

The cholinergic influence on the mesenteric ganglion affects the liberation of ovarian steroids and nitric oxide in oestrus day rats: characterization of an *ex vivo* system

Adriana Vega Orozco, Zulema Sosa, Verónica Fillipa¹, Fabian Mohamed¹ and Ana María Rastrilla

Laboratorio de Biología de la Reproducción (LABIR), ¹Área de Morfología, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Chacabuco 917, 5700 San Luis, Argentina

(Requests for offprints should be addressed to A M Rastrilla; Email: amras@unsl.edu.ar)

Abstract

The axons that constitute the ovarian nervous plexus originate mostly in the principal neurons of the superior mesenteric ganglion (SMG) that is part of the sympathetic ganglionic chain and exhibits cholinergic receptors. In order to observe the effect of acetylcholine, the main neurotransmitter in the ganglionic transmission, the purpose of the present work was: first, to standardize an integrated *ex vivo* superior mesenteric ganglion-ovarian nervous plexus-ovary (SMG-ONP-O) system in oestrus day rats; secondly, to determine if the ganglionic cholinergic stimulus modifies the release of nitric oxide and steroids in the ovary compartment in the absence of humoral factors; and thirdly, to investigate if there are differences in the responses between the left and right ovaries caused by the neural stimulus. The *ex vivo* experimental left and right systems were developed and standardized. The systems were incubated in Krebs-Ringer phosphate buffer in a Dubnoff metabolic shaker. The progesterone release was determined to standardize the incubation times, obtaining different responses between the left and right systems, which shows that both systems have their own autonomic tone. Non-specific stimulation with KCl in the ganglion compartment provoked different responses in terms of release of progesterone and oestradiol. Progesterone decreased in the left and right systems.

However, oestradiol diminished at short times and increased at 60 and 120 min in the left ovary, whereas it increases at 30 and 60 min in the right ovary. These different responses show the sensitivity and viability of both systems. When acetylcholine was used in the ganglion compartment, the release of nitric oxide, progesterone, androstenedione and oestradiol was evaluated. The liberation of nitrite increased at 15, 30 and 60 min in the left system and decreased in the right system at 120 min. Progesterone showed a decrease in its release at 15, 30 and 120 min and androstenedione at 15 min in the left ovary compartment. In the right ovary, only progesterone decreased in relation to the control at 120 min while androstenedione did not show significant changes. Oestradiol showed an increase in the left ovary compartment at all the studied times, while in the right ovary it did not show any changes. These results indicate that the neural stimulus from the superior mesenteric ganglion through the ovarian nervous plexus is one of the factors modulating the secretory activity of the ovarian steroids and nitric oxide. The system is viable and also shows a different sensitivity of the left ovary in relation to the right one at least in this cycle stage, characterized by marked irrigation and profound structural changes in the ovary.

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Introduction

Gerendai *et al.* (1998), using a viral tracing technique, have provided the first morphological evidence of the existence of a neuronal multi-synaptic way between the ovary and various centres of the central nervous system through the sympathetic ganglionic pathway. Experiments carried out in our laboratory have shown that coeliac ganglion stimulus with cholinergic agents and an adrenergic agent modifies the ovarian steroids release through the superior ovarian nerve (Sosa *et al.* 2000, 2004, Casais *et al.* 2001, Delgado *et al.* 2004). On the other hand, the intracerebroventricular injection of epinephrine modifies rapidly the concentration of progesterone in the ovarian vein of

dioestrus day 1 and dioestrus day 2 rats (De Bortoli *et al.* 1998). This may provide physiological evidence of the participation, at least in part, of the sympathetic ganglionic pathway in the responses observed, acetylcholine being the classic preganglionic neurotransmitter.

The ovarian nervous plexus (ONP), whose fibres originate mostly in the neurons of the mesenteric ganglia, reaches the ovary and accompanies the ovarian artery in its entrance through the hilum (Baljet & Drukker 1979, Klein & Burden 1988a). The plexus innervates the blood vessels musculature, serving an important function in the regulation of the blood flow (Wallis *et al.* 1978), and its veins form a net around the ovary cortex and do not have direct relationship with the

corpora lutea and the granulosa cells (Stefenson *et al.* 1981, Burden *et al.* 1985).

In relation to the mesenteric ganglia, it constitutes a component of the sympathetic prevertebral ganglionic pathway. The superior mesenteric ganglion (SMG) has the specific structures to respond to cholinergic stimulus, such as nicotinic and muscarinic receptors in the ganglionic neurons called principal neurons (Prud'Homme *et al.* 1999); muscarinic receptors are also present in the interneurons, such as the small intensely fluorescent cells and peptidergic cells (Jarvi 1989). Neuropeptide Y, substance P, norepinephrine, and nitric oxide (NO) constitute the main postganglionic neurotransmitters released by the ganglion (Klein & Burden 1988b).

The study of the indirect immunofluorescence reveals that in the mesenteric ganglion, the distribution of nitric oxide synthase (NOS), synthesis enzyme of NO, has one possible colocalization with immunoreactive neuropeptides and with two enzymes of the catecholamine synthesis pathway, tyrosine hydroxylase and dopamine β -hydroxylase, as well as the enzyme for the acetylcholine synthesis pathway, choline acetyltransferase (Lars-Gösta *et al.* 1997). The nitric oxide is considered as a neurotransmitter and biological mediator of the neuroendocrine axis and there is abundant evidence of its participation in reproduction control (Brann *et al.* 1997, Motta *et al.* 2001, Delgado *et al.* 2004). However, there is little information about the role played by the neural factors in NO release in cyclic rats. Considering the increasingly recognized influence of the ovary innervation in the control of the diverse organ functions in different reproductive states (Aguado & Ojeda 1984, Sosa *et al.* 2000, 2004, Casais *et al.* 2001, Garraza *et al.* 2004), and the participation of NO in steroidogenesis, as observed in prepubertal rats (Delgado *et al.* 2004), and also in the biochemical and vascular changes that occur in the ovulating period, development and regression of the corpus luteum (Acosta & Miyamoto 2004), we also thought it could be interesting to study the neural influence of the superior mesenteric ganglion on the NO ovarian liberation during oestrus. The oestrus is the stage of the oestrous cycle when there is more ovarian irrigation (Acosta & Miyamoto 2004), and the nervous plexus is related with the blood flow (Lawrence & Burden 1980). With this purpose in mind, this work intends (1) to standardize an integrated *ex vivo* superior mesenteric ganglion-ONP-ovary system in oestrus day rats; (2) to determine if the ganglion cholinergic stimulus modifies the release of nitric oxide and steroids in the ovary compartment in the absence of humoral factors; and (3) to investigate if there are differences in the responses between the left and right ovaries caused by the neural stimulus.

Materials and Methods

Animals

Virgin Holtzman strain adult female rats on the day of oestrus (E) weighing 250 ± 50 g were used in all the experiments. The rats

were kept in a light (lights on from 0700 to 1900 h) and controlled-temperature room (24 ± 2 °C). Animals had free access to food (Cargill, SACI, Saladillo, Buenos Aires, Argentina), and tap water was available *ad libitum*. Vaginal smears were taken daily, and only the rats exhibiting at least two 4-day consecutive oestrous cycles were used. Groups of six animals in oestrus (E) were used for the experimental procedure. This stage was chosen for three important reasons: first, because it is the period of the oestrous cycle when there is more ovarian irrigation, and the increased values of oestrogen cause a rapid dilation of blood vessels by activating endothelial NOS (Acosta & Miyamoto 2004); secondly, because the ONP fibres are mainly perivascular (Lawrence & Burden 1980) and could take part in the vascularization changes that occur in this stage; and thirdly, because it is a stage of deep biochemical and structural changes (Mayerhofer & Fritz 2002). The experiments were performed according to the procedures approved in the UFAW Handbook on the Care and Management of Laboratory Animals (Poole 1999) and the Guide for Animal Use and Handling of the National University of San Luis, Argentina.

Reagents

The following drugs: L-acetylcholine hydrochloride (Ach), ascorbic acid, bovine serum albumin fraction V (BSA), sulphanilamide and *N*-1-naphthyl-ethylenediamine were purchased by the Sigma Chemical Co. Other reagents and chemicals were of analytical grade. $1,2,6,7$ -[3 H] Progesterone (107.0 Ci/mmol), $1,2,6,7$ -[3 H] androst 4-ene-3,17 dione (115.0 Ci/mmol) and 17 - β -2,4,6,7-[3 H] oestradiol (102.0 Ci/mmol) were provided by New England Nuclear Products (Boston, MA, USA).

Surgical procedure

The procedure was carried out between 1500 and 1600 h, taking account of previous descriptions of the anatomical trajectory of this neural pathway as a guide (Lawrence & Burden 1980, Klein & Burden 1988a; Fig. 1a). Rats were anaesthetized with ether under a bell, and the left and right systems were immediately removed by dissection. Each system is conformed by the ovary, the fibres that constitute the ovarian nervous plexus parallel to the ovarian artery and the superior mesenteric ganglion accompanied by some small ganglia surrounding it. The total surgical procedure was completed in 1–2 min. The strip of tissue was carefully dissected avoiding contact between the surgical instruments, the nerve fibres and the ganglion. This is essential in order to prevent spontaneous depolarizations of the nerves which might be caused by an inappropriate contact. The left and right mesenteric ganglion-ONP-ovary systems were washed with incubation solution and immediately placed in one cuvette with two compartments. The superior mesenteric ganglion was placed in a compartment and the ovary in the other one, both joined by the ONP which had to remain humid with the working solution (Fig. 1b).

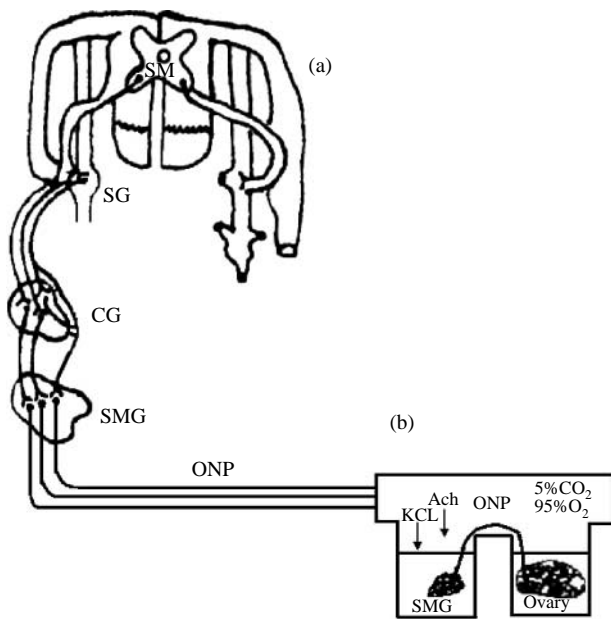


Figure 1 (a) Schematic representation of the neural connection between the superior mesenteric ganglion and the ovary joined by the ovarian nervous plexus. (b) The cuvette utilized for the subsequent incubation of the system. SM, spinal marrow; SG, sympathetic ganglion; CG, coeliac ganglion; SMG, superior mesenteric ganglion; ONP, ovarian nervous plexus; KCl, potassium chloride; Ach, acetylcholine.

Histological control of the superior mesenteric ganglion

Immediately before and after incubation (120 min), the presence of the superior mesenteric ganglion was confirmed in the system by the application of routine histological techniques. The presence of the ganglion was verified in the removed system before the incubation in similar conditions to the systems that will be used later. The material was fixed with Bouin's fluid. Serial cuts of 5 mm thickness were carried out utilizing a sliding Reichert-Jung HN-40 microtome. Preparations were dyed with haematoxylin-eosin and coated with sintete balsam. The micrographic image was captured using a Leitz-Dialux photomicroscope, equipped with a Leica camera. This system, using a twenty-five times objective, was used to examine tissue sections.

Standardization of incubation times

Rats on oestrus day were anaesthetized with ether under a bell for the surgery. The standardization tests were carried out measuring progesterone since it is a stage when the transformation of follicles to corpora lutea starts, and, consequently, the response to the neural stimulus in the progesterone liberation could be more sensitive. The surgery was carried out between 1500 and 1600 h, taking advantage of the stable progesterone concentrations that occur at that time of the day. The *ex vivo* system was removed, cleaned with incubation medium and immediately placed in a cuvette with

two compartments and incubated in Dubnoff metabolic shaker in an atmosphere of 5% CO₂ in 95% O₂ at 37 °C. Each compartment contained 2 ml Krebs-Ringer bicarbonate buffer, pH 7.4, with the addition of dextrose (0.1 mg/ml) and albumin (0.1 mg/ml) in both compartments, which has been described for ovary incubation in other *in vitro* systems (Aguado *et al.* 1982, Sosa *et al.* 2000).

A previously standardized volume (20 µl) of incubation medium was extracted from the ovarian compartment for the determination of progesterone every 1 min for the first 5 min, then every 5 min up to and including the 60th min, and from then on every 10 min up to and including the 180th min. The preincubation time needed for its stabilization was recorded. It was observed that stabilization was achieved at 15 min (see Fig. 3a and b), which was then considered to be incubation time 0. It is emphasized that during the first 15 min of incubation there is a great variation in the progesterone values obtained and after that time, the values fall within a similar range. After 15 min of the system preincubation, the Krebs-Ringer solution was changed in both compartments, and ascorbic acid (0.1 mg/ml in Krebs-Ringer) was added as an antioxidant agent (Koh & Hille 1997) in the ganglion compartment whereas the Krebs-Ringer solution was added in the ovarian compartment. The times of extraction of the ovarian liquid were established at 15, 30, 60 and 120 min. The greatest variations in the liberation of hormones in the right and left systems were observed at these times.

Once the system was extracted and the stabilization period had passed, the substances to be tested were added in the ganglion compartment at incubation time 0.

Experimental procedure

Rats in oestrus were used. In order to observe the viability and functionality of the system, groups with (experimental group) and without (control group) non-specific depolarizing stimulus KCl 56 mM (Koh & Hille 1997) in the ganglion compartment were used. The samples of liquid from the ovarian compartment (250 µl) were collected at 15, 30, 60 and 120 min, and the progesterone and oestradiol levels were measured under these conditions in the ovary compartment.

The ganglionic cholinergic effect on the release of nitric oxide, progesterone, androstenedione and oestradiol in the ovary compartment was evaluated in relation to the control group. The cholinergic agent employed was acetylcholine (Ach), which was dissolved in a working solution at 10⁻⁶ M final concentration (Delgado *et al.* 2004, Sosa *et al.* 2004). The steroids were determined by RIA and the nitrites, by the Griess method. The respective corrections were made in all cases considering the volume extracted in each test period.

Nitrite assay

Levels of nitrite, a water-soluble metabolite of nitric oxide, were measured spectrophotometrically by the Griess method

and were expressed in nmol of nitrite per milligram of ovarian tissue per ml (nmol/mg ovary per ml) (Egami & Taniguchi 1974). The samples (50 μ l) were immediately mixed with Griess reagent (sulphanilamide with *N*-1-naphthyl-ethylenediamine/HCl). After 10 min incubation period at room temperature, it was read for absorbance at 540 nm, and nmols of nitrite were determined using a standard curve. The assay sensitivity was <2.5 nmol/ml. The intraassay coefficients of variation for all the assays was <10.0%.

Progesterone, androstenedione and oestradiol assay

Steroids were measured in duplicate by RIA. The antisera were kindly provided by Dr R Deis (Laboratorio de Reproducción y Lactancia, Mendoza, Argentina). Progesterone was measured in nanogram per milligram ovary per ml and assay sensitivity was <5 ng progesterone/ml serum. The technique sensitivity was measured in serum although in our laboratory the corresponding assays were carried out in our working solution resulting in the same sensitivity; thus, progesterone concentration is expressed as nanogram per milligram ovary per ml of working solution. Androstenedione and oestradiol were expressed as picograms per milligram of ovarian tissue per ml (picogram/milligram ovary per ml), all against incubation time on the left and right systems. The assay sensitivity was <10 pg androstenedione/ml and <2.2 pg oestradiol/ml. The inter- and intraassay coefficients of variation in all the assays were <10.0%.

Statistical analysis

All data are presented as means \pm S.E.M. in each group of six rats. Differences between two groups were analysed with Student's *t*-test. Analysis of variance (ANOVA I) followed by the Duncan's multiple range test was used for several comparisons. A value of $P < 0.05$ was considered statistically significant (Snedecor & Cochran 1976).

Results

Histological control

The morphological study confirmed the presence of the mesenteric ganglion in the isolated *ex vivo* system. The ganglion was extracted from the area proximal to the coeliac ganglion and from the area proximal to the renal and aortic artery.

Figure 2a and b is a photomicrograph of the morphologic characteristics of the mesenteric ganglion, showing numerous neuronal somas (arrowheads). The ganglion is surrounded by a thin capsule of connective tissue (Fig. 2a). Figure 2b higher magnification of Fig. 2a, showing the several forms of the mesenteric neuronal somas.

Standardization of the incubation times

Figure 3a and b shows the progesterone values obtained in the left and right superior mesenteric ganglion-ONP-ovary system during 180 min under basal conditions in the presence of

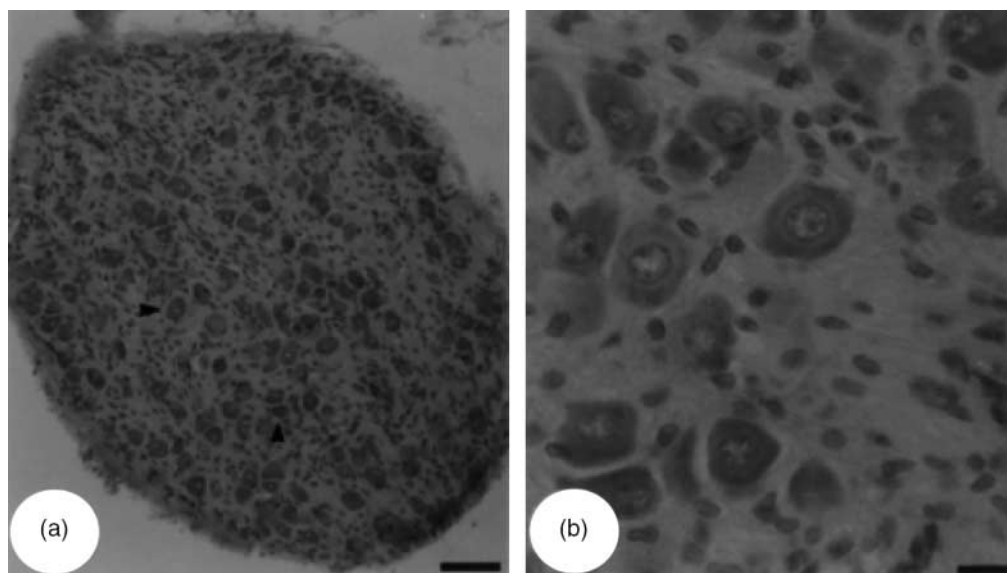


Figure 2 (a) Photomicrograph showing the morphologic characteristic of a mesenteric ganglion, with numerous neuronal somas (arrowheads). The ganglion is surrounded by a thin capsule of connective tissue, $\times 100$. Scale bar: 100 μ m. Haematoxylin–eosin. (b) Higher magnification of (a), showing several forms of the mesenteric neuronal somas, $\times 400$. Scale bar: 25 μ m. Haematoxylin–eosin.

ascorbic acid in the ganglion incubation medium, demonstrating that both systems have their own neural tone when progesterone is released. In both systems, the preincubation time was at 15 min, when stabilization was reached. It should be emphasized that before this time there is a great variation in the values obtained, and after 15 min, considered incubation time 0, the values fall at different times within a similar range. The variation is due likely to the cut or section of the nerve that

causes liberation of abundant quantities of the classic preganglionic neurotransmitter, acetylcholine.

Effect of the addition of KCl 56 mM in the ganglion compartment on the ovarian release of progesterone and oestradiol on oestrus day

In order to determine if non-specific stimulation of the mesenteric ganglion affected the release of progesterone on the oestrus day, KCl 56 mM was used. The KCl is considered to be a non-specific stimulating agent on the preganglionic nerve fibres (Koh & Hille 1997). In this case the progesterone release observed during oestrus (E) day in the different groups was: the addition of KCl to the ganglion compartment in the left and right systems decreased the liberation of progesterone at all the studied times in relation to their controls. In the left system the liberation decreased at 15 and 60 min ($\dagger P < 0.01$) and at 30 and 120 min ($*P < 0.001$), whereas in the right system, it decreased in relation to the controls at all the studied times ($*P < 0.001$) (Fig. 4a and b).

The oestradiol release measured in the left system, when KCl was added to the ganglion compartment decreased the liberation of oestradiol at 15 min ($\dagger P < 0.05$), but it significantly increased at 60 and 120 min ($*P < 0.001$) under the same conditions (Fig. 5a).

In the right system, the liberation of oestradiol increased at 30 and 60 min ($*P < 0.001$), and it reaches control value at 120 min (Fig. 5b).

Effect of the addition of cholinergic agents to the ganglion compartment on ovarian release of nitric oxide on oestrus day

On the day of oestrus the presence of acetylcholine as a physiological cholinergic agonist in the ganglion compartment modified the release of nitric oxide at most of the studied times, increasing in the left system in relation to the control group at 15, 30 and 60 min of incubation ($*P < 0.001$) (Fig. 6a), while in the right system it significantly decreased at 120 min ($\dagger P < 0.05$) (Fig. 6b).

Effect of the addition of cholinergic agents to the ganglion compartment on ovarian steroids release on oestrus day

When the progesterone release was analysed, acetylcholine caused a significant decrease in relation to the controls at 15, 30 min ($\dagger P < 0.05$) and 120 min ($*P < 0.001$) of incubation in the left system (Fig. 7a).

In the right system, the decrease only occurred at 120 min ($\dagger P < 0.05$) while there were no significant changes in relation to the control at the other studied times (Fig. 7b).

The presence of cholinergic agents in the ganglion compartment diminished the release of ovarian androstenedione compared with the control group at 15 min ($*P < 0.001$) in the left system. In the right system, androstenedione did not show any variations in relation to the control group (Fig. 8a and b).

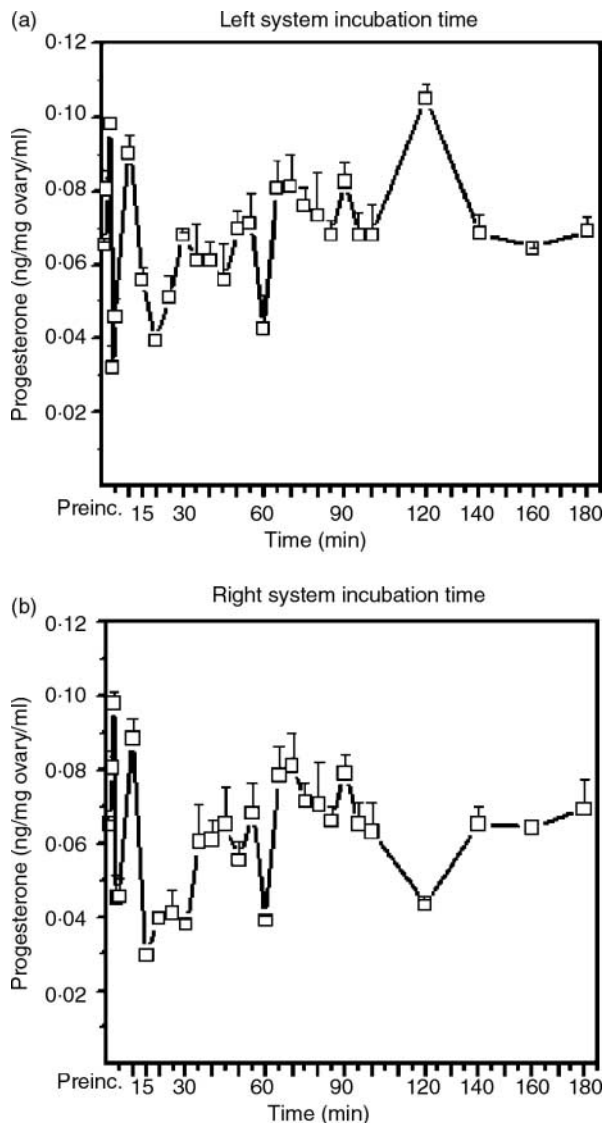


Figure 3 (a and b) Incubation times. Release of progesterone by the left (a) and right (b) ovaries in the superior mesenteric ganglion-ONP-ovary system obtained from the oestrus(E) day. The systems were incubated in Krebs-Ringer solution at 37 °C in an atmosphere of 95% O₂-5% CO₂ for 180 min. Progesterone concentrations were measured every 1 min for the first 5 min, then every 5 min up to including the 60th min, and from then on every 10 min up to and including the 180th min. Values are the means for three experiments \pm S.E.M. (Student's *t*-test).

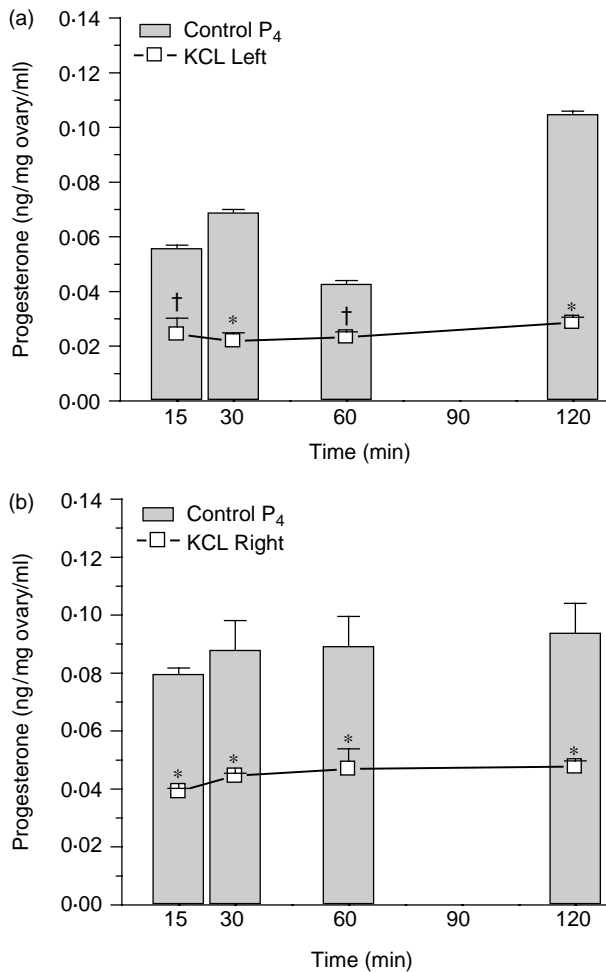


Figure 4 (a and b) Effect of non-specific stimulus of KCl in the ganglion compartment on the left and right systems. Ovarian progesterone (P₄) release was evaluated in the ovarian compartment in the superior mesenteric ganglion-ONP-ovary system obtained from rats on oestrus day. The systems were incubated in Krebs–Ringer buffer, plus ascorbic acid (0.1 mg/ml in Krebs–Ringer) solution, at 37 °C in an atmosphere of 95% O₂–5% CO₂ for 120 min without (control) and with KCl 56 mM concentration added to the ganglionic compartment (experimental group). Values are the mean \pm S.E.M. of six animals per experimental group. Left (a) and right (b), * P <0.001 and † P <0.01 compared with the control group. (Student's *t*-test, ANOVA-Duncan).

When oestradiol release was analysed, acetylcholine caused a significant increase at 15, 60 and 120 min (* P <0.001) and at 30 min († P <0.01) in the left system. In the right system, oestradiol did not show any variations in relation to the control group (Fig. 9a and b).

Discussion

The ovarian nervous plexus and the vagus nerve have been involved only in the regulation of the blood flow

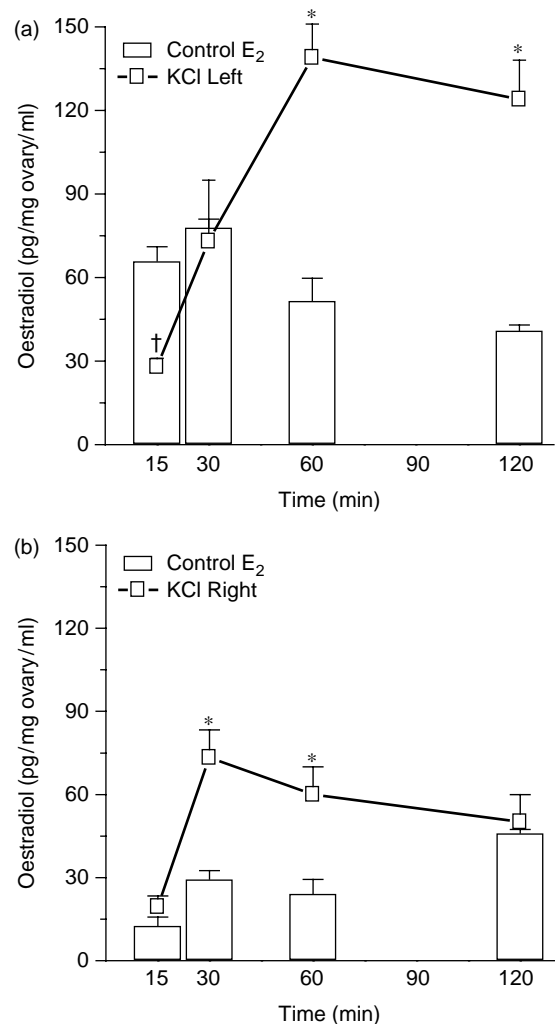


Figure 5 (a and b) Effect of non-specific KCl stimulus in ganglion compartment on the left (a) and right (b) systems. Ovarian oestradiol (E₂) release was evaluated in this ovary compartment in the superior mesenteric ganglion-ONP-ovary system obtained from rats on oestrus (E) day. The system was incubated in Krebs–Ringer buffer, plus ascorbic acid (0.1 mg/ml in Krebs–Ringer) solution, at 37 °C in an atmosphere of 95% O₂–5% CO₂ for 120 min without (control) and with KCl 56 mM concentration added to the ganglion compartment (experimental group). Values are the mean \pm S.E.M. of six animals per experimental group. Left and right * P <0.001 and † P <0.05 compared with the control group. (Student's *t*-test; ANOVA-Duncan).

(Burden & Lawrence 1978, Lawrence & Burden 1980). However, their importance in the ovarian functioning has not yet been determined. It is important to consider that this plexus not only ends around the blood vessels but also surrounds the theca cells and, in the oestrus stage, it surrounds the layer of the thecaluteinica cells (Lawrence & Burden 1980, Vega *et al.* 1998).

The strict relationship of the fibres running from the superior mesenteric ganglion with the blood vessels within

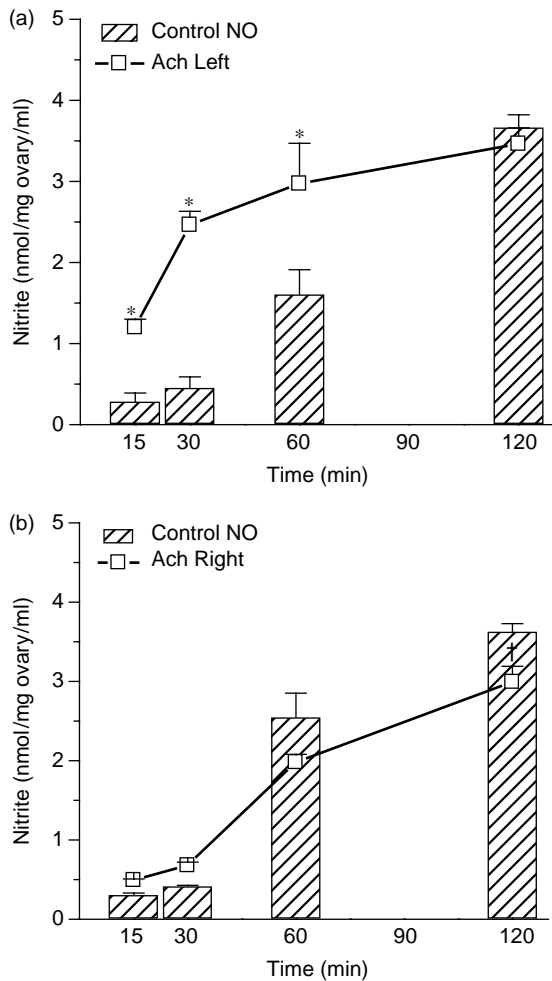


Figure 6 (a and b) NO presence in the incubation liquid of the left (a) and right (b) ovarian compartment in the superior mesenteric ganglion-ONP-ovary system of rats in oestrus, expressed as nmol/mg ovary per ml. Determinations were carried out under basal conditions and after the addition of acetylcholine (Ach) 10^{-6} M in the ganglion compartment. Values are the mean \pm S.E.M., $n=6$, of two experiments. * $P<0.001$. (Student's *t*-test; ANOVA-Duncan).

the ovary suggests that the nervous system might control the ovarian activity by regulating the ovarian vascular bed. Without attempting to oversimplify the complex problem of ganglionic functioning and regulation, the aim of this work was threefold: (1) to standardize an integrated superior mesenteric ganglion-ONP-ovary system in oestrus day rats; (2) to determine if the ganglionic cholinergic stimulus modifies the release of nitric oxide and steroids in the ovary compartment in the absence of humoral factors and (3) to investigate if there are differences in the responses between the left and right ovaries in this *ex vivo* system caused by the neural stimulus.

This system permits *in vitro* emulation of *in vivo* conditions, preserving innervation and paracrine and autocrine

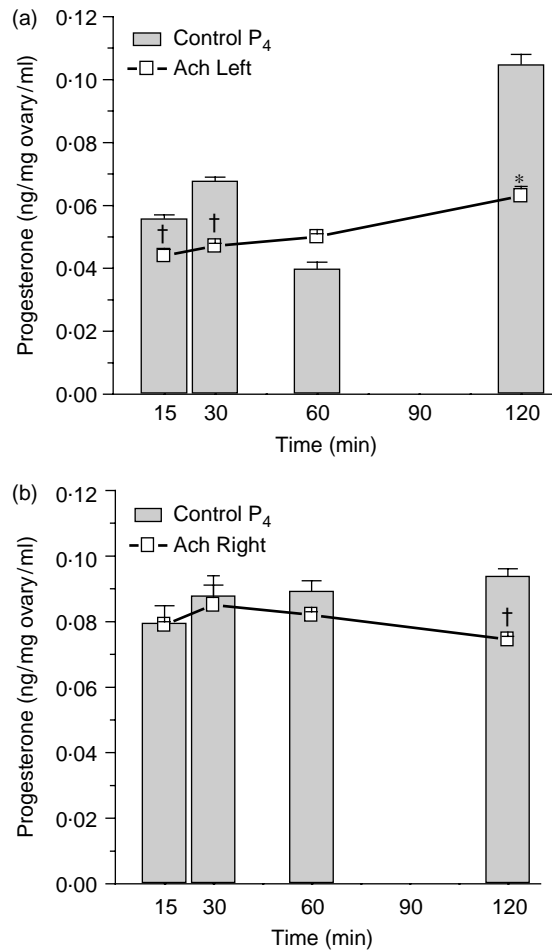


Figure 7 (a and b) Effect of cholinergic agonist in ganglion compartment on ovarian progesterone (P_4) release in the left (a) and right (b) superior mesenteric ganglion-ONP-ovary system removed from oestrus day rats. The system was incubated in Krebs-Ringer buffer, plus ascorbic acid (0.1 mg/ml in Krebs-Ringer) 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 ganglion compartment (experimental group). Values are the mean \pm S.E.M. of six animals per experimental group. Ach: acetylcholine; * $P<0.001$ and † $P<0.05$ compared with the control group. (Student's *t*-test. ANOVA-Duncan).

regulation within the gland without the humoral influence. A further advantage of this system is that it possesses its own neural tone, as can be observed in the different responses of the left system in relation to the right system in the standardization of the incubation times without any ovarian or ganglionic stimulation. This is also in agreement with the results observed in another *ex vivo* system (Sosa *et al.* 2000). The levels of progesterone release in the ovarian compartment on oestrus day are different between the left and right systems showing each ovary's autonomic tone. The sensitivity of the left and right ovaries is also different. This is in agreement with the findings of other authors who have shown an

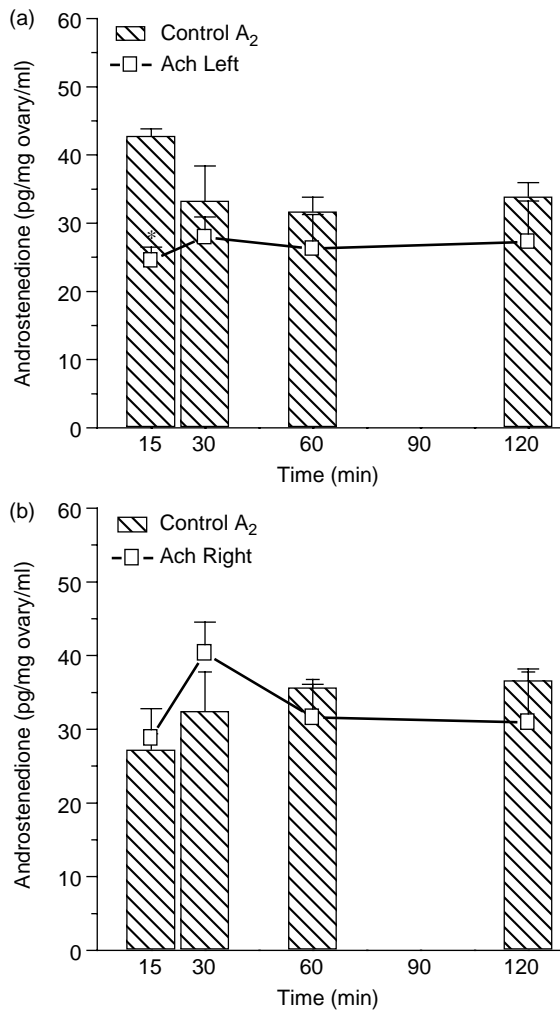


Figure 8 (a and b) Effect of cholinergic agonist in ganglion compartment on ovarian androstenedione (A₂) release in the left (a) and right (b) superior mesenteric ganglion-ONP-ovary system removed from rats on oestrus day (E). The system was incubated in Krebs-Ringer buffer, plus ascorbic acid (0.1 mg/ml in Krebs-Ringer) solution, at 37 °C in an atmosphere of 95% O₂-5% CO₂ for 120 min without (control) and with acetylcholine (Ach) 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. **P*<0.001 compared with the control group (Student's *t*-test; ANOVA-Duncan).

asymmetry in the behaviour of the left ovary in relation to the right one according to the characteristics of the neural stimulus applied (Dominguez *et al.* 1989, Barco *et al.* 2003, Morán *et al.* 2005). Besides, the ganglion presence in the preparation and good conservation of its structure is kept until the end of the incubation period, which shows the excellent viability of the incubation system. This has already been observed in our previous works by using the celiac ganglion-superior ovarian nerve (SON)-ovary system (Sosa *et al.* 2000, Delgado *et al.* 2004).

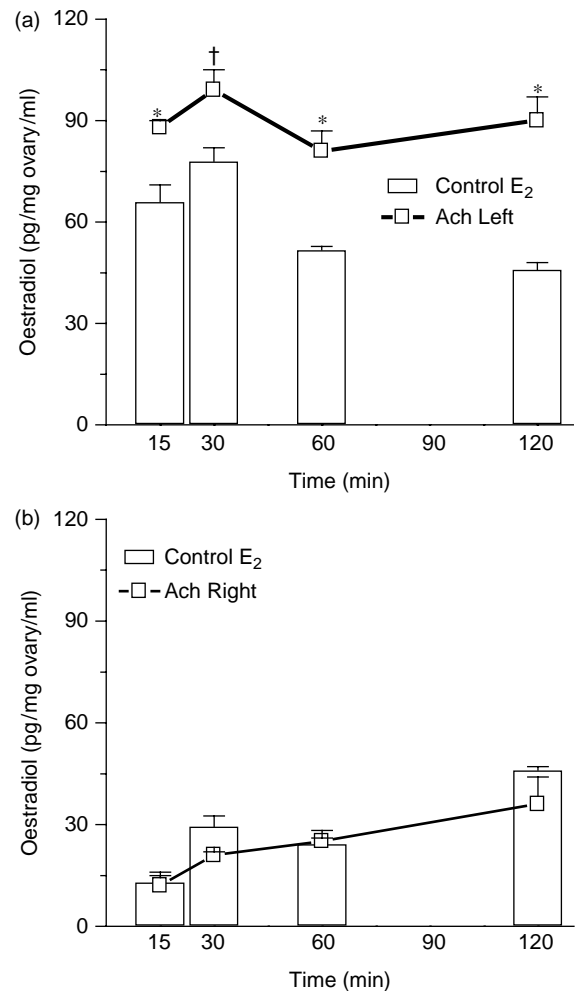


Figure 9 (a and b) Effect of cholinergic agonist in ganglion compartment on ovarian oestradiol (E₂) release in the left (a) and right (b) superior mesenteric ganglion-ONP-ovary system removed from rats on oestrus day (E). The system was incubated in Krebs-Ringer buffer, plus ascorbic acid (0.1 mg/ml in Krebs-Ringer) solution, at 37 °C in an atmosphere of 95% O₂-5% CO₂ for 120 min without (control) and with acetylcholine (Ach) 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. **P*<0.001 and †*P*<0.01 compared with the control group. (Student's *t*-test; ANOVA-Duncan).

When KCl was added as a non-specific agent to the ganglion compartment, different responses were observed on the liberation of progesterone and oestradiol, again showing the viability of the system. The presence of KCl in the ganglion compartment in left and right systems causes an inhibitory effect on the liberation of progesterone at all the studied times. A different effect was observed when another *ex vivo* coeliac ganglion-SON-ovary system was incubated with the non-specific agent in oestrus and dioestrus 1 (D1) stage in the oestrous cycle when the corpora lutea have just been formed (Sosa *et al.* 2000).

In relation to oestradiol, the presence of the non-specific agent in the mesenteric ganglion caused a stimulatory effect on the liberation of the steroid in the left and the right systems but with a different secretion profile along the incubation time. The results show a difference in the physiological response and sensitivity between the left and right ovaries through time as can be observed in the basal values obtained. This would be in agreement with the studies of Morán *et al.* (2005), who found an apparent asymmetry in the behaviour and activity of the neural connections between the ovaries and the prevertebral coeliac-mesenteric ganglia.

On the other hand, this different reactivity of the system and the basal tone found, permit us to add different substances in the ganglion compartment such as the cholinergic agent.

The existence of an interaction between the parasympathetic and sympathetic nerves in prevertebral ganglia is well known, providing evidence of a vagal innervation of the ganglion cells in the rat (Berthoud & Powley 1993, 1996). This vagal contact may either directly modulate the postganglionic outflow or else gate some or all the potential modulatory inputs to these postganglionic neurons, thus allowing the vagal system to exert a more selective influence on sympathetic outflow (Berthoud & Powley 1993). These vagal projections form varicose terminal-like structures, highly suggestive of synaptic contacts surrounding individual ganglion cells, thus demonstrating the presence of cholinergic receptors in the ganglion (Berthoud & Powley 1996). In the present experimental scheme, we used acetylcholine as cholinergic agonist because it is considered to be the classical preganglionic neurotransmitter of the sympathetic ganglionic pathway (Eccles & Libet 1961, Berthoud & Powley 1993). Carbacol was not used because it is a synthetic cholinergic agonist that, though more stable than acetylcholine, has activity only on a specific type of cholinergic receptors (Schroff *et al.* 1999), and our purpose in this work is always to emulate in an *ex vivo* system of the physiological conditions. Besides, the use of carbacol in other experimental schemes has shown a response with the physiological agonist higher than expected (Miller *et al.* 1997, Ghisdal *et al.* 2005). An inhibitor of the acetylcholinesterase was not used because it produces dysfunction in rats by selective complement-mediated destruction of preganglionic sympathetic nerve terminals (Miller *et al.* 1997). The cholinergic agent in ganglion acts in different ways depending on the stage of the oestrous cycle and constitutes one of the physiological factors governing the secretory activity of the ovarian steroids and NO, as observed in another *ex vivo* system (Delgado *et al.* 2004, Sosa *et al.* 2004).

In recent years nitric oxide has been recognized as a paracrine molecule that evokes cell function via gas diffusion, a truly new concept in cell to cell signalling (Moncada & Higgs 1993). NO participates in a variety of general physiological and pathophysiological processes and also acts as a luteotrophic and luteolytic factor in the corpus luteum (Fridén *et al.* 2000) as well as in apoptosis and steroidogenesis (Schukovsky & Tsafiriri 1994, Snyder 1996, Masuda *et al.* 1997). This gaseous

neurotransmitter has also been involved in the regulation of the blood flow in the ovary (Thom *et al.* 1985).

The most relevant results concerning the action of acetylcholine on NO release was observed in the left system on oestrus day. This day is characterized by reordering and structural organization that involves follicular atresia. This atresia also involves apoptosis mechanisms serving physiologic and homeostatic functions in the development of corpora lutea (Vega *et al.* 1998). This is the reason why we decided to elucidate if the addition of acetylcholine to the ganglion mesenteric has an effect on the ovarian NO release in this cycle stage.

In this work, an increase in the liberation of NO in the ovarian compartment was observed with the addition of acetylcholine in the ganglion compartment in the left ovary up to 120 min whereas in the right ovary no variations were observed. This different pattern of liberation of NO is possibly due to variations in the innervation between the right and left ovaries (Dominguez *et al.* 1989). In other words, the ganglionic cholinergic activity provides evidence of a higher sensitivity of the left ovary to the neural stimulus, at least for the NO liberation in these oestrous cycle stages. It is important to emphasize that future studies will be carried out to determine if the nervous peripheral system through the ovarian nervous plexus, at least in oestrus, would be involved in the apoptosis of granulosa cells that lead to the process of luteogenesis in rats, as has already been observed in culture of luteal cells in human beings (Vega *et al.* 1998, Motta *et al.* 1999). In this study, it was not possible to elucidate if NO originated in ganglion by neuronal NOS (nNOS) isoenzyme stimulation under acetylcholine influence, as has already been demonstrated in other experimental schemes (Furchgott & Zawadzki 1980), or if NO in ovary compartment is synthesized by isoforms, inducible NOS (iNOS) or endothelial NOS (eNOS) stimulation via the nerves. Besides, we have demonstrated that the cholinergic agent in ganglion stimulates NO liberation from the prepubertal ovary, and the NO is involved in the steroidogenesis in this stage via the superior ovarian nerve (Delgado *et al.* 2004).

In relation to the liberation of progesterone and androstenedione, the presence of the cholinergic agent in mesenteric ganglion in this experimental scheme produced an inhibition in the liberation of progesterone at 15, 30 and 120 min and of androstenedione at 15 min in the left ovary whereas in the right ovary only progesterone decreased in relation to controls at 120 min while androstenedione did not show significant changes.

The effects observed might be partly related with the liberation of NO. Indeed, there is evidence that NO decreases steroidogenesis although in different experimental schemes (Olson *et al.* 1996, Masuda *et al.* 1997, Vega *et al.* 2000). The mechanisms involved may be related to the inhibition produced by the NO of the activity of steroid synthesis-limiting enzyme, cytochrome P₄₅₀, of the two isoforms of adenylyl cyclase, the second intracellular messenger (McVey *et al.* 1999), and/or of the inhibition of the steroidogenic

acute regulatory protein (StAR) protein (Devoto *et al.* 2001). In our case it should be emphasized that the terminals of the ovarian nervous plexus reach the steroidogenic cells where the inducible NOS or eNOS isoenzymes have been found (Dunnam *et al.* 1999). On the other hand, the effect observed might also be due to the presence of other neurotransmitters such as the substance P described in this nervous pathway, which may be liberated by the action of the cholinergic agent on the mesenteric ganglion. In previous studies, it has been observed that the presence of P substance, one of the neuropeptides described as a neurotransmitter in this neural pathway and with a sensitive function in the ovary, exhibits an inhibitory effect on the liberation of progesterone during dioestrus 1 (D1) when incubating ovaries *in vitro* with such neurotransmitter (Garraza *et al.* 2004). In addition, our work group has demonstrated that the cholinergic agent in coeliac ganglion inhibits the liberation of progesterone in D1 by decreasing noradrenaline liberation in the sympathetic terminal at ovarian level (Sosa *et al.* 2004).

In relation to androstenedione, it only exhibits an inhibition of its release for very short times in the left system, an effect that has also been observed in the liberation of testosterone when the inferior spermatic ganglion is stimulated by acetylcholine in a similar *ex vivo* system (Zhu *et al.* 2002). These results are in agreement with the findings reported by other researchers who have found that NO inhibits the ovarian release of androstenedione, although in different experimental schemes (Olson *et al.* 1996, Dunnam *et al.* 1999). In other words, numerous factors at the ovarian and neural levels may be interacting in the inhibition of the liberation of progesterone and androstenedione in oestrus in order to modulate the physiological processes occurring in the ovary, and may be leading to the inhibition of the steroids liberation in this oestrous cycle stage.

In relation to oestradiol, the ganglionic cholinergic action caused a significant increase at all the studied times in the left system whereas no changes were observed in the right system. The same stimulatory effect on the liberation of oestradiol was observed by other authors, who have proposed that the oestradiol increase would favour the angiogenesis, the local vasodilatation and the increase of the blood flow, facts which favour the follicular and corpus luteum development by eONS stimulation (Masuda *et al.* 2001, Acosta & Miyamoto 2004). Thus, D'Albora *et al.* (2002) have stated that the changes in the irrigation can be caused by the NO, which, acting in a paracrine way, causes a vasodilatation and partly mediates in the blood flow increase occurring during the follicular development, the preovulatory period and the corpus luteum formation. However, other authors have suggested that the *in vitro* synthesis of oestradiol was inversely regulated by NO (Olson *et al.* 1996). Snyder *et al.* (1996) have stated that an increase in the production of nitric oxide inhibits the production of oestradiol in *in vitro* human granulosa-luteal cells cultures, being the NO capable of directly inhibiting the activity of the aromatase enzyme or indirectly decreasing the levels of the mRNA of the enzyme.

These results are likely to be due to differences in the experimental schemes used or to the lack of neural influence on those schemes. We suggest that in oestrus the innervation, in this case from the mesenteric ganglion via the ovarian nervous plexus, would participate favouring the angiogenesis and the formation and irrigation of the corpus luteum, with an increase in the production of oestradiol and NO. The luteotropic effect of oestradiol and NO has already been observed by other authors in the oestrous cycle (Vega *et al.* 1998, Fridén *et al.* 2000, Acosta & Miyamoto 2004).

Another neurotransmitter probably involved in the described processes is the acetylcholine liberated to the ovarian incubation liquid by ganglion stimulus. It has been suggested that acetylcholine may play a role in the cellular proliferation (Gutkind *et al.* 1991) and in the changes of the constantly remodelling ovarian tissue histoarchitecture (Lauder & Schambra 1999, Mayerhofer & Fritz 2002), both of which occur in this oestrous stage. It has also been described that cholinergic agents modify the secretion of hormones from human granulosa cells in culture (Bodis *et al.* 1993).

Although Burden & Lawrence (1978) found typical sympathetic nervous fibres reaching the ovary and showing acetylcholinesterase activity, acetylcholine was not found at ovarian level. However, the presence of acetylcholine receptors in the ovary, such as the M1 and M5 muscarinic receptors, has been demonstrated (Batra *et al.* 1993, Arellano *et al.* 1999, Mayerhofer *et al.* 2003). Luck (1990) reported the stimulation in the production of steroids in bovine granulosa cells by cholinergic effect in ovary whereas Morley *et al.* (1992) did not find any increase in the progesterone production in chicken granulosa cells. Mayerhofer *et al.* (1992) did not find modifications in the liberation of progesterone in human granulosa cells culture by acetylcholine effect. On the contrary, Bodis *et al.* (1993) have stated that the cholinergic neurotransmission may have a physiological significance in the intraovarian regulatory pathways.

Although having controversial results, all these studies have suggested that the receptor of acetylcholine exists in ovarian cells and that this neurotransmitter might be involved in the complex regulation of the ovarian function in the production of steroids. These facts open the way to investigate if this pathway, the ovarian nervous plexus, participates in the liberation of acetylcholine since it is the entrance of the vagal pathway reaching the ovary, and it has been shown to have influence on the ovarian functioning. Thus, Chavez *et al.* (1989) by cutting the vagus nerve found a different response of the left ovary in relation to the right one, indicating that the vagal system is partly responsible for the physiological mechanisms taking place in the ovary.

In our case, the ganglion cholinergic stimulus provides evidence of a greater sensitivity of the left ovary to the neural stimulus in relation to the right ovary in every case in oestrus stage, and of an apparent asymmetry in their responses when there is a marked irrigation and profound structural changes. It cannot be elucidated if the results obtained in this study might be caused by the existence of communication between

both ovaries, which would be via multiple pathways (Dominguez *et al.* 1989, Morales *et al.* 1993), or by the existence of some kind of blood-borne information between the ovaries, as was previously proposed (Morales *et al.* 1998). The results obtained might also be due to an apparent asymmetry in the activity of neural connections between the ovaries and the prevertebral sympathetic chain in addition to the fact that the number of active neurons of these connections varies during the oestrous cycle (Morán *et al.* 2005). We are able to state that the innervation by cholinergic stimulus of the superior mesenteric ganglion participates in the control of the corpus luteum development in a stimulatory and asymmetrical manner. This might indicate that the ovarian nervous plexus, besides regulating the blood flow, contributes to the control of the ovarian physiology with the other neural pathway entering the ovary, the superior ovarian nerve.

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