Effects of Levonorgestrel on Reproductive Hormone Levels and Their Receptor Expression in Mongolian Gerbils (*Meriones unguiculatus*)

Xiao-Hui LV and Da-Zhao SHI

College of Agriculture and Biotechnology, China Agricultural University, Beijing 100193, China

**Abstract:** The effects of levonorgestrel (LNG) on serum levels of reproductive hormones and their receptor mRNA expression in the ovary and uterus of Mongolian gerbils were examined. The results show that serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) increased, whereas serum estradiol (E2) and progesterone (P4) decreased profoundly after LNG treatment. LNG down-regulated the mRNA expression of follicle-stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR), estrogen receptor (ER) β and progesterone receptor (PR) in the ovary, and ERα and PR in the uterus of Mongolian gerbils. The down-regulated effects were time-dependent and dose-dependent. LNG had no obvious effects on ERα mRNA expression in the ovary. The findings suggest that LNG impairs reproductive hormone receptor expression at the molecular level in Mongolian gerbils. Also, the two ER subtypes may play different roles in the ovary, and ERβ may not be the predominant ER subtype in the uterus of Mongolian gerbils. The ovary and uterus may be the important sites of action of LNG through its direct progesterone-like effects in Mongolian gerbils.

**Key words:** levonorgestrel, reproductive hormone receptors, reproductive hormones

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**Introduction**

The Mongolian gerbil (*Meriones unguiculatus* Milne Edwards, 1867) belongs to the subfamily *Gerbillinae*, and is mainly distributed across the arid steppes, semi-deserts and adjacent farming-pastoral areas of North China, Mongolia, and the Baikal Lake region of Russia [17, 25]. The Mongolian gerbil has been extensively used as an experimental animal model in neuroscience, physiology, reproduction, and behavioral research [25]. Additionally, Mongolian gerbils cause serious damage to crops in farming areas when they are present in large numbers and they are the main reservoir host of *Yersinia pestis*, which causes plague [17].

Levonorgestrel (LNG) is a synthetic progestin widely used for hormonal contraception, alone or combined with estrogen. The mechanisms of LNG include actions affecting follicular development, ovulation, corpus luteum formation, sperm motility, fertilization, blastocyst implantation and endometrial function [7, 20, 22]. The activity of the substance varies with species, the dose given and the route of administration. Rats, mice, and other experimental animals have been used as a model to study the mode of action of LNG [1, 5, 11, 24, 29].
In rats, LNG inhibits ovulation whereas it has no effect on fertilization and implantation. In mice, decidualization of stromal cells, atrophy of glandular epithelium, down-regulation of ERα and PR in all cellular components of the grafted endometrium have been observed after LNG treatment. In rabbits, LNG inhibits ovulation and stimulates the endometrium. In cynomolgus monkeys, LNG inhibits ovulation associated with higher levels of serum FSH and LH and lower levels of serum E2. In dogs, LNG is a weak progestin and is more rapidly metabolized than in other species. However, the mode of action of LNG in Mongolian gerbils has not been reported. Reproductive hormones, such as follicle-stimulating hormone, luteinizing hormone, estrogen and progesterone are involved in various reproductive processes. Their actions are mediated through their corresponding receptors [35, 36], and the levels of the receptors directly affect hormonal actions. Therefore, we can gain an understanding of the mode of action of LNG in Mongolian gerbils by investigating the effects of LNG on the reproductive hormone levels and their receptor expression. The aims of this study were, first, to confirm whether LNG has effects on reproductive hormone levels and their receptor expression in the ovary and uterus of the Mongolian gerbil; second, to analyze whether the effects are time-dependent and dose-dependent; and third, to assess the possible effects of LNG on the ovary and uterus of the Mongolian gerbil.

**Materials and Methods**

**Animals**

The Mongolian gerbils used in this study were from an indoor colony bred from animals captured in the Xilinguole League of Inner Mongolia. The gerbils were maintained at 23 ± 1°C with automatically controlled lighting from 0700 to 2100 h (14 h light: 10 h dark). They were provided with a food mixture containing equal parts of corn and sunflower seeds, and they were given water *ad libitum*. Forty 4-month-old virgin female gerbils (55–65 g) were used in this study. The study was conducted according to Guidelines for Animal Experiments and approved by the Animal Care and Use Committee of the China Agricultural University.

**Experimental design**

Levonorgestrel (Zizhu Medicine Co., Ltd., Beijing, China) was dissolved in peanut oil.

In the time-dependent experiment, twenty gerbils were given LNG once at 100 µg/g body weight (BW) intragastrically. At 0, 2, 4, or 6 days following treatment, the gerbils were sacrificed by ether inhalation. The control group was sacrificed on day 0. The ovaries and uteri were collected for RNA extraction. Each experimental group consisted of five gerbils.

In the dose-dependent experiment, another twenty gerbils were intragastrically given LNG once at 0, 1, 10, or 100 µg/g BW. The control group was given peanut oil. The gerbils were sacrificed by ether inhalation 6 days after administration. The ovaries and uteri were collected for RNA extraction. Each experimental group consisted of five gerbils.

**Hormone assays**

Blood samples (0.5 ml) were collected via orbital venous puncture following light ether anesthesia [18] before euthanasia. Serum was separated by centrifugation at 1,000 × g for 20 min at 4°C and stored at –80°C until assayed.

Concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured using rat ELISA kits (EIAab Science Co., Ltd., Wuhan, China), as described and validated previously [27]. The minimum detectable dose was 0.078 mIU/ml for FSH and 0.195 mIU/ml for LH. The intra-assay variations for FSH and LH were 4.8%, while the inter-assay variations were 7.4%. Serum estradiol (E2) and progesterone (P4) levels were determined by a chemiluminescence immunoassay (CLIA) [18] using CLIA kits (North Institute of Biological Technology, Beijing, China). The minimum detectable dose was 4.0 pg/ml for E2 and 0.1 ng/ml for progesterone. The intra-assay and inter-assay variations for both were less than 15 and 20%, respectively.

**Quantitative real-time PCR analysis**

Total RNA was extracted from the ovaries and uteri using the RNAprep pure Tissue Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer’s directions. The concentration and purity of the
isolated RNA in each sample were measured using a nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was verified by visualization of distinct 18S and 28S rRNA bands after ethidium bromide staining in a 1.0% agarose gel. The extracted total RNA was stored at –80°C until used.

Levels of mRNA for reproductive hormone receptors in the ovary and uterus were measured by two-step quantitative real-time PCR. Complementary DNA was reverse-transcribed from total RNA samples in a 20 µl reaction mixture with the Quantscript RT Kit (Tiangen Biotech Co., Ltd.) following the manufacturer’s specifications.

Quantitative real-time PCR was carried out using RealMasterMix (SYBR Green I) (Tiangen Biotech Co., Ltd.) in 20 µl of reaction mixture on an ABI PRISM 7300 sequence detector system (Applied Biosystems, Foster City, CA, USA). The primers were designed using Primer Premier 5.0 software based on the sequences of Mongolian gerbils, corresponding receptors for β-actin (accession number AB177844.1; forward 5'-CCA TCT ATG AGG GCT ACG C-3', reverse 5'-ATG TCA CGC ACG ATT TCC-3'), FSHR (accession number AB303968.1; forward 5'-CGT CCT GAT GAG CAA GTT TGG-3', reverse 5'-TGG GCT GAT TGA CTT AGA GG-3'), LHR (forward 5'-CGA TTA TGG CTT CTG CTC AC-3', reverse 5'-CCA AGG ATG AGT AGG ATG TT-3'), ERα (forward 5'-GGA CAG GAA CCA GGG CAA G-3', reverse 5'-ACC CCC AGT TAA GCA AAA TGA-3'), ERβ (forward 5'-CGA GGA CAG TAA GAG CAA AGA GG-3', reverse 5'-AGG GTT ACA TGA CCA AGG CAG-3'), and PR (forward 5'-CTC CCT GTG CCT TAC CAT-3', reverse 5'-GGC TCC TCA GTC CTT CCA-3'). The thermal cycling conditions were 2 min at 95°C for initial denaturation, then 20 s at 95°C, 20 s at 60°C, and 33 s at 68°C for 40 cycles, followed by 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C for ramp dissociation. The expected product sizes for β-actin, FSHR, LHR, ERα, ERβ and PR were 145, 168, 135, 143, 153 and 128 base pairs respectively. The PCR products were electrophoresed through a 2.0% agarose gel and visualized with ethidium bromide. The melting curve was also analyzed to confirm the specificity of each primer. The relative amount of each mRNA was determined by the 2^ΔΔCT method [16] and normalized to an endogenous reference gene, β-actin. All the quantitative real-time PCR studies were repeated three times with triplicate determination of each sample.

Statistical analysis

Data distributions were analyzed for normality by the one-sample Kolmogorov-Smirnov test. The data were analyzed by one-way ANOVA with Tukey’s test for post hoc multiple comparison analysis. Values were considered statistically significant at P<0.05. The analyses were performed using SPSS 16.0 for Windows. Data are presented as means ± SEM.

Results

Levonorgestrel effects on serum reproductive hormone levels

In the time-dependent experiment (Fig. 1), serum FSH and LH increased after 100 µg/g BW LNG treatment and were significantly higher by day 6 (3.40 ± 0.35 mIU/ml, P<0.05, Fig. 1a) and by day 2 (5.54 ± 0.19 mIU/ml, P<0.05, Fig. 1b), respectively. Serum LH did not increase after day 2. In the control group, serum FSH and LH were 2.04 ± 0.10 and 3.09 ± 0.88 mIU/ml, respectively. Serum E2 and P4 decreased over time after 100 µg/g BW LNG treatment and were significantly lower by day 2 (49.31 ± 2.85 pg/ml, P<0.05, Fig. 1c) and by day 4 (3.09 ± 0.55 ng/ml, P<0.05, Fig. 1d), respectively, but did not subsequently decrease further. In the control group, serum E2 and P4 were 63.40 ± 4.36 pg/ml and 6.75 ± 0.78 ng/ml, respectively.

In the dose-dependent experiment (Fig. 2), serum FSH, LH and E2 had not changed significantly by day 6 (Fig. 2a–c) after different doses of LNG treatment. However, serum P4 had decreased significantly by day 6 after 100 µg/g BW LNG treatment (2.89 ± 0.44 ng/ml, P<0.05, Fig. 2d). Serum P4 had not changed significantly by day 6 after 1 and 10 µg/g BW LNG treatment. In the control group, serum FSH, LH, E2 and P4 were 2.82 ± 0.02 mIU/ml, 5.43 ± 0.25 mIU/ml, 74.84 ± 8.43 pg/ml and 4.99 ± 0.36 ng/ml, respectively.
Fig. 1. Serum (a) FSH, (b) LH, (c) E2, and (d) P4 levels on different days after 100 µg/g BW LNG was administrated to Mongolian gerbils. Data are the mean ± SEM (n=5). Bars with different superscripts are significantly different (P<0.05).

Fig. 2. Serum (a) FSH, (b) LH, (c) E2, and (d) P4 levels 6 days after different LNG doses were administrated to Mongolian gerbils. Data are the mean ± SEM (n=5). Bars with different superscripts are significantly different (P<0.05).
Levonorgestrel effects on reproductive hormone receptor mRNA expression in the ovary

In the time-dependent experiment, real-time PCR analysis revealed that the FSHR, LHR, ERβ and PR mRNA levels gradually decreased over time in the ovary after 100 µg/g BW LNG treatment (Fig. 3). The FSHR mRNA levels had decreased significantly by day 6 (P<0.05, Fig. 3a). The LHR mRNA levels had decreased significantly by day 4 (P<0.05), but did not subsequently decrease further (Fig. 3b). The ERα mRNA levels did not significantly differ over time in the ovary after 100 µg/g BW LNG treatment (Fig. 3c). The ERβ mRNA levels had decreased significantly by day 2 (P<0.05), but did not subsequently decrease further (Fig. 3c). The PR mRNA levels had decreased significantly by day 6 (P<0.05, Fig. 3d).

In the dose-dependent experiment, real-time PCR analysis showed that with increasing LNG doses, the FSHR, LHR, ERβ and PR mRNA levels gradually decreased in the ovary by day 6 (Fig. 4). Expression of FSHR and LHR mRNA decreased significantly in response to the LNG dose of 100 µg/g BW (P<0.05, Fig. 4a and 4b). The ERα and ERβ mRNA levels had not changed significantly 6 days after different LNG doses treatment (Fig. 4c). PR mRNA levels had decreased significantly 6 days after 100 µg/g LNG treatment (P<0.05, Fig. 4d).

Levonorgestrel effects on ER and PR mRNA expression in the uterus

The expected ERβ PCR product was not satisfactorily generated from reverse transcribed mRNA from the uterus.

The time-dependent experiment, real-time PCR analysis revealed that ERα and PR mRNA levels gradually decreased over time in the uterus after 100 µg/g LNG treatment (Fig. 5). The ERα and PR mRNA levels had decreased significantly by day 4 (P<0.05), but did not subsequently decrease further (Fig. 5a and 5b). In the dose-dependent experiment, real-time PCR analysis showed that the mRNA expression of ERα and PR gradually decreased with increasing LNG dose. The ERα mRNA levels had decreased significantly in the uterus by day 6 in response to the LNG dose of 100 µg/g
BW \( (P<0.05, \text{Fig. } 6a) \). The PR mRNA levels did not change significantly after treatment with different doses of LNG \( (\text{Fig. } 6b) \).

**Discussion**

To our knowledge, this is the first study of the expression of reproductive hormone receptors by real-time quantitative RT-PCR in Mongolian gerbils. The partial sequences of reproductive hormone receptors (LHR, ER and PR) in the Mongolian gerbil were showed in the present study. The fertility of Mongolian gerbils was inhibited by treatment with multiple doses of levonorgestrel-quinestrol at 10 \( \mu g/g \) BW [13]. In this study the Mongolian gerbils were given LNG intragastrically at 1, 10, or 100 \( \mu g/g \) BW, in a single-dose treatment. The LNG effects on reproductive endocrinology under physiological tolerance in Mongolian gerbils were revealed...
in this work.

Our results suggest that LNG inhibits ovarian function possibly, via its direct progesterone-like effects on the ovary in Mongolian gerbils. The inhibitive effects of LNG on ovulation have been reported in rats, rabbits and monkeys [11, 24, 29], but it is a weak progestin in dogs [5]. Reproductive hormones play critical roles in folliculogenesis, ovulation and luteinization in the ovary. Hormone actions are determined by hormone levels and the concentration of corresponding receptors [35, 36]. The location and distribution of FSHR, LHR, ER and PR in the ovary have been defined [10, 28, 30, 35] and suggest their importance in intra-ovarian function. follicular growth is demonstrated by serum E2 patterns, while luteal function is measured by serum P4 levels [11]. The low serum E2 and P4 levels observed in the present study indicate impaired follicular development, maturation, rupture, ovulation and luteal activity, which were also observed in previous studies [2, 11, 19, 26, 33]. Exogenous progestin can directly inhibit steroidogenesis [33]. LNG is a synthetic progestin which has strong progesterone-like and anti-ovulatory activities, but it lacks estrogen-like activity [15]. The profound reduction of serum E2 and P4 levels, despite elevated serum FSH and LH levels in the present study, suggests that the ovary of the Mongolian gerbil may be an important site of action for the direct progesterone-like effects of LNG. This is consistent with the results for monkeys [9, 11]. The down-regulated mRNA expression of FSHR, LHR, ERβ and PR may also be the result of the direct progesterone-like effects of LNG on the ovary of Mongolian gerbil. Abnormal follicular development, anovulation, and infertility due to deficiencies of ER or PR have been observed in mice [6, 19]. In this study, LNG effects on the expression of reproductive hormone receptors were time-dependent and dose-dependent whereas effects on the hormone levels were not obvious. The reasons for this are not clear and need further investigation.

The two ER subtypes may play different roles and the estrogen action may be mediated primarily by ERβ in the ovary but by ERα in the uterus of the Mongolian gerbil. ERα is primarily localized in the stromal cells, whereas ERβ is expressed predominantly in the granular cells of developing follicles [10, 30, 34]. The differential expression of the two ER subtypes in the reproductive organs indicates that the mediation of estrogen action in these tissues may be accomplished through the more dominant receptor [23]. ERβ mRNA expression was down-regulated while ERα mRNA expression did not change significantly after LNG treatment. ERβ was expressed almost exclusively in granular cells suggesting the possibility that the estrogen action may be mediated primarily by ERβ in the ovary of the Mongolian gerbil. This result is similar to that reported for rats [3, 8]. Also, the finding that the expected ERβ PCR product was not satisfactorily generated from reverse transcribed mRNA from the uterus of the Mongolian gerbil is consistent with other work [32]. The low level of ERβ expression in the uterus relative to that of ERα may be due to ERβ not being the dominant ER subtype and therefore not playing significant roles in the uterus. This finding is similar to that reported for rats [37]. Effects of LNG on the expression of ER subtypes may be tissue-specific in
Mongolian gerbils.

LNG may have inhibitory effects on uterine growth and endometrial function via its direct progestational effects in Mongolian gerbils. Decreased stromal density and atrophy of glandular epithelium have been observed after LNG treatment in mice [1]. In the uterus, estrogen provides the main proliferative stimulus, while progesterone is more involved in differentiation [14, 19]. Estrogen stimulates uterine growth through binding to ER, while progesterone is antagonistic to estrogen through suppression of ER in the uterus [12, 38]. The reduction of mRNA expression of ERα and PR in the uterus of the Mongolian gerbil is possibly caused by the progestational effects of LNG and the lack of estrogenic stimulation. Estrogen and progesterone are the major regulators of endometrial function, and they act through binding to their nuclear receptors [31]. The hormonal actions on the endometrium and the morphological changes are dependent on the interaction of the hormones with the endometrial ER and PR. The down-regulated expression of the ER and PR by LNG in the endometrium has previously been reported [1, 21, 31], and the possible effects of LNG on the morphology and function of the endometrium have been observed [4].

In summary, our study revealed down-regulation of FSHR, LHR, ERβ and PR expression in the ovaries, and ERα and PR expression at the molecular level in the uterus, of Mongolian gerbils after LNG treatment; serum E2 and P4 decreased despite elevated FSH and LH levels. In Mongolian gerbils, the ovary and uterus may be important sites of action of LNG through direct progestrone-like effects. Although the precise mode of action remains to be investigated, this study provides additional information on LNG actions in Mongolian gerbils.

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References

LNG EFFECTS ON REPRODUCTIVE ENDOCRINOLOGY


