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# Aromatase, 3β and 17β-Hydroxysteroid Dehydrogenase Genes' Expression in the Ovaries varies during the Estrous Cycle, is Asymmetric and depends on the Superior Ovarian Nerve Innervation

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## Abstract

Secretion of steroid hormones by the ovaries is regulated by hormonal and neural signals. In the rat, the right and left ovaries' ability to secrete steroid hormones is different and varies throughout the estrous cycle. Gonadotropins stimulate the synthesis of 3β and 17β hydroxysteroid dehydrogenase and aromatase by activating the expression of genes codifying for each hormone. The aim of the present study was to analyze if the expression of genes codifying for 3β-HSD, 17β-HSD and aromatase in the ovaries varies along the estrous cycle, and its dependence of superior ovarian nerve innervation. At 07.00 h on each day of the estrous cycle, adult cyclic rats were submitted to the unilateral section of the superior ovarian nerve (SON) or kept as control. The animals were killed one hour after surgery. Progesterone, testosterone and estradiol serum levels varies depending the day of the cycle and the SON sectioned. The expression of the genes codifying for each enzyme also varies along the estrous cycle, depending on the ovary studied the day of the cycle and the SON sectioned. The results suggest that the expression of genes codifying for three key enzymes in ovarian steroidogenesis is regulated, among other signals, by those arriving through the ovarian innervations and that such regulation varies along the estrous cycle.

## Introduction

Secretion of steroid hormones by the ovaries is regulated by hormonal signals originating in the hypothalamus, pituitary, adrenal, thyroid and the ovary itself; as well as through nerve signals reaching the ovaries from the Superior Ovarian Nerve (SON), the Ovarian Plexus Nerve (OPN) and vagus nerve [1,2]. In the rat, the right and left ovaries' ability to secrete steroid hormones is different and varies throughout the estrous cycle [1,3-6].

Cholesterol is the precursor of all steroid hormones. Specific enzymes transform cholesterol into particular steroid hormones. These enzymes are mainly stimulated by Follicle Stimulating Hormone (FSH) and luteinizing hormone (LH), whose levels varies along the estrous and menstrual cycles. Other hormones also participate in its regulation [7].

According to Dong et al. [8], 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3β-HSD) and 17β-Hydroxysteroid Dehydrogenase (17β-HSD) are key enzymes catalyzing the conversion of steroids from the active to inactive form, and vice versa. 17β-HSD is a key enzyme involved in testosterone production in females [9]. Aromatase cytochrome P450 (aromatase) is the key enzyme for estrogen biosynthesis from androgens [10,11].

Gonadotropins stimulate the synthesis of 3β-HDS, 17β-HDS and aromatase by activating the expression of genes codifying for each hormone [12,13]. In the rat, estradiol enhances aromatase stimulation by FSH [14].

The ovarian innervation arriving via the SON participates in follicular growth and maturation [15], ovulation [16, 17] and hormone-secretion regulation [18,19].

In cyclic rats on estrus (E), the acute effects resulting from electric stimulation of the peripheral end of the SON resulted in lower testosterone and estradiol levels [20]. Uchida et al [21] showed that pinching stimulation of the hind-paw for 5 min decreased estradiol secretion rates from the ovary; a response associated to reflex activation of the ovarian sympathetic nerves and mediated by supra-spinal structures.

In rats on diestrus-2 (D2), electric stimulation of the peripheral end of the SON also resulted in lower progesterone levels [22]. Aguado and Ojeda [23] showed that in rats on Proestrus (P), transection of the left SON at 11.00 or 16.00 h resulted in lower progesterone and estradiol levels within 4 min of surgery, and these levels remained significantly low during the next 24 min. Neither progesterone nor estradiol secretion were altered by SON sectioning at 11.00 h of E.

Based on the effects of SON sectioning or stimulation on serum levels of progesterone, testosterone and estradiol, we presume that the nerve is involved in regulating the expression of genes encoding enzyme synthesis used by the ovary during hormonal synthesis.

Then, the aim of the present study was to analyze if the expression of genes codifying for 3β-HSD, 17β-HSD, and aromatase in the ovaries varies along the estrous cycle. In addition, the present study assessed how the unilateral sectioning of SON, performed on rats in each day of the estrous cycle, modifies progesterone, testosterone and estradiol levels and evaluated if these changes are related to modifications in the expression of the genes codifying for each hormone.

## Material and Methods

All experiments were carried out in strict accordance with the Mexican Law of Animal Treatment and Protection Guidelines, and followed the Mexican Official Standard NOM-062-ZOO-1999 specifications. The Institutional Committee for the Care and Use of Animals of the Facultad de Estudios Superiores Zaragoza approved all experimental protocols. All possible efforts were made to minimize the number of animals used and their suffering.

This study was performed with adult female rats from the CIIZ-V strain from our own stock (195-225 g body weight) that had shown at least two consecutive 4-day cycles, monitored by cytological examination of daily vaginal smears. All animals were housed in an artificial light-dark cycle (lights on from 05:00 to 19:00 h), with free access to food (Purina S.A., Mexico) and tap water ad libitum. All surgeries were performed under ether anesthesia, between 07.00-07.15 h on diestrus-1 (D1), D2, P, or estrus (E). Rats were randomly allotted to unilateral, right or left, SON sectioning treatment or kept untouched (control). Animals with surgical treatment were sacrificed 1 h after surgery. Control animals on each day of the estrous cycle were sacrificed at 08.00-08.15 h.

Rats were sacrificed by decapitation. The blood of the trunk was collected, allowed to clot at room temperature for 30 minutes, and centrifuged at 3,000 rpm during 15 minutes. Serum was stored at -20°C, until progesterone, testosterone and estradiol concentrations were measured using Radio-Immuno-Assay (RIA); with kits purchased from Diagnostic Products (Los Angeles, CA). Analytical results are expressed in ng/ml (progesterone) and pg/ml (testosterone and estradiol).

Ovarian 3β HSDs, 17β HSDs and P450 aromatase mRNA levels were measured in the right (R) and left (L) ovaries using real time RT-PCR. Data was normalized with CYCA (cyclophilin A) mRNA values.

### Total RNA extraction, reverse transcription and quantitative real-time PCR assay

Relative gene expression analysis was conducted using Real-Time Quantitative PCR. For this analyses, a phenol-chloroform of total RNA extraction was performed from the rats' ovaries, which had been previously frozen with liquid nitrogen and homogenized in a morter. RNA was extracted with Trizol reagent and chloroform-isoamyl alcohol [24,25]. The extracted RNAs were precipitated with isopropanol and the pellet was re-suspended on nuclease-free water (Fermentas). A DNAse treatment with DNAse I (Fermentas, Glen Burnie, MD, USA) was performed on each extract, following the manufacturer's instructions. The total RNA quantity was measured using Nanodrop UV/Vis Spectrophotometer. cDNA was synthesized from 1 µg of total RNA with RevertAid First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific), following the manufacturer's instructions and using specific primers for *cyp19a1* (aromatase) and *hsd3b* (3-β-5-old dehydrogenase) and *hsd17b3* (17-β-hidroxyesteroid dehydrogenase).

Reverse-transcribed cDNA samples (20 ng) were subjected to PCR amplification in 10 µl of reaction with Maxima SYBR Green qPCR Master Mix kit (Fermentas, ThermoScientific), 900 nM of forward and reverse primers. The primers used are listed in Table 1. The PCR was preceded by a one step at 50 °C for 2 min, 95 °C for 10 min. Subsequently, 30 PCR cycles were carried out at 95 °C for 15 seconds, at 56 °C for 30 seconds, and at 72 °C for 30 seconds. The primers used are listed in Table 1. Normalization was based on the expression of the CYCA housekeeping gene. All reactions were performed in triplicate. Dissociation curves were constructed at 95 °C for 15 seconds, 60 °C for 30 seconds, 72 °C for 1 min, 45 °C for 15 seconds and 60 °C for 15 seconds. Ct (threshold cycle) and amplification efficiencies for target (enzymes) and CYCA genes were tested simultaneously and compared to demonstrate their equivalence. Expression of *hsd3b* and *hsd17b3* genes were quantified by the comparative method 2-ΔΔCT. *cyp19a1* expression was quantified by the standard curve method [26,27].

### Statistical analysis

Data on hormone concentrations in serum and genes in the ovaries were analyzed using independent Analysis Of Variance (ANOVA), followed by Tukey's test. The interaction between hormone serum levels, gene concentrations in the left or right ovary of rats with SON sectioning treatment, and day of the cycle when treatment was performed were analyzed by a two-way analysis of variance. A p value of less than 0.05 was considered significant.

**Table 1:** Primers used to amplify cyclophilin A, aromatase, 3-β-HSD and 17β-HSD by real-time PCR.

| Gene | Primer (5'–3') | Sequence | Size (nt) | Tm |
|---|---|---|---|---|
| **Cyclophilin A** | Forward<br>Reverse primer | TCATGTGCCAGGGTGGTGACTT<br>GAGCAAGTCTTTCAGTCCTGTC | 22<br>24 | 61.1 °C<br>60.1 °C |
| **Aromatase** | Forward primer Reverse primer | TCATGTGCCAGGGTGGTGACTT<br>GAGCAAGTCTTTCAGTCCTGTC | 24<br>22 | 61.2 °C<br>61.2 °C |
| **3-β-HSD** | Forward primer Reverse primer | TCATGTGCCAGGGTGGTGACTT<br>GAGCAAGTCTTTCAGTCCTGTC | 23<br>23 | 60.4 °C<br>60.9 C |
| **17β-HSD** | Forward primer Reverse primer | TCATGTGCCAGGGTGGTGACTT<br>GAGCAAGTCTTTCAGTCCTGTC | 23<br>22 | 60.9 °C<br>61.1 °C |

## Results

### Hormone Levels (Figure 1)

Progesterone levels varied along the estrous cycle (F (3, 18) = 13.25, P < 0.0001). Rats on D1 or D2 showed higher progesterone levels than rats on P or E. Left SON sectioning treatment to rats on D1, P or E resulted in higher progesterone levels than in control rats. Right SON sectioning treatment to rats on P or E also yielded higher progesterone levels (Figure 1a).

The two-way ANOVA confirmed that, regardless of the estrous cycle day of treatment, animals with left or right SON sectioning yielded higher progesterone levels than control rats. The results depend on the day of the cycle (F (3, 64) = 3.815, P = 0.01, the SON sectioned (F (2, 64) = 36.42, P < 0.0001) and the interaction between day of the cycle and SON sectioned (F (6, 64) = 2.417, P<0.05).

Testosterone levels also varied along the estrous cycle (ANOVA F (3, 18) = 3.651, P < 0.05). Left SON sectioning treatment to rats on D1 or D2 resulted in higher testosterone levels than in control animals. A similar result was observed in rats with right SON sectioning treatment on D2 (Figure 1b). The two-way ANOVA confirmed that, regardless of the estrous cycle day of treatment, animals with left or right SON sectioning yielded higher testosterone levels than control rats (F (3, 48) = 7.862 P = 0.0001. No differences were observed between which SON was sectioned (F (2, 48) = 2.410 P < 0.1006) and the interaction between day of the cycle and the sectioned SON (F (6, 48) = 0.3077, P=0.9399).

Estradiol levels (Figure 1c) fluctuated along the estrous cycle (F (3, 20) = 12.01, P < 0.0001). Estradiol levels were higher in control animals and in rats with unilateral SON sectioning treatment on D1, P or E. Left or right SON sectioning treatment to rats on D1 yielded lower estradiol levels than control rats. The two-way ANOVA showed that, regardless of the estrous day cycle of treatment, animals with left or right SON sectioning yielded lower estradiol levels than control rats (day of the cycle (F (3, 56) = 29.04 P = 0.0001, the SON sectioned (F (2, 56) = 23.85 P < 0.0001) and the interaction between day of the cycle and SON sectioned (F (6, 56) = 3.849, P=0.001. Compared to control rats, unilateral (left or right) SON sectioning treatment to rats on D1, and left SON sectioning treatment to rats on P resulted in lower estradiol levels.

### Gene Expression

No difference in the 3β-HSD, 17β-HSD, or aromatase gene expression was observed between the left and right ovary (3β-HSD, 28.77±4.54 *vs.* 26.80±3.02, non-significant (n.s.); 17β-HSD 0.567±0.09 *vs.*0.513±0.23, n.s., and 0.549±0.2 *vs.* 0.183±0.05, n.s.

**3- β -Hydroxysteroid Dehydrogenase:** 3β-HSD gene expression in each ovary varied along the estrous cycle (F (7, 16) = 7.891 P = 0.001). Greater variations were observed in the right ovary (F (3, 8) = 35.83 P < 0.0001) than in the left (F (3, 8) = 7.560, P = 0.01) (Figure 2a).

The 3β-HSD gene expression in the left ovary was modified by sectioning the right or left SON. The results depend on the interaction between the nerve sectioned and the day of the cycle (interaction F (6, 24) = 2.873, P = 0.05), but not of the SON sectioned F (2, 24) = 2.738, P = 0.0849) or day of the cycle (F (3, 24) = 1.934, P = 0.1511) (Figure 3). In the right ovary, such effects were not observed (interaction F (6, 24) = 1.379, P = 0.2633; SON sectioned F (2, 24) = 0.7539, P = 0.4814; day of the cycle F (3, 24) = 0.9286, P = 0.4421) (Figure 3).

**17 β -Hydroxysteroid Dehydrogenase:** 17β-HSD gene expression in the left and right ovary varies along the estrous cycle (F (7, 16) = 3.356, P<0.05) (Figure 2b). A two-way ANOVA on gene expression in the left and right ovary between control rats and animals with ipsilateral or contralateral SON sectioning treatment show that the differences are explained by the day of the estrous cycle when treatment was performed (Figure 4). No interaction between the day of the cycle and treatment was observed (interaction F (3, 16) = 1.918, P<0.1674; treatment F (1, 16) = 0.04757, P = 0.8301; day of the cycle F (3, 16) = 6.218, P< 0.001.

**Aromatase:** ANOVA on aromatase gene expression between left and right ovaries showed significant differences along the day of the estrous cycle, and the differences were higher in the right ovary (right ovary F (3, 8) = 35.83, P < 0.0001; left ovary F (3, 8) = 7.560, P= 0.01). The two-way analysis of variance showed an interaction between aromatase gene expression in each ovary and the day of the cycle (by ovary F (1, 16) = 9.352, P< 0.001; by day of the cycle F (3, 16) = 12.2, P< 0.001; interaction F (3, 16) = 4.737, P<0.05) (Figure 2 c).

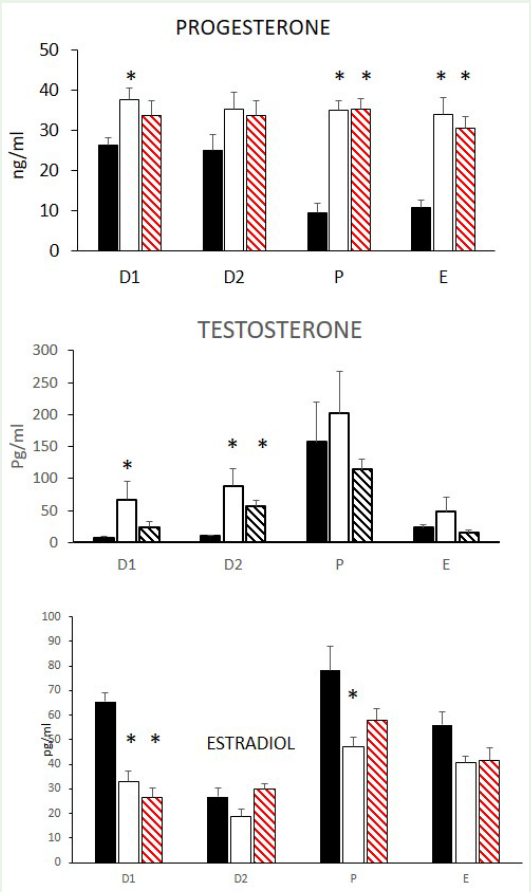


**Figure 1:** Progesterone (ng/ml), testosterone and estradiol (pg/ml) in cyclic control rats on each day of the estrous cycle and in rats with unilateral section of the superior ovarian nerve (SON) performed at 07.00 at each day of the estrous cycle, sacrificed one hour after surgery. Control; Section of the left SON. Section of the right SON.
* p<0.05 *vs.* control

In the left ovary, aromatase gene expression varied depending on the day of the cycle and the unilateral SON sectioning (ipsi-lateral or contralateral) treatment; and showed a significant interaction between the day of the cycle and the sectioning treatment (by sectioning treatment $F(3, 24) = 5.572$, $P < 0.01$; by day of the cycle $F(2, 24) = 23.70$, $P < 0.0001$; by interaction $F(6, 24) = 13.92$, $P < 0.0001$) (Figure 5). Such effects were not observed in the right ovary of rats with unilateral SON sectioning treatment (by SON sectioning treatment $F(2, 24) = 2.235$, $P = 0.1288$; by day of the cycle $F(3, 24) = 1.499$, $P = 0.2402$; by interaction $F(6, 24) = 1.051$, $P = 0.4141$) (Figure 5).

**Estradiol/Testosterone Ratio:** The estradiol/testosterone ratio during the estrous cycle is considered an index of aromatase activity (Chen et al. 2015). In the present study, the highest estradiol/testosterone ratio was observed on D1 (8.27) and the lowest on P (0.5). Unilateral sectioning of the SON resulted in significantly lower aromatase activity, since testosterone levels increased (control = 57, SON sectioning = 114), while estradiol levels were similar (control = 64, SON sectioning = 66).

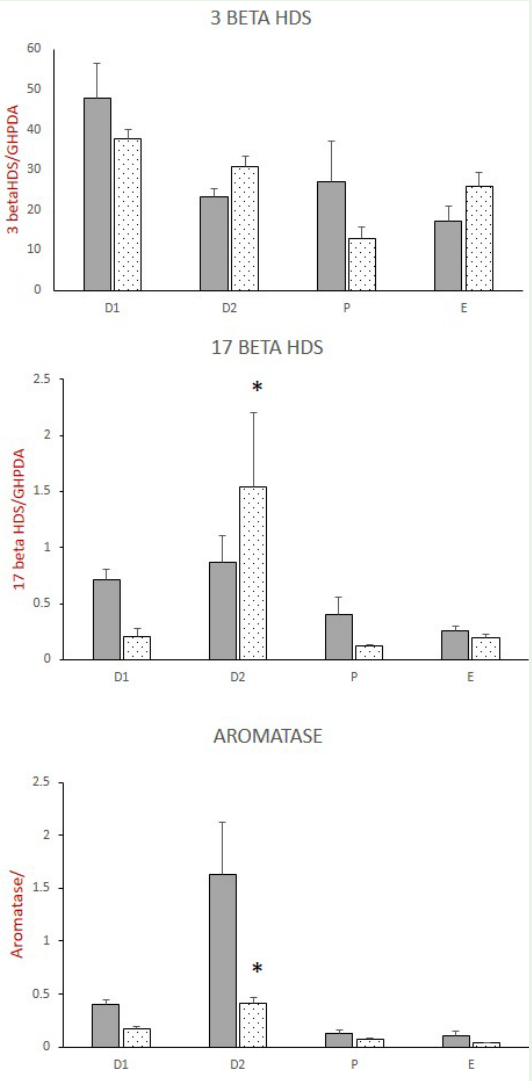


**Figure 2:** 3β-HSD, 17β-HSD, or aromatase gene expression in the left and right ovary from groups of untouched control rats sacrificed at 08.00 on each day of the estrous cycle. Left ovary right ovary.

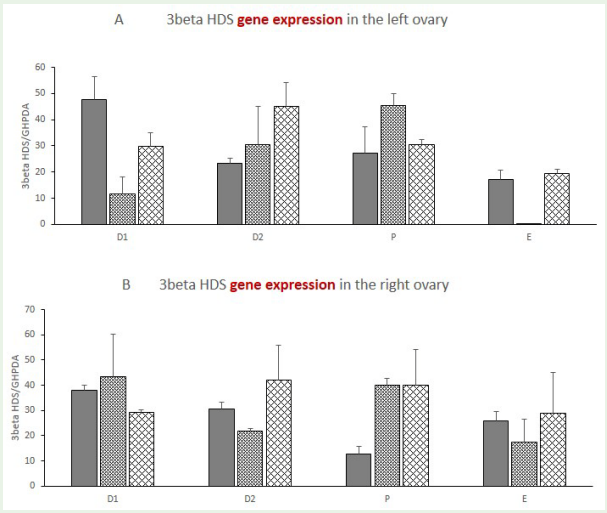


**Figure 3:** Effects of the section of ipsi or contralateral SON to the left (A) or right (B) ovary performed at 07.00 h on each day of the estrous cycle and sacrificed at 08.00 h, on the 3β-HSD gene expression. Control Section of the ipsilateral SON Section of the contralateral SON.
*p<0.05 *vs.* control.

**Analysis of percent changes in hormone levels and genes expression:** Analysis of the parallel changes between hormone levels and genes' expression resulting after unilateral SON sectioning showed two patterns: A) an increase in hormone levels followed by a decrease in gene expression, and vice versa; and B) an increase in both, hormone levels and gene expression, and vice versa (Figure 6).

Pattern A occurred in: 7/16 analysis on the progesterone-3β HDS relation; 11/16 for the testosterone-17β HDS relation; and 12/16 for the estradiol-aromatase relation.

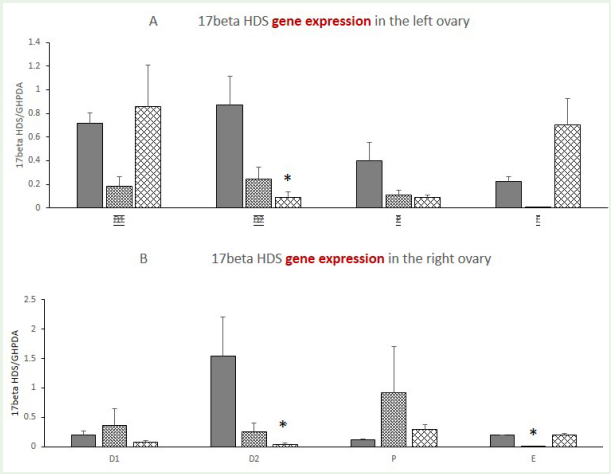


**Figure 4:** Effects of the section of ipsi or contralateral SON to the left (A) or right (B) ovary performed at 07.00 h on each day of the estrous cycle and sacrificed at 08.00 h, on the 17β-HSD gene expression. Control Section of the ipsilateral SON Section of the contralateral SON.
*p<0.05 *vs.* control.

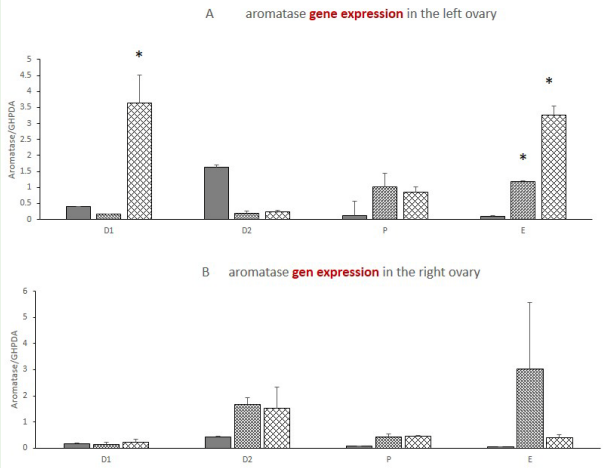


**Figure 5:** Effects of the section of ipsi or contralateral SON to the left (A) or right (B) ovary performed at 07.00 h on each day of the estrous cycle and sacrificed at 08.00 h, on the aromatase gene expression. Control Section of the ipsilateral SON Section of the contralateral SON.
* p<0.05 *vs.* control.

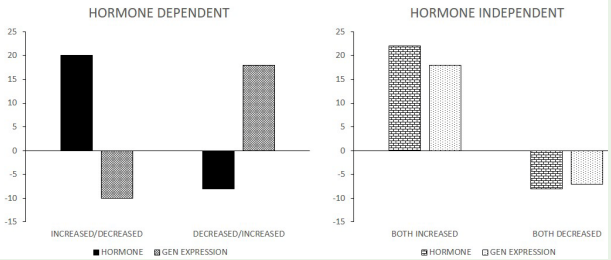


**Figure 6:** Summary of results of percentage changes in hormone levels and gene expression.

## Discussion

The results obtained in the present study show that the expression of 3βHDS, 17β HDS and aromatase genes vary along the estrous cycle. The results also showed that unilateral SON sectioning treatment modifies gene expression depending on the nerve sectioned, the gene studied, and the day of the cycle treatment was performed. The effects of unilateral SON sectioning on progesterone, testosterone and estradiol serum levels depends on the hormone evaluated, the nerve sectioned and the day of the cycle.

Morales et al. [19] suggested that the ovaries' SON innervation regulate the effects of gonadotropin in a stimulatory way that results in ovulation. On proestrus, SON sectioning resulted in lower progesterone and estradiol levels than in control rats. These effects were not observed in rats treated on E [23]. In estrus-day, electrical stimulation of the peripheral end of the right SON, at a supramaximal intensity for C fibers, reduced the right ovary's estradiol secretion rates. Estradiol's secretory response to SON activation is mediated by alpha 2-adrenoceptors, while alpha 1- adrenoceptors mediates the ovarian vascular response [28].

One hour after treatment, unilateral SON sectioning at 13.00-h resulted in hormone levels variations that were dependent of the estrous cycle's treatment day and the treated SON [18] and were similar to those observed in the present study. However, the modification patterns in hormone levels presented by Flores et al [18] do not parallel the changes observed in the present study, suggesting that the effects of neural denervation also depend on the hour of the day treatment is performed.

Morales-Ledesma et al. [19] suggested that in the pre-pubertal rat, the neural signals arriving to the ovaries via the SON regulate the enzymes participating in progesterone, testosterone and estradiol synthesis in a non-parallel way. They also suggested that the mechanisms regulating the synthesis of these hormones are not regulated by the same neural signals arriving to the ovaries via the SON and that the changes in steroids hormone levels are not explained by modifications in gonadotropin secretion. Present results support such interpretation.

Genes codifying for 3-β-HDS, 17-β-HDS and aromatase expression are stimulated by gonadotropins and growth hormone [13,29]. In the ovaries of gilts, expression of the aromatase (CYP19A1) protein was up regulated in response to increases in estradiol levels resulting from the prenatal or neonatal treatment with an antiandrogen (flutamide). The authors concluded that the higher estradiol levels observed in response to flutamide treatment was the result of the intensified aromatization and local estradiol action at the ovary levels [30]. Such relationship, between aromatase gene expression and estradiol levels, was not observed in the present study. The differences between the studies may be explained by the species studied, the age of the treated animals, and the time lapse between treatment and data collection (acute in the present study and several months in [30] study).

Compared to the control system, adding noradrenaline to the ovary incubation fluid of the ex-vivo system (coeliac ganglion-superior ovarian nerve- ovary) increased -HSD gene expression [31]. In the present study, unilateral sectioning the ipsilateral SON modified gene expression depending on the day of the cycle, the ovary (left or right) and the gene analyzed. These results suggest that each gene expression is regulated differently by the SON innervation. Rosas et al [32] showed that each ovary has different sensitivities to VIPergic stimulation, which depends on the endocrine status of the animal.

Sectioning the contralateral nerve to the left or right ovary modified the ovarian genes expression, supporting the idea of a neural communication between the ovaries [33]. According to Gerendai et al [34] neural fibers arising from the ovaries carry neural information to the central nervous system, including the hypothalamus, and such information participates in regulating gonadotropins secretion. It is well known that sectioning any nerve provokes an acute neural stimulus that travels through the proximal and distal ends of the nerve [35]. The ovary with the sectioned SON still receives neural information through the OPN and the vagus nerve, and the results obtained in the present study may reflect an imbalance between the neural signals arriving to the ovaries and the circulating gonadotropins, as proposed previously [19].

Taken together, present results suggest that the expression of genes codifying for three key enzymes in ovarian steroidogenesis is regulated, among other signals, by those arriving through the ovarian innervations and that such regulation varies along the estrous cycle.

## Authors' Note

NHRA and RD planned the experiments. MLS and AZ performed the surgeries and hormones measurements. MAP performed the genes measurements. RD, NHR, AF and SECM devised the study, participated in the discussion of the results, and co-wrote the manuscript. All authors read and approved the final manuscript. All experiments were carried out in strict accordance with the Mexican Law of Animal Treatment and Protection Guidelines and followed the Mexican Official Standard NOM-062-ZOO-1999 specifications.

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