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Characterization of the ovarian and reproductive abnormalities in prepubertal and adult estrogen non-responsive estrogen receptor α knock-in (ENERKI) mice

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Abstract

Estrogen non-responsive estrogen receptor alpha (ER α) knock-in (ENERKI) mice have a mutation (glycine 525 to leucine, G525L) in the ligand-binding domain of ER α . The mutant ER α protein has a significantly lower affinity and response to endogenous estrogens, while not altering growth factor activated ligand-independent pathways. ENERKI females demonstrated signs of early follicle development as determined by a significant increase in antral follicle formation by 20 days of age. Adult ENERKI females were infertile, had hemorrhagic ovarian follicular cysts, and failed to develop corpora lutea in response to a superovulation regimen. These results illustrate the importance of ER α ligand-induced signaling for ovarian development and for estrogen feedback on the hypothalamus and pituitary. Although ER α ligand-induced signaling by endogenous estrogens is lost in ENERKI females, the ER α selective agonist propyl pyrazole triol (PPT), a synthetic nonsteroidal compound, is still able to activate G525L ER α *in vivo* to increase uterine weight. To test whether PPT could restore ligand-dependent receptor activation, ENERKI females were treated with PPT and evaluated for spontaneous ovulation, ovarian hemorrhagic cysts, and LH serum levels. Daily PPT treatments beginning on day 4 of life prevented formation of ovarian hemorrhagic cysts in adult ENERKI animals. In accordance with this result, preputial gland weight and LH levels were also lowered in these animals, indicating PPT treatments most likely led to restoration of ER α negative feedback of the hypothalamic-pituitary axis.

Keywords

estrogen receptor; ovary; hypothalamic-pituitary axis; reproduction; ovarian function; estrogen

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Introduction

The ovary houses individual follicular units that each contains an oocyte and the surrounding cells necessary for maturation and ovulation. Follicles are the primary source of estradiol production, as well as a target of action by estradiol. Estradiol binds to the estrogen receptor (ER), which is a ligand-inducible transcription factor that plays a crucial role in the growth, development, and differentiation of the female reproductive tract [1,2]. The two ER subtypes, ER α and ER β , are encoded on different chromosomes and have different tissue expression patterns [3]. Ovarian granulosa cells primarily express ER β and proliferate as the follicle matures, while theca cells express ER α and are the primary source of androstendione biosynthesis [4-6]. Estradiol regulates the pulsatile release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) via a classical negative feedback mechanism acting on both the hypothalamus and the pituitary gonadotrope cells. Estrogen also regulates positive feedback to induce the LH surge through stimulation of estrogen receptors in neurons of the rostral periventricular region of the brain that then innervate GnRH neurons in the hypothalamus [7].

The particular roles of ER α and ER β have been studied by disrupting one or both of the receptors in ER α knockout (α ERKO), ER β knockout (β ERKO), and ER α /ER β knockout ($\alpha\beta$ ERKO) mice [8-13]. Analyses of these mice revealed the critical role of ER in mediating folliculogenesis, ovulation, and negative feedback inhibition on the hypothalamus to reduce circulating levels of FSH and LH [10,14]. The phenotype of the α ERKO animals shows the importance of hypothalamic ER α expression for mediating the effects of negative feedback and LH suppression [15]. The α ERKO mouse develops preovulatory follicles that fail to spontaneously ovulate and progress into large, cystic, hemorrhagic structures, demonstrating the critical interplay between estrogen production and ovarian folliculogenesis [10,16,17]. Nonclassical ER α knock-in (NERKI) mice, which have a mutation in ER α that disrupts DNA binding, have also been generated [18]. The NERKI mice had less ovarian follicular cysts and lower LH than α ERKO animals, indicating the importance of nonclassical signaling via protein-protein interactions for aspects of negative feedback inhibition [19]. Neuronal and pituitary specific estrogen receptor knockout mice have both been generated and illustrate that mice deficient for ER α in either GnRH innervating neurons or the pituitary gonadotropes renders mice infertile [7,20].

We previously generated mice with an ER α mutation (glycine to leucine, G525L) that significantly reduced the ability of endogenous estrogens to activate transcription from ligand-induced G525L ER α pathways [21]. Because the ligand-binding pocket mutation in these estrogen non-responsive ER α knock-in (ENERKI) mice did not affect growth factor activation of the receptor, they could be used to distinguish between ligand-induced and ligand-independent ER α actions *in vivo*. ENERKI females developed immature and hypoplastic uterine tissues and only rudimentary mammary gland ductal trees [21]. The ENERKI ovaries contained no corpora lutea (CL), and the ovaries contained large, hemorrhagic, cystic follicles [21]. The phenotypes of ENERKI ovaries and uteri were similar to those of the α ERKO and NERKI mice [18,21], confirming ligand-induced activation of ER α is crucial in female reproductive tract development.

Although the G525L mutation significantly reduces ER α interaction with and response to endogenous estrogens, the ER α selective agonist propyl pyrazole triol (PPT), a synthetic nonsteroidal compound, is still able to activate G525L ER α *in vivo* [21]. Therefore, ligand-induced ER α signaling pathways may be regulated in these mice through PPT administration or withdrawal. ENERKI females injected with 10,000 μ g/kg PPT every fourth day from 4 days to 8 weeks of age exhibit uterine and mammary gland ductal development, but retain hemorrhagic cystic follicles in the ovary [21, data not shown]. The objectives of this study

were to characterize the ovarian phenotype of immature 3 week old ENERKI females to determine if ligand-induced signaling is required before puberty, formally examine if adult ENERKI females are infertile by performing continuous mating studies and superovulation experiments, and determine if daily administration of PPT could reverse the adult ENERKI ovarian phenotype.

Experimental

Animals

The generation of ENERKI mice has been previously described [21]. All procedures involving animals were conducted in accordance with the policies of the Institutional Animal Care and Use Committee at the University of Chicago. Animals were group housed in a barrier facility with light/dark cycles of 14:10 hours and provided food and water *ad libitum*. Mice were maintained on a regular diet (2918 Charles River Laboratories, Wilmington, MA).

Sample Collection and Histology

Female mice were sacrificed by CO₂ inhalation, followed by cervical dislocation, at 3 or 12 weeks of age. The ovaries were dissected, weighed, and fixed overnight in 10% neutral buffered formalin. Tissues were embedded in paraffin and sectioned at the Human Tissue Research Center (The University of Chicago, Chicago, IL). Standard hematoxylin and eosin (H&E) staining was then performed. Follicle counts were performed on 3 sections per ovary at equivalent depths each separated by 50 microns and the follicle and cyst numbers were averaged between the three sections to generate the average follicle number per section. Between 3-12 ovaries were evaluated in each group. Duplicate follicle counts were avoided by only counting the follicle if the oocyte was present in that section. Follicle counts were similar to those performed in other estrogen receptor mouse models [19,22]. Theca cell hypertrophy was evaluated as either present or not present in each section based on morphology of multiple layers of theca cells after H&E staining.

Serum Gonadotropin and Steroid Hormone Assays

Blood was collected by cardiac puncture from 3-week-old sacrificed animals. Coagulation was prevented by treating blood with heparin. Blood was centrifuged and plasma frozen at -80°C for later use. Serum radioimmunoassays (RIAs) were performed by Brigitte Mann at Northwestern University. Serum LH RIAs were performed using iodinated standards (rLH-RP3) and antisera (anti-rLH-S11) from the National Institute of Diabetes and Digestive and Kidney Diseases. Serum progesterone RIA was performed with the antibody coated tube kit (MP Biomedicals, Solon, OH) according to the manufacturer's directions.

Continuous Mating Studies

For the continuous mating studies, two 12-week-old female mice, one of which was a wild type proven breeder, were housed with a wild type male. The first visibly pregnant female in each cage was removed and placed in a separate cage to give birth so the cages would not become overcrowded. This female was returned to the harem mating cage after giving birth. Mice were mated for 2 months and the number of litters, pups per litter, and date of each birth were recorded. A female was considered fertile if she had one litter. At the end of the 2 month mating period, the remaining animals were sacrificed.

Superovulation

Superovulated mice (12 weeks) were injected intraperitoneally with 5 IU/mouse pregnant mare serum gonadotropin (PMSG) (Sigma-Aldrich) to promote follicle maturation, followed by 5 IU/mouse human chorionic gonadotropin (hCG) (Sigma-Aldrich) 48 hrs later to induce

ovulation. Mice were sacrificed 12-16 hrs after the second hormone injection and the ovaries, including fat pad, oviduct, and partial uterine tube, were fixed in formalin and processed as described above. Immature 3 week old mice were similarly superovulated, oviducts removed, flushed with PBS, and the oocytes counted 12-16 hours post hCG.

Long-Term Injection Studies

Mice were maintained on a soy-free diet (2919, Charles River Laboratories) for all experiments. Compounds were prepared in a vehicle of 2% ethanol, 10% Cremophor EL (Sigma-Aldrich, St Louis, MO), and 88% 1x PBS at defined concentrations, so that the treatment volume was 0.01 mL/g body weight. 4-5 animals were used for each treatment group. Female mice were subcutaneously injected with 0-100,000 µg/kg PPT (Obiter Research, Champaign, IL). Mice were injected daily from 4 days or 3.5 weeks of age to 8 weeks of age. Mice were then sacrificed and reproductive tissues were excised for analysis. Injections were started on day 4 to allow time for genotyping and to provide a large enough pup for injection.

Quantitative Real-Time PCR

Total RNA was prepared with TRIzol reagent according to the manufacturer's directions (Invitrogen). One microgram of RNA was treated with DNase I (Invitrogen) before being reverse transcribed using the Superscript III First-Strand Synthesis System (Invitrogen). Random hexamers were used to prime the cDNA synthesis reaction. The resultant cDNA products were diluted to 100 µl, and 5 µl cDNA was used in RT-PCR using the QuantiTect SYBR Green PCR kit (Qiagen). RT-PCR was performed with QuantiTect primers for ribosomal protein L13A (RPL13A) and ERα (Qiagen). The reactions were performed using the ABI 7300 Real-Time PCR System (Applied Biosystems) for 45 cycles (95C for 15 sec, 55 C for 30 sec, 72 C for 40 sec) after an initial 15-min incubation at 95 C. RNA levels were determined for ERα and RPL13A by comparison with standard curves generated from reference RNA (Stratagene, La Jolla, CA). ERα expression was then normalized to the reference gene RPL13A, and the relative expression was determined by normalizing to the WT control. The reported results represent the average $\pm$ SEM of triplicate samples and are representative of two independent experiments.

Statistical Analysis

All reported values represent the mean $\pm$ SEM. Differences were considered significant at $p < 0.05$ using factorial analysis of variance (ANOVA) with appropriate *post hoc* tests or the Fisher Exact test (Sigma Stat 3.5).

Results

ENERKI females exhibit early antral follicle development

αERKO follicles mature to the antral stage prior to puberty and mice exhibit elevated serum LH levels [22]. In order to determine whether prepubertal ENERKI mice develop similarly, ovaries from 18-20 day old females were isolated and evaluated for follicle class and number. Secondary follicle number did not differ significantly between the genotypes (Figure 1). A significant increase in the number of antral follicles was seen in the ENERKI animals (Figure 1). The antral structures were determined to be pre-cystic based on signs of granulosa cell atresia and a degenerate oocyte reminiscent of the hemorrhagic follicular structures seen in the older ENERKI mice (Fig. 1C). These early cysts in general had only one or two layers of granulosa cells. Serum LH levels from mice of all three genotypes were measured and on average ENERKI had higher LH levels than wildtype females (0.83 $\pm$ 0.29 vs. 0.44 $\pm$ 0.07 ng/mL) but this difference did not reach statistical significance, most likely due to an increase

in the variation of LH pulse amplitude and frequency in ENERKI animals in the absence of estrogen ligand signaling.

ENERKI females are infertile

Adult ENERKI female ovaries contain no corpora lutea [21]. Vaginal smear cytology analysis revealed ENERKI mice were in a persistent diestrus (data not shown) consistent with other models of ER α insufficiency and consistent with a lack of endometrium responsiveness due to the receptor mutation [19]. Continuous mating studies were performed to test this hypothesis, and confirmed female ENERKI infertility. While all of the wild type and heterozygous females were fertile, with a similar number of pups per litter and days until the arrival of the first litter, none of the ENERKI females gave birth (Table 1). In order to test whether ENERKI mice are capable of induced ovulation, a superovulation regimen was implemented. Adult female mice (12 weeks) were treated with exogenous pregnant mare serum gonadotropin (PMSG) to promote follicle maturation. 48 hours later, mice were injected with human chorionic gonadotropin (hCG) to induce ovulation. All of the wild type ovaries contained multiple corpora lutea, while the ENERKI ovaries contained none (Figure 2), indicating they did not respond to superovulation treatments. Next superovulation of immature animals was performed. Females 21-25 days of age were similarly superovulated and 14-16 hours following hCG the ovaries and oviducts were collected and the number of oocytes counted. Ovaries examined histologically contained corpora lutea from wildtype animals while ENERKI mice had mostly unruptured antral follicles or cystic lesions (Figure 2). ENERKI mice had an average of 4 +/- 2 oocytes per mouse while wild type animals had significantly more oocytes 31 +/- 12 (p = 0.02).

Daily PPT treatments prevent formation of ENERKI hemorrhagic cystic follicles

ENERKI females were previously injected with 10,000 $\mu\text{g/kg}$ PPT every fourth day beginning at 4 days or 3.5 weeks of age in an attempt to induce normal reproductive tract development [21]. While this PPT treatment schedule restored the development of normal uterine and mammary gland tissues, it did not prevent the formation of ENERKI hemorrhagic cystic follicles [21]. Therefore in the present study, daily PPT injections were implemented. Mice were injected daily (10,000 $\mu\text{g/kg}$) from either day 4 or 3.5 weeks of age until 8 weeks of age with vehicle or PPT. A subset of these animals was injected every fourth day with a higher dose, 100,000 $\mu\text{g/kg}$ PPT, to mimic the estrous cycle surge. As anticipated, mice treated with vehicle developed the typical ENERKI ovarian phenotype (Table 2 & Figure 3A). Daily PPT treatments started at day 4 of life successfully prevented hemorrhagic cyst formation (Table 2 & Figure 3B). When daily injections were not started until week 3.5 of life, hemorrhagic structures remained present in the ovaries (Figure 3C). Females injected daily from 3.5 weeks of age but with an additional 100,000 $\mu\text{g/kg}$ PPT surge every fourth day did not develop hemorrhagic cysts (Table 2 & Fig. 3D). The absence of corpora lutea indicated none of the treatments induced spontaneous ovulation (Fig. 3A-D). Theca cell hypertrophy, normally seen in ENERKI and ERKO females [21,23], was eliminated with both these treatments. ENERKI females from all three PPT treatment groups had significantly smaller preputial glands than vehicle treated animals (Table 3), indicating their testosterone levels may have been lower. Supporting this hypothesis, daily PPT injected females also had lower serum LH levels than vehicle treated mice (Table 3). Previously it was demonstrated that adult ENERKI females normally have significantly elevated LH, E2, and T levels, but normal FSH levels [21]. No significant difference was found between wild type (30.9 +/- 9.4 ng/mL, n=6) and ENERKI (49.2 +/- 9.4 ng/mL, n=11) serum progesterone levels. Quantitative real-time PCR showed relative mRNA levels of mutant ER α were significantly lower in ENERKI (4.4 +/- 0.5) versus wild type animals (15.9 +/- 3.6, p < 0.01). As anticipated, all PPT treatments reduced ENERKI body and fat pad weights (data not shown). Studies performed in ENERKI mice injected with 100,000 $\mu\text{g/kg}$ PPT every fourth day demonstrated some toxicity as mice appeared

malnourished and unhealthy. In agreement with this finding, high E2 treatments have been shown to induce anorexia and weight loss in mice by poorly understood mechanisms [24].

Discussion

Analysis of ENERKI mice confirms the importance of ligand-induced ER α signaling in the development and fertility of the female reproductive system. Previously, elimination of the receptor revealed its importance in regulating LH levels through negative feedback [16,17, 23,25,26]. Chronic administration of gonadotropin releasing hormone (GnRH) antagonists demonstrated that hemorrhagic cysts of the ovary in α ERKO mice could be prevented by blocking LH secretion [22]. In addition, NERKI mice, which have a mutant form of ER α that fails to bind DNA, had lower serum LH levels as compared to α ERKO mice, suggesting that ER α mediated negative feedback depends both on estrogen ligand binding and nonclassical protein-DNA interactions [19]. The results from the α ERKO, NERKI, and ENERKI studies indicate that ER α must bind estradiol in order for LH levels and hemorrhagic cysts to be reduced. By treating ENERKI females with PPT, an ER α specific agonist, the ovarian phenotype was reversed and cysts were eliminated in the present study. Therefore, the role of ER α that prevents ovarian hemorrhagic follicular cysts and mediates negative feedback requires ligand-induced activation.

ENERKI females injected daily with PPT from 4 days of age or with 100,000 μ g/kg surges from 3.5 weeks of age did not develop hemorrhagic cysts, demonstrating that an ER α -specific and potent estrogen can reverse the ENERKI ovarian phenotype. ENERKI preputial glands, which produce pheromones that are secreted in the urine, are most likely large and masculinized due to elevated serum testosterone levels. Therefore, the drastic reduction in preputial gland weights to wild type levels in the daily PPT-treated mice may be due to lowered testosterone levels and lower LH [27]. These data indicate daily PPT treatments most likely led to restoration of ER α negative feedback of the hypothalamic-pituitary axis and negated hemorrhagic cyst formation in these females [7,28]. In addition, the α ERKO neuron specific knockout does not spontaneously ovulate while the pituitary specific knock mouse does spontaneously form corpora lutea, pointing toward neuronal regulation as critical estrogen targets for permitting spontaneous ovulation [7,20,28]. The absence of hemorrhagic cysts in pituitary and neuron specific α ERKO animals but present in the ENERKI and α ERKO suggests that the presence of a functional estrogen receptor in the ovary may properly regulate vascularization by preventing hemorrhaging when an antral follicle is differentiating into the CL [7,20,23,28]. Interestingly, NERKI mice have LH levels that are higher than wildtype mice but significantly lower than ER α KO mice, as well as hemorrhagic cysts but significantly fewer than ER α KO. This may further suggest ligand induced ERE-independent signaling is critical for blocking hemorrhaging in the ovary after ovulation. Absence of hemorrhagic follicles in animals injected daily with a 100,000 μ g/kg surge of PPT every fourth day likely represents activation of the mutant receptor by the ER α agonist. A relatively high dose of PPT was needed to reverse the ENERKI ovarian phenotype because PPT is as efficacious as E2 in animals, but much less potent [29]. Similar PPT doses were needed to elicit a uterotrophic response in immature ENERKI females as well [21]. In addition, mature ENERKI animals had significantly lower ovarian ER α transcript levels than wild type animals, which may have also contributed to the reason such high levels of PPT were needed to prevent ovarian hemorrhagic cysts.

The lack of spontaneous ovulations was confirmed to render the animals sterile based on the continuous mating experiments. Although PPT treatments were able to reverse ovarian cysts formed in ENERKI females, the formation of corpora lutea was never detected in these animals. This inability to spontaneously form corpora lutea is likely due to the lack of positive feedback [7,28]. Regulation of the intra-follicular feedback loop by PPT might have resulted in the reduction of theca cell hypertrophy in the ovary [30], but failed to initiate corpora lutea

formation [31]. Gonadotropin induced superovulation was able to generate corpora lutea as previously demonstrated in α ERKO and pituitary specific α ERKO mice [20,23]. However, the presence of an estrogen responsive splice variant might be responsible for corpora lutea formation in the original α ERKO engineered by Korach and colleagues because ER α KO mice that lack all forms of the receptor fail to produce corpora lutea [10]. All splice variants in an ENERKI mouse would contain the point mutation that blocks estrogen binding, so these data suggest that the inability for full ER α or any splice variants to bind estradiol in the ENERKI mouse blocks corpora lutea formation in the ovary. All ER α mutated models that lack positive feedback either due to global ER deletion, neuronal deletion, or loss of ERE-dependent signaling block corpora lutea formation [7,10,19,20]. In the neuron specific ER knockout mice despite having normal LH levels, ovaries appear hyperstimulated and do not form corpora lutea in the context of lacking positive feedback. LH hypersecreting mice have ovarian cysts but an abundance of corpora lutea indicating that functional ovarian ER α even in the presence of high LH can form a CL [32]. The formation of the corpus luteum must partially rely on the proper timing and dose of PPT to generate positive feedback and future studies should be conducted to determine if positive feedback and corpora lutea formation could be restored in the ENERKI mouse.

The ENERKI mouse reproductive phenotype sheds some light on the critical role for ER α to bind estradiol. Because the ovarian phenotype of the ENERKI mouse so closely resembles both ERKO α mouse models, these data suggest that ligand independent activation of the receptor is not critical for ovarian function. Also, recent generation of a membrane only estrogen receptor mouse (MOER) illustrates that the loss of nuclear receptor, loss of ligand binding, or total elimination of ER produces mice that are infertile with ovarian hemorrhagic cysts, and high circulating LH [33]. Recently the total deletion of ER β was reported to induce infertility in mice [9]. The lack of oocytes present after superovulation of ER β but present after superovulation of ER α mice demonstrates that ER β might play a role in ovulation while ER α facilitates CL formation [9]. ER β receptor mRNA was upregulated in the ENERKI uterus providing evidence that the resulting problems with fertility reflect the absence of ER α function [21].

In summary, ENERKI females demonstrated signs of precocious puberty and were infertile. Superovulation generated oocytes in the oviduct but did not produce CLs in the ovary. Daily PPT treatments prevented formation of ovarian hemorrhagic cysts in adult ENERKI animals, but also failed to generate corpora lutea. These studies have therefore led to an improved understanding of the role of estrogen- and growth factor-induced ER α signaling in prepubertal and adult ENERKI ovarian tissues.

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Figure 1.

Ovarian follicle development in prepubertal ENERKI mice. Ovarian tissue H&E staining from immature 18-20 day old wild type (A), heterozygous (B), and ENERKI (C) mice (100X). The number of secondary follicles, antral follicles, and early cysts were counted in both ovaries in three representative sections per animal. (D) Average number of follicles per section examined for each genotype. ENERKI females had a higher number of antral and cyst-like follicles than their wild type (WT) and heterozygous (HET) littermates (D). a, ENERKI value significantly different from WT value ($p < 0.01$). b, ENERKI value significantly different from WT and HET value ($p < 0.01$).

Figure 2. Superovulation Experiments

ENERKI ovaries fail to develop corpora lutea after superovulation. 12-week-old animals were injected with PMSG and hCG in a standard superovulation regime. Ovarian tissue H&E staining from superovulated 12 week old animals indicated wild type (A) ovaries contained many corpora lutea (CL) and healthy follicles, while ENERKI (B) ovaries had no corpora lutea and the majority contained large hemorrhagic cysts (100X). Immature animals were also superovulated. Wild-type animals generated corpora lutea (C), while immature ENERKI mice (D) has partially luteinized, hemorrhagic structures that produced oocytes in the oviduct but did not fully form a corpora lutea.

Figure 3. Long-Term PPT Injections

Long-term daily PPT injections prevent formation of ovarian hemorrhagic cysts in ENERKI mice. Groups of 4-5 ENERKI females were injected subcutaneously with vehicle from 4 days of age (A), 10,000 µg/kg PPT from 4 days of age (B), or 10,000 µg/kg PPT from 3.5 weeks of age (C), everyday until 8 weeks of age. A subset of animals injected with 10,000 µg/kg PPT from 3.5 weeks of age was injected with 100,000 µg/kg PPT every fourth day to mimic the estrous cycle surge (D). Ovarian tissue H&E staining (100X) revealed daily vehicle (A) and 10,000 µg/kg PPT treatments from 3.5 weeks of age (C) exhibited the same phenotype. Hemorrhagic cyst formation was prevented in ENERKI mice treated with 10,000 µg/kg PPT from 4 days of age (B) or 100,000 µg/kg PPT surges every fourth day (D).

Table 1

Continuous Mating Studies

12 Week Mice	% Fertile	Pups per Litter	Days to First Litter
WT, n=5	100 +/- 0	7.8 +/- 0.6	26.4 +/- 4.4
HET, n=5	100 +/- 0	7.6 +/- 0.9	23.2 +/- 1.0
ENERKI, n=5	0 +/- 0 ^a	N/A	N/A

Data expressed as mean (+/- SEM).

^aENERKI value significantly different from WT and HET value (p<0.001).

Table 2**PPT Injections**

Ovary characteristics of treated ENERKI females

Mice	Treatment	Injection Schedule	Injection Period	# Cystic Follicles
ENERKI n=4	Vehicle	daily	4 days to 8 weeks	3.75 +/- 1.44
ENERKI n=3	10,000 µg/kg PPT	daily	3.5 weeks to 8 weeks	2.00 +/- 1.00
ENERKI n=5	10,000 µg/kg PPT	daily	4 days to 8 weeks	0.00 +/- 0.00 ^a
ENERKI n=4	10,000/100,000 µg/kg PPT	daily/every 4th day	3.5 weeks to 8 weeks	0.00 +/- 0.00 ^a

Data expressed as mean (+/- SEM).

^aValue significantly different from all other values (p < 0.01).

Table 3
PPT Injections
Preputial gland weights and LH levels of treated ENERKI females

Mice	Treatment	Injection Schedule	Injection Period	Preputial Gland (% body weight)	LH (ng/mL)
ENERKI n=4	Vehicle	daily	4 days to 8 weeks	0.14 +/- 0.01 ^a	5.66 +/- 1.67 ^a
ENERKI n=3	10,000 µg/kg PPT	daily	3.5 weeks to 8 weeks	0.01 +/- 0.00	0.17 +/- 0.07
ENERKI n=5	10,000 µg/kg PPT	daily	4 days to 8 weeks	0.01 +/- 0.00	0.13 +/- 0.03
ENERKI n=4	10,000/100,000 µg/kg PPT	daily/every 4th day	3.5 weeks to 8 weeks	0.01 +/- 0.00	< 0.10 [*]

Data expressed as mean (+/- SEM).

^a Value significantly different from all other values (p < 0.01).

^{*} Limit of detection for assay.