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Effect of the relation between neural cholinergic action and nitric oxide on ovarian steroidogenesis in prepubertal rats☆

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Abstract

The coeliac ganglion and the ovary are related by the superior ovarian nerve, which penetrates into the ovary by the hilium and innervates mainly the ovarian stroma. On the other hand, it is known that the gaseous neurotransmitter nitric oxide (NO) and the two isoforms of its synthesis enzyme, the nitric oxide synthetase (NOS), are present in the ovary. Both innervation and NO participate in ovarian steroidogenesis. Therefore, the purposes of this work were (a) to standardize an in vitro coeliac ganglion–superior ovarian nerve–ovary integrated system in prepubertal rats; (b) to determine the presence of NO in the ovary and analyze the ganglionic cholinergic effect on the ovarian release of androstenedione, progesterone and NO; and (c) to assess the steroids/NO relationship. The system was incubated in buffer solution for 120 min, with the ganglion and ovary located in different compartments and linked by the superior ovarian nerve. From the results obtained, it is concluded that the system is viable and functional. The presence of basal NO is stimulated by the cholinergic action, while the release of the steroids is inhibited, which might indicate that the ganglionic cholinergic effect is probably mediated by NO. To our knowledge, this work constitutes the first study of the relationship between the neural cholinergic action and NO on the ovarian steroidogenesis of prepubertal rats.

Keywords: Coeliac ganglion; Ovarian innervation; Nitric oxide; Prepubertal rats

1. Introduction

Many researchers have reported evidence of a direct functional neural connection between the central nervous system and the ovary that contributes to the functioning of this gland [1–5]. The coeliac ganglion has proved to be one of the most relevant structures of this pathway, since most of the axons of its principal neurons constitute the superior ovarian nerve, which is considered as the main neural pathway related to ovarian steroidogenesis. The superior ovarian nerve penetrates into the ovary through the hilium and innervates the ovarian stroma, especially the theca and secondary interstitial cells, both responsible for androgen synthesis [6–12]. Previous studies have revealed that coeliac ganglion stimulation [13,14] as well as section or electric stimulation of the superior ovarian nerve, produces modifications in the secretion of ovarian steroids in rats at different stages of the reproductive life [15–19]. The fact that the preganglionic fibers reaching the coeliac ganglion are of cholinergic nature might indicate that there exists a cholinergic modulation over the sympathetic postganglionar output acting in the ovary [9,20].

A further factor related to the synthesis of ovarian steroids is nitric oxide (NO), a diffusible gas considered to act as a neurotransmitter and biological mediator of the neuroendocrine axis. A great deal of evidence has been reported of NO participation in reproduction control [21–23].

The synthesis enzyme of NO, nitric oxide synthetase (NOS), is present in the ovaries in its constitutive and inducible isoforms. The inducible isoform (iNOS) is expressed in the granulosa cells of the immature follicles and is absent in the mature follicles or those destined for atresia [24]. This indicates that, in these cells, the NO might act as a cytostatic factor. On the other hand, the constitutive isoform, more specifically, the endothelial nitric oxide synthetase (eNOS) is expressed in ovarian theca and stromal cells, in mural

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granulosa cells of maturing follicles and in the endothelial and steroidogenic cells of the theca–luteal region of corpora lutea [25]. The eNOS is stimulated by substances of neural origin such as acetylcholine and by platelet products such as serotonin, thrombin and adenosyldiphosphate [26].

Even though both the coeliac ganglion, through the superior ovarian nerve, and NO participate in ovarian steroidogenesis, no evidence has been reported, at least to our knowledge, of the effect of the interrelationship of these two factors on steroid synthesis during the rat prepubertal stage.

Therefore, and considering that this relationship may be studied in the in vitro coeliac ganglion–superior ovarian nerve–ovary integrated system previously developed in our laboratory [13], the purposes of this work were:

(a) to standardize the coeliac ganglion–superior ovarian nerve–ovary system in prepubertal rats measuring the androstenedione release profile, and to investigate possible histological modifications produced in the ovary throughout the incubation period;
(b) to investigate the presence of NO in the ovarian cuvette in basal conditions in the coeliac ganglion–superior ovarian nerve–ovary system;
(c) to study the ganglionic cholinergic influence on the ovarian release of androstenedione, progesterone and NO, and the possible steroid/NO relationship.

2. Materials and methods

2.1. Animals

Virgin Holtzman strain female prepubertal rats of 30 days of age (60 ± 10 g body weight) were used in all the experiments. The rats were kept under controlled conditions with lights on from 07:00 to 19:00 h and at a temperature of 24 ± 2 °C. Animals had free access to food (Cargill SACI, Saladillo, Buenos Aires, Argentina), and tap water was available ad libitum. Groups of six animals were used for the experimental procedure.

The experiments were performed in accordance with the revised Guide for the Care and Use of Laboratory Animals [27] and the Guide for Animal Use and Handling of the National University of San Luis.

2.2. Reagents

The following drugs: L-acetylcholine hydrochloride, L-hexametonium, atropine, dextrose, ascorbic acid, bovine serum albumin fraction V, sulfanilamide y N-1-naphthylethylenediamine, were purchased by the Sigma Chemical Co. (St. Louis, MO, USA). 1,2,6,7-[^{3}H]-Progesterone (107.0 Ci/mmol) and 1,2,6,7-[^{3}H]-androst-(4-ene-3,17)-dione (115.0 Ci/mmol) were provided by New England Nuclear Products (Boston, MA, USA). Other reagents were of analytical grade.

2.3. Surgical procedure and characterization of the coeliac ganglion–superior ovarian nerve–ovary system

The surgical procedure used for removing the system, its characterization and histological control were performed as described previously [13]. Briefly, a piece of tissue containing the left ovary, the fibres that constitute the superior ovarian nerve and the coeliac ganglion were removed. The strip of tissue was carefully dissected avoiding contact between the surgical instruments and the nerve fibres or the ganglion in order to prevent spontaneous depolarisation of the nerves. The total surgical procedure was completed in 1–2 min. In order to verify the existence of the ganglion, routine histological techniques were followed throughout the characterization of the system.

2.4. Standardization of incubation times

Rats on day 30 were anaesthetised with ether and the surgery procedure was performed between 09:00 and 10:00 h. The coeliac ganglion–superior ovarian nerve–ovary system was removed, cleaned with incubation medium and placed immediately in a cuvette with two compartments. The ganglion was placed in one compartment and the ovary in the other, connected by the superior ovarian nerve, which had to be kept moist with the work solution.

Each compartment contained 1 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) with the addition of glucose (0.1 mg/ml) and albumin (0.1 mg/ml) as has been described for the incubation of ovaries in other in vitro systems [28]. The system was immediately put in a metabolic bath at 37 °C in an atmosphere composed of 95% O_2–5% CO_2, and the preincubation time necessary for its stabilisation was noted. A previously standardized volume (80 μl) of incubation medium was extracted from the ovarian compartment for the determination of androstenedione every 5 min for the first 30 min, then every 30 min up to and including the 150th min. It was observed that stabilisation was achieved at 15 min (Fig. 1) which was then considered to be incubation time 0. At this time, the buffer was changed in both compartments and ascorbic acid (0.1 mg/ml in Krebs–Ringer) was added as an antioxidant agent in the ganglion compartment [29]. For all future experiments, extraction times of ovarian liquid were established at 15, 30, 60 and 120 min after incubation time 0.

2.5. Histological control of ovarian viability

Immediately after incubation (120 min), the ovarian viability was confirmed by the application of routine histological techniques. The material was fixed with Bouin's fluid. Serial cuts of 5 mm thickness were carried out utilising a sliding Reichert–Jung HN-40 microtome. Preparations were stained with haematoxylin–eosin and coated

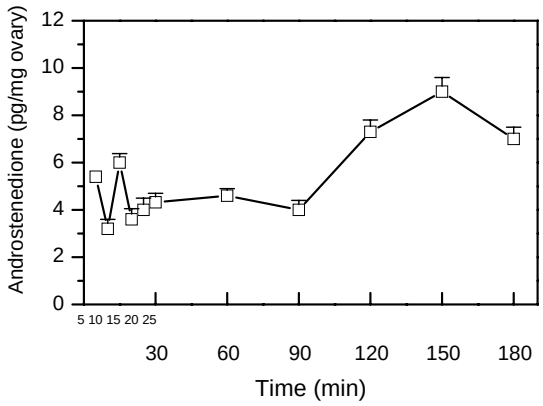

Fig. 1. Standardization of incubation times. Release of androstenedione by ovary in the coeliac ganglion–superior ovarian nerve–ovary system obtained from prepubertal rats. The system was incubated in Krebs–Ringer solution at 37 °C in an atmosphere of 95% O_2–5% CO_2 for 150 min. Androstenedione concentrations were measured every 5 min for the first 30 min, then every 30 min up to and including the 150th min. Values are the means for three experiments ± S.E.M.

with sintetle balsam. The micrographic image was captured using a Leitz–Dialux photomicroscope, equipped with a Leica camera. This system, using a 25′ objective, was used to examine tissue sections (total magnification ×250; Fig. 2).

2.6. Experimental procedure

The coeliac ganglion–superior ovarian nerve–ovary system was removed and placed in the above-described cuvette. The values of the androstenedione, progesterone and nitrite (a water-soluble metabolite of nitric oxide) released under these conditions were considered to be the control (control group). For the experimental groups, the cholinergic agents used were added to the ganglion compartment, and androstenedione, progesterone and nitrite release in ovarian comparment were measured. Acetylcholine was used as cholinergic agonist, atropine as muscarinic antagonist and hexametonium as nicotinic antagonist. The different substances were dissolved in equal concentrations (10^{-6} M) [29] and volumes (1 ml) of Krebs–Ringer solution plus ascorbic acid.

The samples of liquid from the ovarian compartment (250 μl) were collected at the times established during the standardization stage. These were kept at −20 °C until the determination of androstenedione and progesterone by radioimmunoassay (RIA) and nitrite by Griess method.The results were expressed as picograms of androstenedione per milligram of ovarian tissue (pg/mg ovary). The results of progesterone were expressed as nanograms of progesterone per milligram of ovarian tissue (ng/mg ovary), and nitrite as nanomol of nitrite per milligram of ovarian tissue (nmol/mg ovary) all against time of incubation. Corresponding corrections were made in all cases, taking into consideration the volume extracted in each period tested.

2.7. Androstenedione assay

The androstenedione contents were measured in duplicate by RIA. The antiserum were kindly provided by Dr R. Deis (Laboratorio de Reproducción y Lactancia, Mendoza, Argentina). These assays have previously been validated [30].

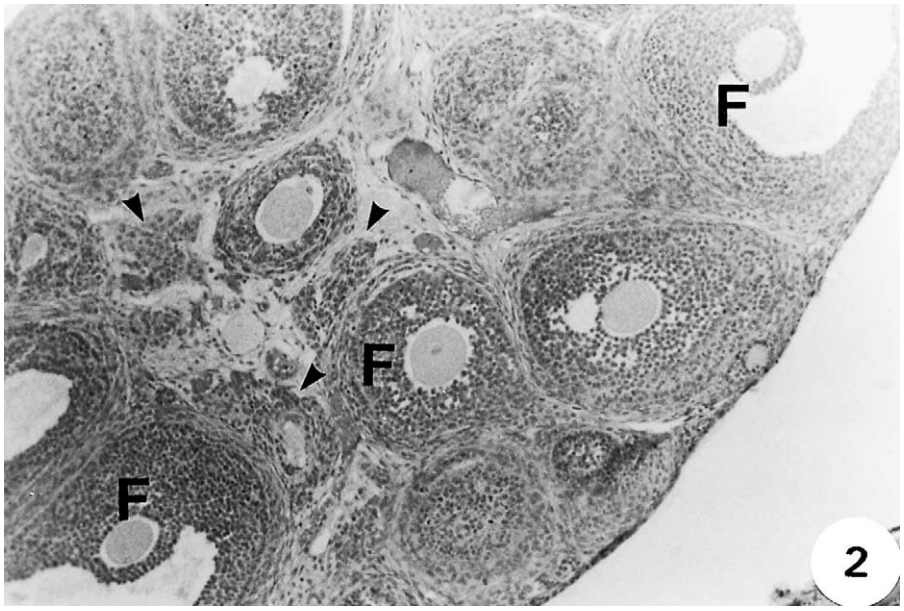

Fig. 2. Survey photomicrograph of ovary after 120 min of incubation, showing follicles in different stages of development (F) and interstitial tissue (Arrowheads). Stained with haematoxylin–eosin (original magnification ×250).

The assay sensitivity was less than 0.01 ng/ml. The intraassay coefficients of variation for all the assays were less than 10.0%.

2.8. Progesterone assay

Progesterone was measured by RIA using antiserum raised against progesterone-11-bovine serum albumin conjugate in rabbits, provided by Dr. R. Deis (Laboratorio de Reproducción y Lactancia, Mendoza, Argentina). The sensitivity, of the assay is less than 5 ng/ml and the inter and intraassay coefficients of variation were less than 10%. The variability and cross-reaction of this RIA has been reported previously [31]. This assay has been validated previously [30].

2.9. Nitrite assay

Levels of nitrite, a water-soluble metabolite of nitric oxide, were measured spectrophotometrically [32]. Briefly, the sample was mixed with Griess reagent (sulfanilamide with *N*-1-naphthyl-ethylenediamine/HCl). After a 10 min incubation at room temperature, it was read for absorbance of 540 nm, and nmol of nitrite were determined using a standard curve. The assay sensitivity was less than 2.5 nmol/ml. The intraassay coefficients of variation for all the assays were less than 10.0%.

2.10. Statistical analysis

Results are presented as mean ± S.E.M. in each group. Student's *t*-test was used to assay significant differences between means of two groups. Analysis of the variances (ANOVA) followed by Tau's multiple range test was used for several comparisons. A values of $P < 0.05$ was accepted as statistically significant [33].

3. Result

3.1. Histological study

Abundant follicles in different maturation stages are observed. The follicles are covered by highly developed theca cells. Blood vessels are abundant.

The interstitial tissue is arrayed in the form of cell strings. All the structures are well preserved. Neither control ovaries nor ovaries stimulated with cholinergic agents showed structural modifications after 120 min of incubation (haematoxylin–eosin staining ×250; Fig. 2).

3.2. Presence of nitric oxide in the ovarian compartment

3.2.1. Effect of addition of cholinergic agents to the ganglion compartment on release of nitrite

The presence of cholinergic agents in the ganglion compartment increased the release of nitrite compared with the control group.

Acetylcholine caused a significant increase in the release of nitrite at all times studied (15, 30 and 60 min, $P < 0.001$; 120 min, $P < 0.01$) (Fig. 3a).Hexametonium only increased the release of nitrite at 120 min ($P < 0.01$), and atropine until 60 min (15 min, $P < 0.001$; 30 min, $P < 0.01$; 60 min, $P < 0.05$) (Fig. 3b).

3.2.2. Effect of addition of cholinergic agents to the ganglion compartment on release of ovaric steroids

The presence of cholinergic agents, in 10^{-6} M concentration, in the ganglion compartment diminished the release of ovarian androstenedione and progesterone, compared with the control group.

When the release of androstenedione was analyzed, acetylcholine inhibited it, at all times studied (15 min, $P < 0.05$; 30 min and 60 min, $P < 0.01$; 120 min, $P < 0.001$) (Fig. 4a). Hexametonium and atropine only caused

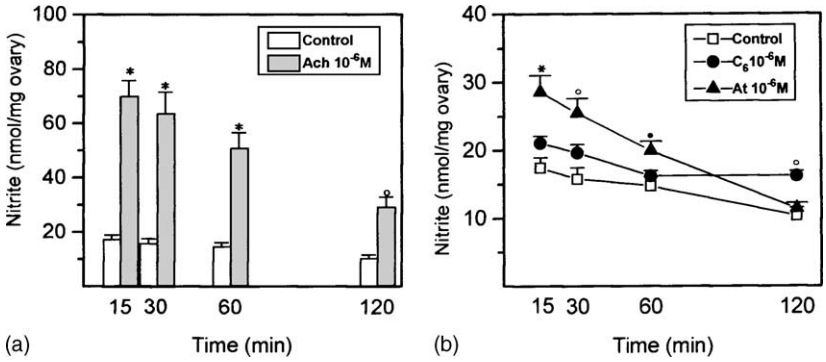

Fig. 3. Effect of agonist (a) and antagonist (b) cholinergic in ganglion compartment on ovarian nitrite release in the coeliac ganglion–superior ovarian nerve–ovary system obtained from prepubertal rats. The system was incubated in Krebs–Ringer solution, at 37 °C in an atmosphere of 95% O_2–5% CO_2 for 120 min without (control) and with cholinergic agents in 10^{-6} M concentration added to the ganglionic compartment (experimental group). Values are the mean ± S.E.M. of six animals per experimental group. Ach: acetylcholine; C6: hexametonium; At: atropine. *$P < 0.001$, °$P < 0.01$ and ●$P < 0.05$ compared with the control group.

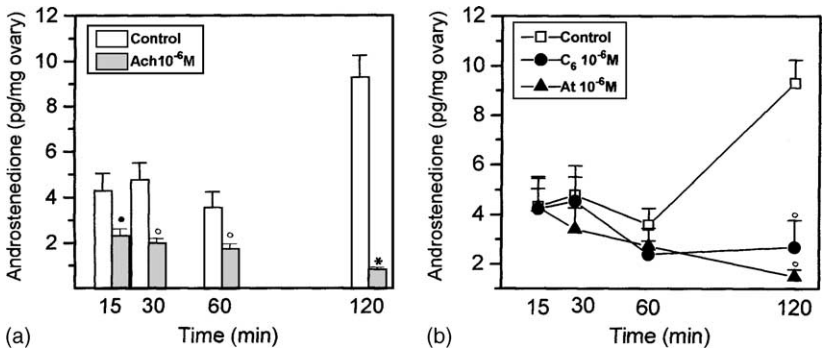

Fig. 4. Effect of agonist (a) and antagonist (b) cholinergic in ganglion compartment on ovarian androstenedione release in the coeliac ganglion–superior ovarian nerve–ovary system obtained from prepubertal rats. The system was incubated in Krebs–Ringer solution, at 37 °C in an atmosphere of 95% O_2–5% CO_2 for 120 min without (control) and with cholinergic agents in 10^{-6} M concentration added to the ganglionic compartment (experimental group). Values are the mean ± S.E.M. of six animals per experimental group. Ach: acetylcholine; C_6: hexametonium; At: atropine. $^*P < 0.001$, $^\circ P < 0.01$ and $^\bullet P < 0.05$ compared with the control group.

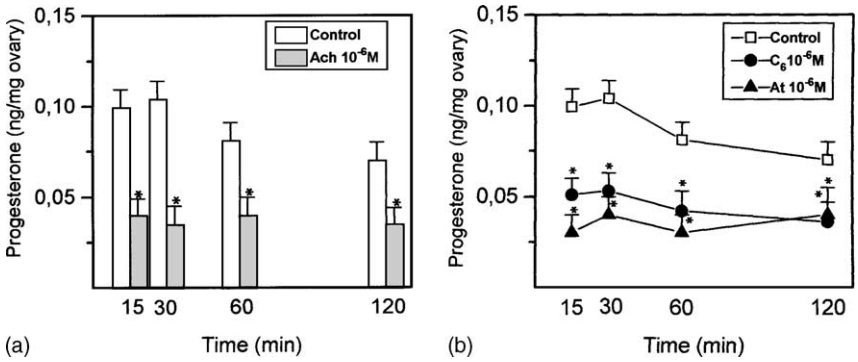

Fig. 5. Effect of agonist (a) and antagonist (b) cholinergic in ganglion compartment on ovarian progesterone release in the coeliac ganglion–superior ovarian nerve–ovary system obtained from prepubertal rats. The system was incubated in Krebs–Ringer solution, at 37 °C in an atmosphere of 95% O_2–5% CO_2 for 120 min without (control) and with cholinergic agents in 10^{-6} M concentration added to the ganglionic compartment (experimental group). Values are the mean ± S.E.M. of six animals per experimental group. Ach: acetylcholine; C6: hexametonium; At: atropine. $^*P < 0.001$ compared with the control group.

a significant decrease of androstenedione at 120 min ($P < 0.01$) (Fig. 4b).

When the release of progesterone was analyzed, acetylcholine, hexametonium and atropine caused a significant decrease of progesterone, at all times studied ($P < 0.001$; Fig. 5a and b).

4. Discussion

It is well known that the ovary is controlled by different hormonal [34], immunologic [35], paracrine–autocrine [22] and neural [13] factors.

At present, there is strong evidence that SON is the way most highly involved in the ovary functioning by its participation in cell maturation and differentiation phenomena, as well as in steroidogenesis [4,7,8,13,14,16,17,19,28]. The participation of NO in these phenomena has also been demonstrated [21–23]. In this context, it is interesting that NO synthesis enzymes in their different isoforms have been characterized and located in the ovary [24,36].

Considering the increasing interest on the direct neural influence on steroidogenesis and that NO also participates in follicle maturation by modulating the synthesis of steroids, we studied the relationship between the neural cholinergic action and NO, and its effect on ovarian steroidogenesis in prepubertal rats, in an in vitro coeliac ganglion–superior ovarian nerve–ovary integrated system.

In prepubertal rats, the main functionally active structures are immature follicles [37]. These follicles are formed by granulosa and theca cells, the latter being in close relation with the superior ovarian nerve fibers. On the other hand, considering that androstenedione is the main secretion product of the theca cells [10], that it is the substrate for estradiol synthesis in granulosa cells [38], and that it has been shown to significantly increase with neonatal section of the superior ovarian nerve in prepubertal rats [16], the system was standardized for secretion of this androgen.

The coeliac ganglion–superior ovarian nerve–ovary system provides an in vitro simulation of in vivo conditions [13]. In this work, the system proved to be functional during the incubation period because the basal level of androstene-

dione release is maintained by effect of the autonomous ganglionic tone. This result seems to be an indication of the greater androstenedione availability as estrogen substrate to favor follicular maturation [38]. Besides, the histological study showed that ovarian structures are preserved after 120 min of incubation, which is an indication of the viability of the coeliac ganglion–superior ovarian nerve–ovary system in the prepubertal stage, as has already been demonstrated for other stages of the rat reproductive life [13,14].

On the basis of these results, and also considering evidence for the participation of NO in reproduction control [21–23], we investigated whether NO is present in the ovarian cuvette of the system under study. It was shown that, under basal conditions, the profile of NO release is maintained throughout the experiment, which, as in the case of androstenedione, is a result of the neural ganglionic tone. These findings match reports by Srivastava et al. [36], who provided evidence for the expression of the constitutive and inducible isoforms of the NO synthesis enzyme in the prepubertal ovary, indicating that the NO/NOS system plays a physiological role in the ovarian function. In turn, Matsumi et al. [24] found that iNOS is predominantly localized in granulosa cells of healthy immature follicles in the rat ovary, whereas granulosa cells of either healthy mature follicles or follicles destined to be atretic are devoid of iNOS. These finding suggest that iNOS is pivotal for immature follicles to remain dormant.

Considering the above results, and knowing that (i) the classical preganglionic neurotransmitter of the sympathetic ganglionic chain is acetylcholine [9,20], and (ii) the coeliac ganglion exhibits nicotinic and muscarinic receptors [20,39] and has its own neural tone, which permits the use of antagonists without the simultaneous addition of the agonist [13], we studied the direct ganglionic cholinergic effect upon the ovarian release of androstenedione, progesterone and NO. Progesterone analysis was included because previous studies have shown that it is the most sensitive steroid to direct neural action [28].

Our results indicated that the presence of acetylcholine in ganglion produced a stimulating effect on NO release, at all the studied times. Considering the effect of the antagonists, it can be concluded that in this case the ganglionic action of acetylcholine takes place through nicotinic receptors. On the other hand, acetylcholine inhibited the release of androstenedione and progesterone in an unspecific way. These results are in agreement with those reported by other researchers who have found that NO inhibits ovarian release of both androstenedione [23] as well as progesterone [22], although in different experimental schemes. The involved mechanisms are probably related to NO inhibition of the activity of steroid synthesis-limiting enzyme, cytochrome P_{450} side chain cleavage, and of the enzyme that participates in androgen aromatization, aromatase [40,41], as well as of two isoforms of adenylyl cyclase, the second intracellular messenger [42].

To our knowledge, this study constitutes the first demonstration of the effect of the relation between neural cholinergic action and NO on ovarian steroidogenesis. Undoubtedly, further experiments are needed to elucidate the mechanisms involved in the obtained responses.

As a whole, this study permits us to hypothesize that the ganglionic cholinergic effect on ovarian steroidogenesis may be mediated, among other neurotransmitters, by NO. In this way, NO seems to play a role in maintaining the physiology of the prepubertal rat ovary, characterized by the presence of immature follicles.

Acknowledgements

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