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Progesterone Together with Estrogen Attenuates Homologous Upregulation of Gonadotropin-Releasing Hormone Receptor mRNA in Primary Cultured Rat Pituitary Cells

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In a previous study, we clearly demonstrated that an application of gonadotropin-releasing hormone (GnRH) to cultured rat pituitary cells increased the expression of GnRH receptor (GnRH-R) mRNA through transcriptional activation of GnRH-R gene rather than suppression of the turnover rate of GnRH-R mRNA. Along with GnRH, gonadal steroids seem to be an important regulator for GnRH-R expression in the pituitary gland. Recent in vivo studies reported that an application of gonadal steroids to gonadectomized animals modulated GnRH-R mRNA expression in the pituitary gland. However, it has not been clearly understood whether steroids may act directly at the pituitary or indirectly via modulation of hypothalamic GnRH release. Therefore, we assessed the effects of estrogen and progesterone on GnRH-R mRNA expression in primary cultured female rat pituitary cells. Neither estradiol nor progesterone modulates the basal expression of GnRH-R mRNA in primary cultured pituitary cells. When cultured pituitary cells were exposed to different doses of estradiol in combination with GnRH (0.2 n*M*), the GnRH-stimulated increment of GnRH-R mRNA expression was not significantly changed by estradiol at any given doses. However, when different doses of progesterone were added to primary cultured pituitary cells in combination with GnRH (0.2 n*M*), GnRH-induced increases in GnRH-R mRNA levels were reduced in a dose-related manner, showing a significant reduction at 100 n*M* progesterone. Furthermore, the addition of estradiol reinforced the suppressive effect of progesterone on the homologous upregulation of GnRH-R mRNA expression. Collectively, our results clearly demonstrated that progesterone directly attenuates the homologous upregulation of GnRH-R mRNA expression at the pituitary level, and that estradiol potentiates the effect of progesterone.

Key Words: Gonadotropin-releasing hormone receptor; gonadotropin-releasing hormone; progesterone; estradiol; anterior pituitary; mRNA.

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Introduction

It has been reported that the number of gonadotropin-releasing hormone receptor (GnRH-R) and the amount of GnRH-R mRNA in the anterior pituitary gland were changed during the estrous cycle in rats and sheep *(1–6)*. Because endocrine milieus are also altered dramatically during the estrous cycle, many endocrine factors might be potential candidates as a regulator of GnRH-R expression in the pituitary gland. In particular, recent in vivo studies suggested that ovarian steroids might be responsible for the fluctuation of GnRH-R mRNA expression in the pituitary gland *(7–11)*. However, in vivo study does not differentiate whether ovarian steroids act directly at the level of anterior pituitary or indirectly via modulating hypothalamic GnRH release. Indeed, several in vitro studies demonstrated that pulsatile or short-term (6 h) continuous administration of GnRH upregulates its own receptor mRNA expression in the pituitary gland *(12–14)*. The cultured pituitary cells are a useful system to differentiate the action site of ovarian steroids to modulate the expression of GnRH-R mRNA in the pituitary gonadotropes. In a previous study, we clearly demonstrated that continuous administration of GnRH to cultured rat pituitary cells increased the expression of GnRH-R mRNA *(14)* through transcriptional activation of GnRH-R gene rather than stabilization of GnRH-R mRNA *(15)*. Therefore, in the present study, we aimed to investigate the effects of ovarian steroids on the expression of GnRH-R mRNA in primary cultured rat pituitary cells.

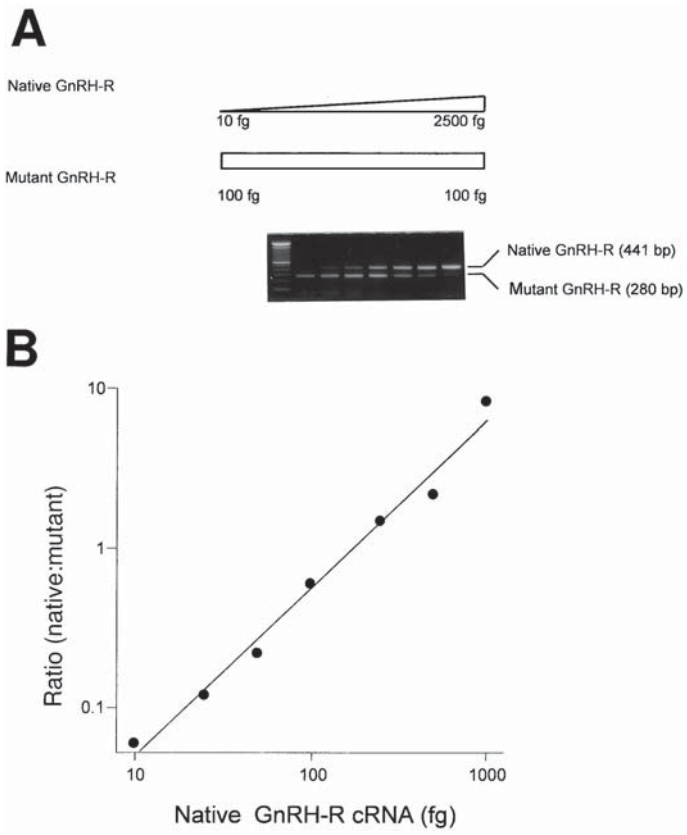

Fig. 1. Standard curve of competitive reverse transcriptase polymerase chain reaction (RT-PCR) for quantitation of GnRH-R mRNA levels. A constant amount of mutant GnRH-R cRNA was coamplified with various concentrations of native GnRH-R cRNA. **(A)** PCR product was separated on a 2.0% agarose gel. **(B)** Plot of ratios of native:mutant GnRH-R signals against different amounts of native GnRH-R cRNA revealed a linear relationship). Mutant GnRH-R cRNA (100 fg) was coamplified with 10, 25, 50, 100, 250, 500, and 1000 fg of native GnRH-R cRNA.

Results

Effect of Estrogen and Progesterone on Basal GnRH-R mRNA Levels

For the construction of the standard curve, 100 fg of mutant GnRH-R cRNA was coamplified with different amounts of native GnRH-R cRNA (10–1000 fg). The plot for ratios of native:mutant signals vs the amounts of native cRNA revealed a linear relationship (Fig. 1). The regression coefficient (r) of the standard curve was above 0.950. GnRH-R mRNA in pituitary total RNA (0.5 mg) competed with 100 fg of mutant GnRH-R cRNA. GnRH-R mRNA levels were calculated based on the standard curve.

We initially examined whether ovarian steroids can modulate GnRH-R mRNA levels, directly acting at the pituitary gonadotropes in the monolayer cultures. When pituitary cells were exposed to different doses of estradiol for 6 h, no change in the basal levels of GnRH-R mRNA was observed at any given concentrations of estradiol (data not shown). In addition, the basal GnRH-R mRNA levels were not changed during the different durations (3–24 h) of estradiol treatment (data not shown). Furthermore, an application of progesterone to pituitary cells for 6 h did not alter the basal levels of GnRH-R mRNA at any given concentrations (data not shown).

Progesterone Attenuates Homologous Upregulation of GnRH-R mRNA Levels

Although ovarian steroids did not modulate the basal GnRH-R mRNA levels, we further delineated whether ovarian steroids are able to modulate GnRH-stimulated GnRH-R mRNA levels. When pituitary cells were exposed to GnRH (0.2 n*M*) for 6 h in the presence of different doses of estradiol, any change in homologous upregulation of GnRH-R mRNA levels was not observed at any given concentrations of estradiol (Fig. 2). However, administration of progesterone attenuated GnRH-stimulated augmentation of GnRH-R mRNA in a dose-dependent manner (Fig. 3). Homologous upregulation of GnRH-R mRNA was significantly reduced at a 100 n*M* concentration of progesterone.

Estrogen Potentiates Suppressive Effect of Progesterone on Homologous Upregulation of GnRH-R mRNA

We further explored whether the addition of estrogen can potentiate the action of progesterone on homologous upregulation of GnRH-R gene expression. When different concentrations of progesterone in combination with estradiol (10 n*M*) were applied to pituitary cells in the presence of GnRH (0.2 n100 n*M*), GnRH-stimulated GnRH-R mRNA levels were significantly reduced at all given concentrations (4–100 n*M*) of progesterone (Fig. 4). Thus, simultaneous administration of estrogen with progesterone potentiated the suppressive effect of progesterone on homologous upregulation of GnRH-R mRNA levels.

Discussion

The results presented herein showed that progesterone reduced GnRH-stimulated GnRH-R mRNA levels in primary cultured rat anterior pituitary cells, although the basal levels of pituitary GnRH-R mRNA were not changed by the administration of progesterone. Furthermore, simultaneous treatment of pituitary cells with estradiol potentiated the suppressive effect of progesterone on GnRH-stimulated GnRH-R mRNA expression. These results suggest that ovarian steroids are able to regulate the homologous upregulation of GnRH-R mRNA expression, but not the basal expression of GnRH-R mRNA in the pituitary gland, through their direct action at the pituitary level.

Recently, some in vivo studies explored the effect of exogenous progesterone on the expression of GnRH-R mRNA in the pituitary of gonadectomized sheep *(11,16)*. In orchidectomized sheep, Sakurai et al. *(16)* observed that the implantation of progesterone significantly diminished GnRH-R mRNA levels in the pituitary, compared with vehicle-implanted wethers. In addition, implantation of

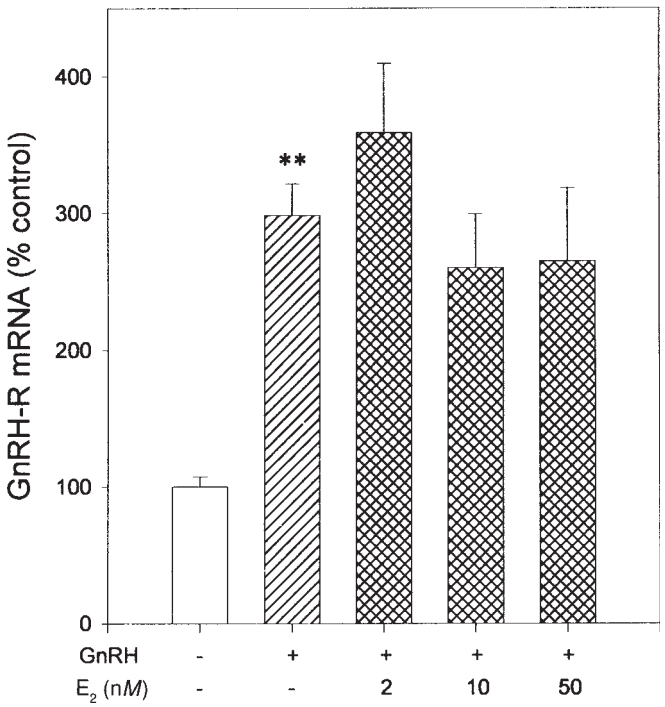

Fig. 2. Effects of estrogen on homologous upregulation of GnRH-R mRNA expression in primary cultures of rat pituitary cells. Pituitary cells were continuously exposed to different doses of estradiol for 6 h in the presence of GnRH (0.2 n*M*). The values of GnRH-R mRNA are expressed as a percentage of the value in control (medium alone) after calculation based on the standard curve. Each bar represents the mean ± SEM ($n = 5$ to 6 from two independent experiments); $^{**}p < 0.01$ (vs control).

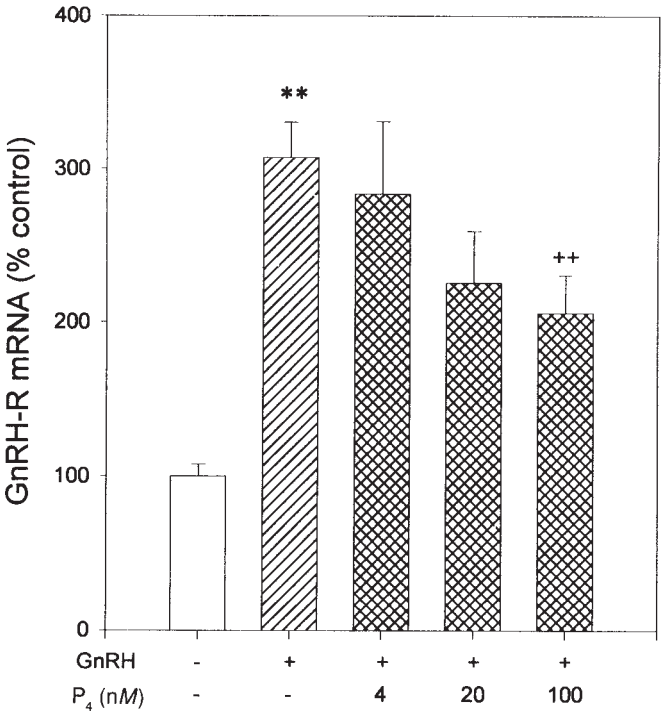

Fig. 3. Effects of progesterone on homologous upregulation of GnRH-R mRNA levels in primary cultures of rat pituitary cells. Pituitary cells were continuously exposed to different doses of progesterone for 6 h in the presence of GnRH (0.2 n*M*). The values of GnRH-R mRNA are expressed as a percentage of the value in control (medium alone) after calculation based on the standard curve. Each bar represents the mean ± SEM ($n = 8$–10 from three independent experiments); $^{**}p < 0.01$ (vs control); $^{++}p < 0.01$ (vs GnRH alone).

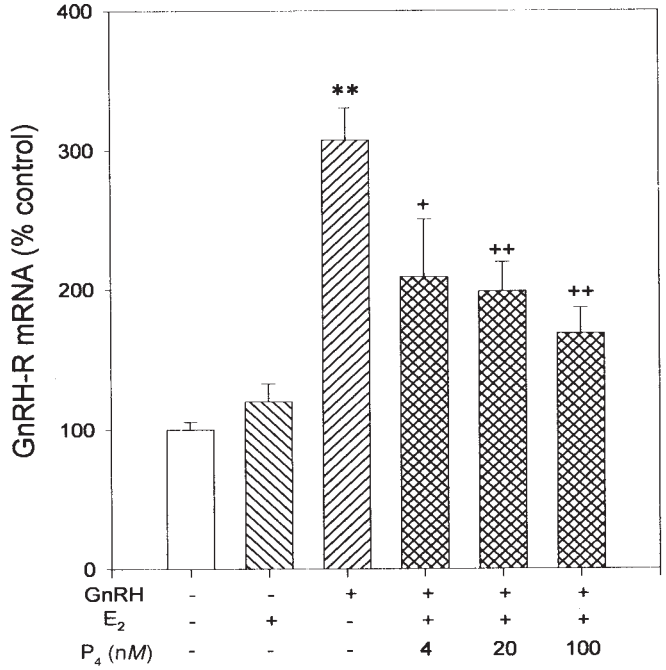

Fig. 4. Effects of estrogen on the suppressive action of progesterone in homologous upregulation of GnRH-R mRNA levels in primary cultures of rat pituitary cells. Pituitary cells were continuously exposed to different doses of progesterone for 6 h in the presence of GnRH (0.2 n*M*) and estradiol (10 n*M*). The values of GnRH-R mRNA are expressed as a percentage of the value in control (medium alone) after calculation based on the standard curve. Each bar represents the mean ± SEM ($n = 8$ to 9 from three independent experiments). $^{**}p < 0.01$ (vs control); $^{++}p < 0.01$ (vs GnRH alone).

progesterone together with estradiol in ovariectomized sheep also reduced the concentration of pituitary GnRH-R mRNA, in comparison with sheep implanted with estradiol alone *(11)*. Furthermore, in cultured ovine pituitary cells, Wu et al., *(17)* showed that progesterone decreased the basal levels of GnRH-R mRNA as well as activator- (inhibin and estradiol) induced GnRH-R mRNA levels. Thus, in sheep it appears that progesterone downregulates the basal and induced expression of GnRH-R mRNA directly at the pituitary gland. However, in rats, the effect of progesterone on pituitary GnRH-R mRNA expression appears to be somewhat different from that in sheep. Yasin et al. *(8)* found that an application of progesterone to estrogen-replaced ovariectomized rats of which GnRH deficiency was induced by α-adrenergic receptor blocker, phenoxybenzamine, did not

modulate GnRH-R mRNA levels in the pituitary gland. On the other hand, Bauer-Dantoin et al. *(7)* demonstrated that pentobarbital prevented estrogen-induced increase in pituitary GnRH-R mRNA levels, and progesterone replacement decreased estrogen-induced augmentation of pituitary GnRH-R mRNA levels in ovariectomized rats. Thus, in rats, the effect of progesterone on pituitary GnRH-R mRNA expression seems to depend on the presence or absence of endogenous GnRH. However, the action site of ovarian steroids was not clearly elucidated because studies were performed only in the in vivo system in rats.

Our data revealed that progesterone attenuated the homologous upregulation of GnRH-R mRNA in primary cultured pituitary cells, although the basal expression of GnRH-R mRNA was not modulated. It seems that progesterone may exert differential effects on the expression of GnRH-R mRNA in the pituitary cells of rats and sheep. In our primary cultured pituitary cells of rats, progesterone significantly decreased GnRH-stimulated augmentation of GnRH-R mRNA levels, although any significant reduction in the basal expression of GnRH-R mRNA was not observed at any given doses of progesterone. However, in cultured ovine pituitary cells, progesterone reduced the basal levels of GnRH-R mRNA as well as activator-stimulated levels of GnRH-R mRNA *(17)*. This discrepancy in the effect of progesterone on the basal levels of GnRH-R mRNA still cannot be explained. This might be owing to the difference in the duration of progesterone treatment. Wu et al. *(17)* exposed ovine pituitary cells to progesterone for 48 h, whereas in our study we exposed rat pituitary cells for 6 h. Because only few studies are available on the effect of exogenous progesterone on pituitary GnRH-R mRNA expression, further studies are required to clarify the role of progesterone in regulating GnRH-R mRNA expression in different species.

The present study showed that the administration of estradiol potentiated the inhibitory effect of progesterone on GnRH-stimulated expression of GnRH-R in cultured rat anterior pituitary cells. Estradiol potentiation of the effect of progesterone was also observed in our previous study *(18)*, in which an application of estradiol potentiated the suppressive effect of progesterone on the decay of luteinizing hormone-β mRNA, although estradiol alone showed no effect. It has been known that the administration of estrogen increases the number of progesterone receptors in the rat pituitary gland *(19–22)* and in primary cultured rat pituitary cells *(23)*. Therefore, in our study it is assumed that estradiol reinforced the suppressive action of progesterone on the expression of GnRH-R mRNA. On the other hand, estrogen alone at any given doses (2–50 n*M*) and any duration of treatment (3–24 h) did not change the basal expression as well as a GnRH-stimulated increment of GnRH-R mRNA in cultured rat pituitary cells. However, Wu et al. *(17)* reported that in cultured ovine pituitary cells, the amounts of GnRH-R mRNA were increased to 190% over the control value after the administration of estradiol alone. In both studies, the discrepancy in the effect of estrogen on GnRH-R mRNA expression may also be owing to the difference in the duration of estrogen treatment and species-specific differences (i.e., very low plasma concentration of estradiol in sheep). From the present study, it is concluded that progesterone could directly attenuate the homologous upregulation of GnRH-R mRNA expression at the pituitary level and that estradiol potentiates the effect of progesterone.

Materials and Methods

Preparation of Anterior Pituitary Cell Cultures

Four-day cycling female Sprague-Dawley rats (150–200 g; provided by Yuhan Research Center, Korea) were used for the preparation of pituitary cell cultures. Pituitary glands were taken at 10 AM at the estrous stage and prepared by enzymatic dispersion, with modifications to previously described methods *(24)*. Following decapitation, the anterior pituitary glands were removed and washed in Spinner's minimal essential medium (S-MEM) (Gibco-BRL, Gaithersburg, MD) containing 0.3% bovine serum albumin (BSA) (fraction V) (Sigma, St. Louis, MO) and 10 m*M* HEPES (Sigma). Anterior lobes were cut into several pieces in S-MEM/BSA and then enzymatically digested with 20 mL of S-MEM/BSA containing 0.25% trypsin (1:250) (Difco, Detroit, MI) and DNase (10 µg/gland) (Sigma) for 1 h at 4°C and a further 30 min at 37°C.

Dispersion was facilitated by repeated aspiration and expulsion of the tissue fragments with a fire-polished Pasteur pipet. Dispersed cells were then centrifuged at 400*g* for 10 min. The pellet was resuspended in 20 mL of S-MEM/BSA containing trypsin inhibitor (5 µg/mL) (Sigma) and filtered through lens paper to remove residual tissue fragments. The cell suspension was briefly centrifuged, and the cell pellet was resuspended in α-MEM (Gibco-BRL) containing 2.5% fetal bovine serum (Gibco-BRL), 10% horse serum (Gibco-BRL), and antibiotics (100 U of penicillin and 100 mg of streptomycin/mL) (Sigma). All sera were dextran-charcoal stripped for the removal of residual steroids. The cells were more than 95% viable, as measured by trypan blue exclusion. Aliquots of these cells (1×10^6 cells/mL) were incubated in multiwell culture plates (Falcon) in 5% CO_2/air at 37°C. Following preincubation, the cells were washed twice with Dulbecco's phosphate-buffered saline (Sigma) to remove serum and nonadherent cells, and thereafter they were further incubated in serum-free medium (α-MEM) containing 0.3% BSA for experiments.

Total RNA Extraction

Total cytoplasmic RNA from the pituitary was extracted by the acid guanidinium thiocyanate-phenol-chloroform method *(25)*. Briefly, 600 µL of denaturing solution containing 4 *M* guanidinium thiocyanate, 25 m*M* sodium citrate (pH 7.0), 0.5% *N*-lauroyl sarcosine, and 0.1 *M* 2-mercaptoethanol was added to each well and collected in a microcentrifuge tube. Sixty microliters of 2 *M* sodium acetate (pH 4.0), 600 µL of water-saturated phenol, and 120 µL of chloroform:isoamyl alcohol mixture (49:1) were added. After cooling on ice for 15 min, the samples were centrifuged (10,000*g*) at 4°C for 20 min and precipitated with ethanol. After washing with 75% ethanol, the RNA pellet was dried under a vacuum and dissolved in 20 mL of sterilized distilled water. RNA content was then quantified at A_{260} absorbance.

Competitive RT-PCR

Competitive RT-PCR to determine GnRH-R mRNA levels was performed as previously described *(14,26)*. GnRH-R primers were synthesized based on the sequence of the rat GnRH-R cDNA *(27)*. The upstream primer is 5'-CTTGAAGCCCGTCCTTGGAGAAAT-3' and the downstream primer is 5'-GCGATCCAGGCTAATCACCACCAT-3'. Primers were designed such that the predicted sizes of PCR products were 441 and 280 bp for native and mutant GnRH-R, respectively. After linearization of plasmids containing native and mutant GnRH-R cDNA, native and mutant GnRH-R cRNAs were synthesized by T7 RNA polymerase using an in vitro transcription system kit purchased from Promega (Madison, WI). Concentrations of native and mutant GnRH-R cRNAs were measured with an ultraviolet (UV) spectrophotometer at A_{260}.

Briefly, native (in the case of biological samples, GnRH-R mRNA in pituitary total RNA) and mutant cRNA templates were co–reverse transcribed by 200 U of RNaseH$^-$ Moloney murine leukemia virus RT (Gibco-BRL). Subsequently, the PCR reaction mixture containing 50 pmol of up- and downstream primers and 2.5 U of *Taq* DNA polymerase (Promega) was added. When native and mutant cDNAs are coamplified in the same tube, the sequence homology between them may cause hybrids of native and mutant DNA at the annealing temperature *(28)*. Therefore, PCR amplification was carried out with a two-step procedure to reduce hybrids (step 1: denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 2 min; step 2: denaturation at 85°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 2 min). To determine GnRH-R mRNA levels, 33 cycles of step 1 and 6 cycles of step 2 were used. Ten-microliter aliquots of PCR products were electrophoresed on a 2.0% agarose gel in TAE buffer, stained with ethidium bromide, and photographed under UV illumination with Polaroid 665-type negative and positive films (Polaroid, Cambridge, MA). Negative film was used for densitometric scanning of native and mutant signals.

Data Analyses

GnRH-R signals on negative film were measured with a densitometric scanner (Hoefer, San Francisco, CA). The amounts of GnRH-R mRNA were calculated from the ratio of native:mutant signals using a standard curve. Data were statistically evaluated using the one-way analysis of variance (ANOVA), followed by Fisher's least significant difference test for a posthoc comparison. Where appropriate, data were evaluated using the student's *t*-test. All values are given as the means ± SEM. Statistical significance was set at $p < 0.05$.

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