of previous studies (Shen et al., 2011; Li et al., 2014) and our study (Chen et al., 2017). Mice were given about 0.8 mL drug solution once by using a reusable, straight, 2 in. long, stainless-steel feeding needle with a 2.25 mm ball with manual restraint. The control males (C) were given sunflower oil in accordance with the same protocol for the treatment groups.

The animals were sacrificed by decapitation between 0900 and 1100 h on day 15 after the end of the gavage treatment. Trunk blood was collected, and serum was extracted by centrifugation at 4000*g* for 15 min at 4 °C before being stored at −20 °C for later hormone analyses. Epididymis, seminal vesicle, and testis were dissected and weighed. Testis were immediately frozen in liquid nitrogen and then stored at −80 °C for later analysis of hormones and receptors levels.

Sperm density was measured by hemocytometer count at ×400 magnification (Qin et al., 2015). One side of epididymal cauda was cut with scissors in 0.9% normal saline (37 °C) and prepared as 10% sperm solution. One drop was placed on a warmed hemocytometer, and a 22 × 22 mm cover slip was added. Microscopic fields were observed at 400 × magnification using a standard light microscope, and the percentage of motile sperm was determined. We evaluated at least 200 spermatozoa in at least five fields of vision. Sperm motility was graded as following: Grade A (rapid progressive motility), Grade B (slow or sluggish progressive motility), Grade C (non-progressive motility), and Grade D (immotility) (Qin et al., 2015; Massawe et al., 2018).

Commercial enzyme-linked immunosorbent assay kits (Shanghai Guyan Biotechnology Company) were used to test levels of testosterone (T), estradiol (E2), luteinizing hormone (LH), luteinizing hormone receptor (LHR), follicle stimulating hormone (FSH), androgen receptor (AR), estrogen receptor α (ERα), estrogen receptor β (ERβ), and aromatase. Determinations and procedures were performed in accordance with the recommended procedures by the manufacturer of commercial kits. For every test, just 10 μL serum or homogenate were used. Intra- and inter-assay variabilities were < 7.5% and 9.6% for all analysis, respectively.

### 2.3. Statistics analysis

All data were analyzed by using SPSS 16.0. Prior to statistical analysis, normality and homogeneity of data were tested using Kolmogorov–Smirnov and Levene tests, respectively. The weights of testis, epididymis, seminal vesicle, levels of hormones (T, E2, FSH, LH), receptors (AR, ERα, ERβ, LHR), and aromatase, sperm motility and density in different groups were analyzed by One-way ANOVA, following by LSD for post hoc multiple comparison analysis. Results were presented as means ± SE, differences at the 5% level or lower were considered significant.

## 3. Results

### 3.1. Reproductive organ weights

There were no differences in body mass of male mice between groups during experiment, so we did not correct organ weights for body mass. The weights of the testis did not significantly differ between the groups ($F_{3,36} = 1.590$, $P = .209$, Fig. 1a). The weights of the epididymis significantly decreased in all of the treatment groups compared with those in the control group (a reduction of 31.6%, 34.0%, and 32.7% for c1(1:2), c2(1:1) and c3(2:1) groups, respectively, Fig. 1b) ($F_{3,36} = 30.578$, $P < .001$). The weights of seminal vesicle also significantly decreased in all of treatment groups compared with those in the control group (a reduction of 69.5%, 70.8%, and 68.0% for c1, c2 and c3 groups, respectively, Fig. 1c) ($F_{3,36} = 94.188$, $P < .001$).

### 3.2. Sperm quality

The sperm density in all of the treatment groups significantly decreased by 27.7%, 42.6%, and 29.8% for the c1, c2 and c3 groups compared with those in the control group, respectively. The sperm density in the c2 group was significantly lower than those in the c1 and c3 groups ($F_{3,36} = 102.508$, $P < .001$, Fig. 2a). The number of rapidly progressive motile sperms ($F_{3,36} = 400.567$, $P < .001$), slowly or sluggishly progressive motile sperms ($F_{3,36} = 72.597$, $P < .001$), and nonprogressive motile sperms ($F_{3,36} = 42.243$, $P < .001$), in all of the treatment groups decreased compared with that of the control group, while the number of immotile sperms ($F_{3,36} = 525.755$, $P < .001$) significantly increased (Fig. 2b).

### 3.3. Hormone levels in serum

Serum T levels were significantly reduced in all of the treatment

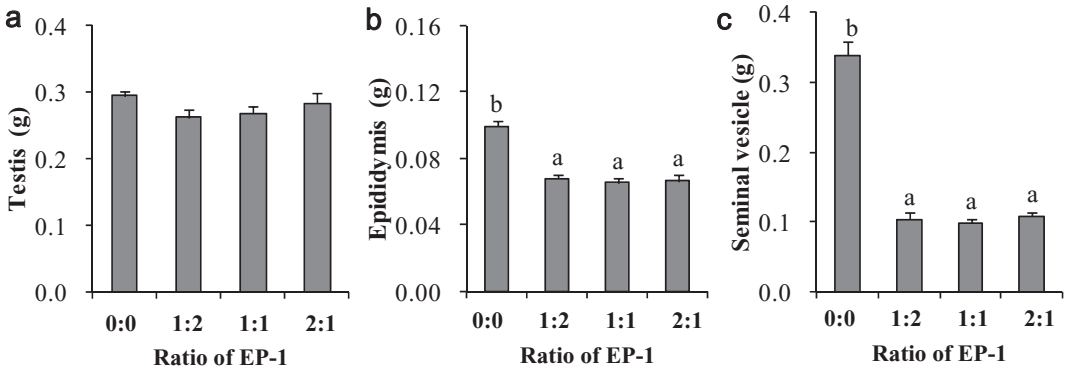


**Fig. 1.** Effects of different ratios of quinestrol and levonorgestrel on the weights (g, Means ± SE) of testis (a), epididymis (b) and seminal vesicle (c) in male adult mice. Different superscripts in each bar indicated significant differences ($P < .05$). For each group, $n = 10$. C (Control, quinestrol / levonorgestrel = 0:0); c1 (quinestrol / levonorgestrel = 1:2); c2 (quinestrol / levonorgestrel = 1:1); c3 (quinestrol / levonorgestrel = 2:1).

groups compared with those in the control group (a reduction of 10.7%, 5.8%, and 7.5% for the c1, c2, and c3 groups, respectively) ($F_{3,36} = 5.868$, $P = .002$, Fig. 3a). The serum LH level decreased near significantly in the c3 group compared with that in the control group (a reduction of 8.4%) ($F_{3,36} = 2.833$, $P = .052$, Fig. 3b). The serum LH levels in the c1 and c2 groups were not significantly different from that of the control group. The difference in the serum FSH level was not significantly different in all of the groups ($F_{3,36} = 1.632$, $P = .199$, Fig. 3c).

### *3.3.1. Hormone and receptor, aromatase levels in the testis*

Testicular T levels in the c1 and c2 groups increased by 17.9% and 24.3% compared with those in the control group ($F_{3,36} = 3.569$, $P = .023$, Fig. 4a). Testicular E2 levels in the c1 and c2 groups were reduced significantly by 6.9% and 9.3% compared with those in the control group ($F_{3,36} = 5.619$, $P = .003$, Fig. 4b). Testicular aromatase levels in the c2 group were reduced significantly by 8.2% compared with those in the control group ($F_{3,36} = 4.383$, $P = .010$, Fig. 4c). The testicular ERβ levels in the c1 and c2 groups were reduced significantly by 11.8% and 16.8%, but the testicular ERβ level in the c3 group increased significantly by 29.3% compared with that in the control group ($F_{3,36} = 53.344$, $P < .001$, Fig. 4d). No significant difference was observed in the testicular ERα levels ($F_{3,36} = 1.236$, $P = .311$) and AR levels ($F_{3,36} = 1.827$, $P = .160$) among different groups. The testicular LHR levels in the c2 group significantly reduced by 8.0% compared with those in the control group ($F_{3,36} = 8.669$, $P < .001$, Fig. 4e).

## 4. Discussion

This study showed that the weights of the epididymis and seminal vesicle but not that of the testes of male mice were significantly reduced by all the combinations of quinestrol and levonorgestrel tested. These findings are generally consistent with previous observations for various rodent species (e.g. Liu et al., 2012a, 2012b; Yang et al., 2012; Massawe et al., 2018). For example, Liu et al. (2012a, 2012b) found that EP-1 (1:2) significantly decreases the weights of the testis, epididymis, and seminal vesicle of male plateau pikas (*O. curzoniae*) in China. Massawe et al. (2018) reported that quinestrol and levonorgestrel combinations (1:1) induced atrophy of the testis, epididymis and seminal vesicle in male *M. natalensis* in Africa. However, studies on male Djungarian hamster (*Phodopus camplli*) (Wan et al., 2006) and Brandt's voles (*L. brandtii*) (Zhao et al., 2007) have shown that the combination (1:2) of quinestrol and levonorgestrel has no or small effect on the weight of male reproductive organs. These differences may be due to species specificity, dosages or methods used. Some studies have suggested that quinestrol accounts for most of antifertility effects on male reproductive organs of some species (e.g, Zhao et al. 2007; Liu et al., 2012a, 2012b; Liu et al., 2013; Massawe et al., 2018).

Estrogen treatment may impair spermatogenesis. A previous study showed that 17$\beta$-estradiol and environmental estrogen treatment negatively affects mammalian sperm function (Adeoya-Osiguwa et al., 2003). In human seminomas, excess estrogens induce an impairment of spermatogenesis (Carreau and Hess, 2010). Progesterone also elicits a regulatory effect on spermatogenesis (Liu et al., 2012a, 2012b; Massawe et al., 2018). In the present study, all EP-1 treatment ratios significantly decreased the sperm density and sperm motility, although the 1:1 EP-1 ratio induced the greatest reduction in sperm density in the male mice. These results support previous findings, such as EP-1 (1:2) treated male gerbils (*Meriones unguiculatus*) (Zhang et al., 2011) and male Yangtze vole (*Microtus fortis*) (Yang et al., 2012), and EP-1 with a 1:1 ratio of quinestrol and levonorgestrel treated on male *M. natalensis* (Massawe et al., 2018). Estrogen suppresses spermatogenesis by blocking the hypothalamic-pituitary-gonadal (H-P-G) axis (Sharpe et al., 2003), and progesterone also weakly affects androgen production

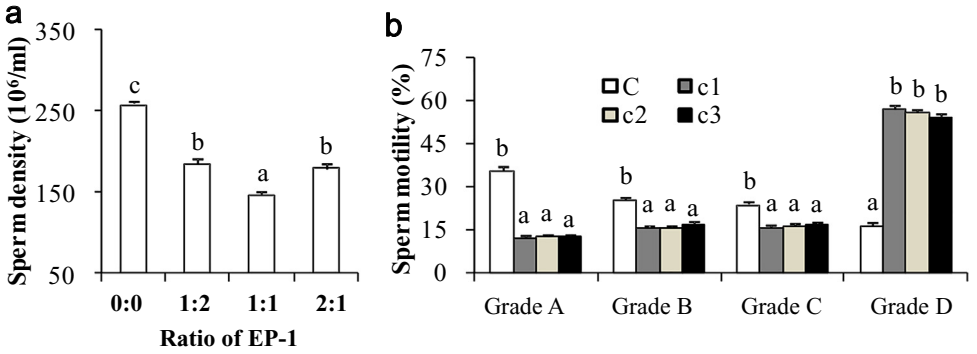


**Fig. 2.** Effects of different ratios of quinestrol and levonorgestrel on sperm density (a) and motility (b) in male adult mice. Results were presented as means ± SE. Different superscripts in each bar indicated significant differences ($P < .05$). Each group had 10 mice. Grade A (rapid progressive motility), Grade B (slow or sluggish progressive motility), Grade C (non-progressive motility), and Grade D (immotility). C (Control, quinestrol / levonorgestrel = 0:0); c1 (quinestrol / levonorgestrel = 1: 2); c2 (quinestrol / levonorgestrel = 1:1); c3 (quinestrol / levonorgestrel = 2:1).

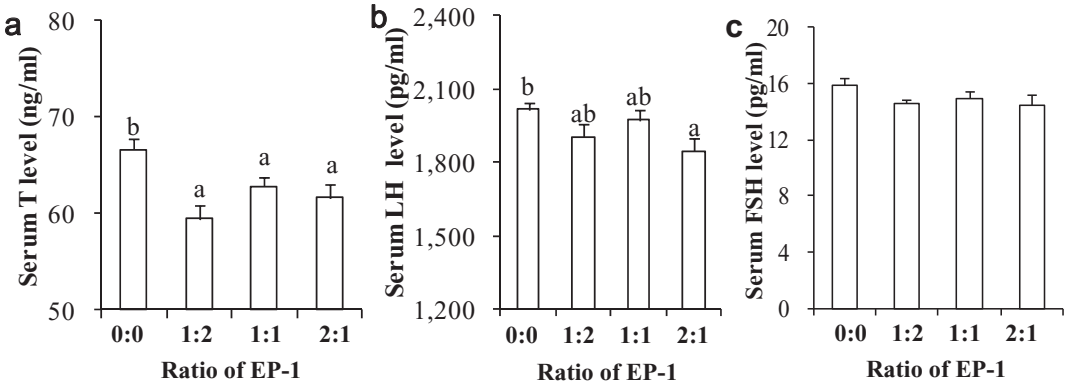


**Fig. 3.** Effects of different ratios of quinestrol and levonorgestrel on the serum T (a), LH levels (b), and FSH levels (c) in male adult mice. Results were presented as means ± SE. Different superscripts in each bar indicated significant differences ($P < .05$). Each group had 10 mice. C (Control, quinestrol / levonorgestrel = 0:0); c1 (quinestrol / levonorgestrel = 1: 2); c2 (quinestrol / levonorgestrel = 1:1); c3 (quinestrol / levonorgestrel = 2:1).

(Anderson and Baird, 2002). This study confirmed that serum T levels of male mice treated with quinestrol and levonorgestrel compounds were significantly decreased compared with those of the control male mice, indicating that the suppression of the H-P-G axis should be the main cause of reduction in sperm density. In another aspect, the serum LH level decreased remarkably at a ratio of 2:1, but this observation was not consistent with our observations of changes in sperm density and motility, suggesting that more complicated mechanism might be involved in the regulation of spermatogenesis among different ratios of quinestrol and levonorgestrel.

Spermatogenesis and sperm maturation are complex processes in the mammalian testis and epididymis, and they are supported by the coordination of steroid hormones. Spermatogenesis and maturation are not only mainly regulated by androgens (Notini et al., 2005), but also affected by estrogen. ERα knockout mice exhibited fluid re-absorption disorder in the epididymis, leading to abnormal sperm morphology and significant decreases in sperm density and motility (Hess et al., 1997). Treating GnRH-deficient mice (hpg mice) with estrogen induces spermatogenesis (Humphreys, 1977). Thus, estrogen benefited spermatogenesis by causing some local effects, and the balance of androgen and estrogen may be a key factor for successful spermatogenesis. Cytochrome P450 aromatase, the enzyme that catalyzes the conversion of androgen to estrogen, plays important roles in producing estrogen and maintaining the balance between androgens and estrogens (Carreau and Hess, 2010). Aromatase inhibition inevitably affects the fertilization ability of sperm (Luconi et al., 2002; Lazaros et al., 2012), and aromatase-deficient men show impaired spermatogenesis and low sperm motility (Rochira and Carani, 2009). Aromatase knockout mice display severe impairment in spermatogenesis and Leydig cell abnormalities (Robertson et al., 1999, 2001). In our study, the testicular T levels of mice treated with compounds at ratios of 1:2 and 1:1 significantly increased. Testicular aromatase, E2, ERβ, and LHR levels were the lowest in EP-1 at a ratio of 1:1. This finding might explain why EP-1 at a 1:1 ratio of quinestrol and levonorgestrel performed the best

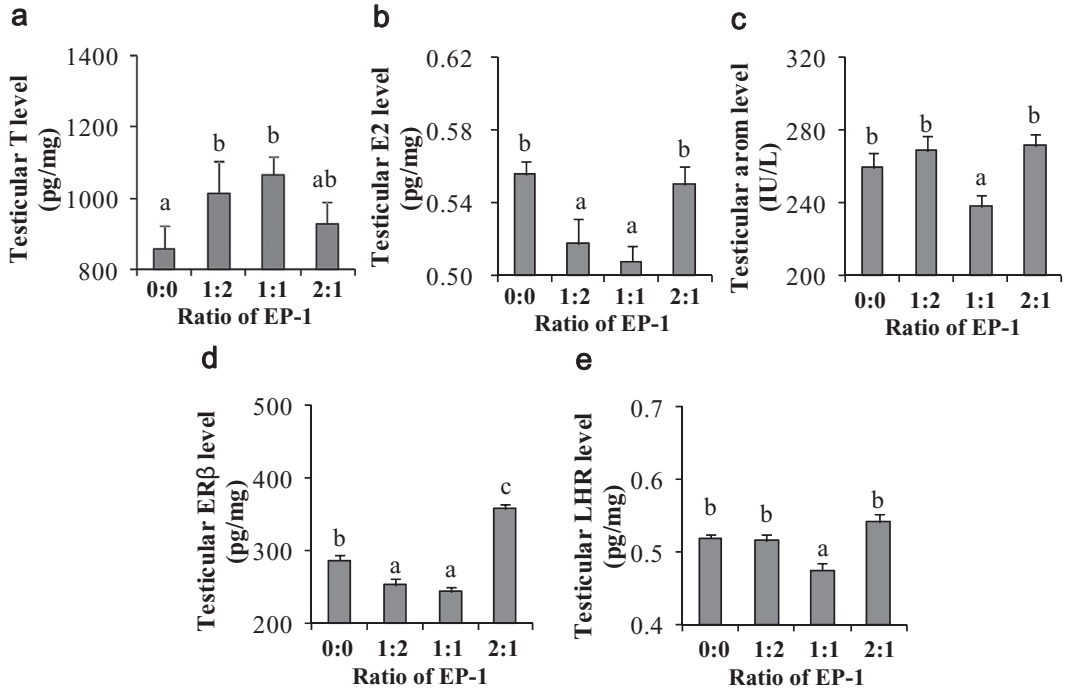


**Fig. 4.** Effects of different ratios of quinestrol and levonorgestrel on testicular T (a), E2 (b), ERβ (c), aromatase (d) and LHR (e) levels in male adult mice. Results were presented as means ± SE. Different superscripts in each bar indicated significant differences ($P < .05$). Each group had 10 mice. C (Control, quinestrol / levonorgestrel = 0:0); c1 (quinestrol / levonorgestrel = 1: 2); c2 (quinestrol / levonorgestrel = 1: 1); c3 (quinestrol / levonorgestrel = 2: 1).

in reducing the sperm density of male mice, that is, by disrupting the testicular hormones, their receptors, and cytochrome P450 aromatase, and by impairing spermatogenesis. However, the knowledge about the physiological effect of progesterone on male reproduction is limited. As such, the detailed mechanisms of EP-1 with different ratios should be further investigated.

The functions of the epididymidis and seminal vesicle should be considered. In higher vertebrates, sperms maturation occurs within the caput and corpus epididymidis. In this process, sperm change from an infertile status to a fertile status (Jones, 2004; Cornwall, 2009). Seminal vesicular secretion is essential not only for semen coagulation, sperm motility, and sperm chromatin stability but also for immune activity suppression in the female reproductive tract (Gonzales, 2001). Several products, such as fructose, potassium, bicarbonate, magnesium, 19-OH-prostaglandin, and prolactin of the seminal vesicles are stimulators of sperm motility (Gonzales, 2001). Steroid hormones regulate gene expression in the proximal mouse reproductive tract (Snyder et al., 2009), but detailed mechanisms are poorly understood except the function of ERα in the epididymidis. Further investigations in this area could contribute to the development of contraception in rodents and humans.

Estrogen and androgens bind to receptors and directly modulate various functions, such as spermatogenesis, maturation, and maintenance of spermatogonia, spermatocytes, round spermatids, Sertoli cells, Leydig cells (Hess, 2000; Luconi et al., 2002; Notini et al., 2005; Carreau and Hess, 2010). ERα knockout mice exhibit testicular dysgenesis, abnormal sperm morphology, and remarkable decrease in sperm density and motility (Hess et al., 1997). However, ERβ knockout mice develop normally, with the increase of age, urogenital duct abnormalities were arising, such as prostate and bladder hyperplasia and hypertrophy (Krege et al., 1998). In our study, EP-1 (1:2) and (1:1) treatment significantly decreased the testicular ERβ levels, whereas AR and ERα levels showed no remarkable differences. However, compound 3 treatment (ratio = 2:1) increased testicular ERβ levels. This study first demonstrated that ERα and ERβ responded differently to quinestrol and levonorgestrel treatment in the male mouse genital tract. Other reports showed that anti-estrogen treatment resulted in a dramatic decrease in ERα levels in the rat efferent duct, but no detectable changes were found in ERβ and AR (Oliveira et al., 2003). Bisphenol A (BPA) treatment decreased the expression of ERα but did not change the expression of ERβ in the testis of rats at the middle and high dosages of BPA or at all the dosages of E2 (Nakamura et al., 2010). Our findings were not consistent with these studies, so further investigation should be performed.

To our knowledge, this study provided clear evidence that different ratios of EP-1 could reduce sperm density and motility, and induce the atrophy of the epididymal and seminal vesicle in male mice. Our study highlighted the finding that EP-1 with quinestrol and levonorgestrel at a ratio of 1:1 showed the most profound effects on the reproductive parameters assessed among the combinations of the two compounds. This result suggested that levonorgestrel could enhance the effects of quinestrol which might be ratio dependent. Our study revealed the EP-1 reduces sperm density mainly by increasing testicular testosterone levels and decreasing testicular estradiol, ERβ, and aromatase levels, thereby interfering with the testosterone and estradiol balance mediated by aromatase. Therefore, EP-1 (at a 1:1 ratio of quinestrol: levonorgestrel) showed the potential for use in fertility control of male rodents.

## Declaration of Competing Interest

The authors have no conflicts of interest.

## Acknowledgements

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