Journal of Steroid Biochemistry & Molecular Biology 120 (2010) 45–52

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Involvement of ganglionic cholinergic receptors on the steroidogenesis in the luteal phase in rat

Adriana Vega Orozco, Zulema Sosa, Silvia Delgado, Marilina Casais, Ana M. Rastrilla*

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

ARTICLE INFO

Article history:
Received 15 May 2009
Received in revised form 8 March 2010
Accepted 10 March 2010

Keywords:
Superior mesenteric ganglion
Steroid
Ovarian nervous plexus
Ovary
Cholinergic agents

ABSTRACT

The ovarian nervous plexus (ONP) is one of the principal extrinsic innervation pathways reaching the ovary from the superior mesenteric ganglion (SMG). The aims of this work were: (a) to determine if acetylcholine (Ach) in the SMG modifies the release of steroids and ovarian nitrites in an *ex vivo* SMG–ONP–ovary system on dioestrus (D) I and II, and (b) to demonstrate if the activities and gene expression of the steroidogenic enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and 20α-hydroxysteroid dehydrogenase (20α-HSD) are modified by cholinergic stimulus. The system was incubated in Krebs–Ringer buffer bicarbonate at 37 °C in metabolic bath. Ach (10^{-6} M) was used as cholinergic agonist. Ach in SMG increased progesterone release at all the incubation times on DI and DII (*$p < 0.001$). Androstenedione increased at 15 and 30 min on DI, and at 30 min on DII whereas nitric oxide (NO) increased at 30 min on DI, and at 15 and 30 min on DII. The activity of 3β-HSD increased whereas the activity of 20α-HSD decreased (*$p < 0.001$) on DI and DII. The gene expression of 3β-HSD showed a significant increase at 120 min on DI and DII (°$p < 0.01$) and 20α-HSD diminished only on DII. The results show the importance of the SMG via the ovarian nervous plexus on the regulation of the steroid secretory activity and on the ovarian release of NO in the luteal phase. The complex synaptic connections in the prevertebral ganglia and the sympathetic ganglionic chain participate in the neuroendocrinological mechanisms that take place during the luteal steroidogenesis.

1. Introduction

The importance of innervation in the gonadal function regulation has been widely demonstrated [1–4]. The rat ovary receives innervation from two principal pathways: the ovarian nervous plexus (ONP) and the superior ovarian nerve (SON), associated to the suspensory ligament. Both plexuses are mainly constituted by sympathetic postganglionic fibres whose neuronal somas are located in the superior mesenteric ganglion and the coeliac ganglion, respectively.

The superior mesenteric ganglion is constituted by specific structures responding to the cholinergic stimulus such as the nicotinic and muscarinic receptors in the ganglionic neurons called principal neurons [5] and the muscarinic receptors present in the interneurons, such as the small intensely fluorescent (SIF) cells, and peptidergic cells [6]. Recent studies have shown that the superior mesenteric ganglion, through the ONP, participates not only in the regulation of the blood flow [7,8] but also in the ovary physiology [9].

The ONP enters the ovary, accompanied by the ovarian artery, through the hilium and is constituted by sympathetic, sensory fibres and, to a lesser degree, parasympathetic fibres. The principal neurotransmitters in this nervous pathway are neuropeptide Y (NPY), substance P (SP) and noradrenaline (NA) [10]. The presence of Ach in the nervous terminals that reach the ovary has not been determined. Studies using histofluorescence suggest that these fibres are of noradrenergic nature. However, Burden and Lawrence [7], found that typical sympathetic ovarian nerve fibres show acetylcholine-esterase activity and suggested that some fibres, possibly from the vagus nerve, may travel along with sympathetic fibres to the ovary. Studies with rats have shown that vagotomy alters oestrous cyclicity [8], which may be due to direct effects of the vagus nerve on the ovary [11]. These results can be taken as evidence for the presence of neurotransmitter Ach in sympathetic ovarian nerve fibres.

The catecholamines and their receptors have been detected in the ovarian tissue [1]. It has been demonstrated that the number of adrenergic receptors in *in vitro* experimental schemes is modulated by the innervation and not by the circulating catecholamines. It is important to emphasize that granulosa cells as well as luteal

* Corresponding author. Tel.: +54 02652 423789x115/426324; fax: +54 02652 431301.
E-mail addresses: asvega_2006@hotmail.com, asvega@unsl.edu.ar (A. Vega Orozco), amras@unsl.edu.ar (A.M. Rastrilla).

0960-0760/$ – see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.jsbmb.2010.03.040

cells have adrenergic receptors [12,13] but they are not directly innervated [14].

As regards the ovarian Ach-receptors, it is well known that Ach interacts with different muscarinic receptor (MR) types [15]. Which receptor types are expressed in the ovary and by which cells is not fully known, but evidence for ovarian MRs has been found [16,17].

On the other hand, indirect immunofluorescence studies have demonstrated the presence of the neuronal nitric oxide synthase (nNOS) in the mesenteric ganglion. The distribution of NOS, a synthesis enzyme of nitric oxide (NO), has one possible colocalization with immunoreactive neuropeptides and with two enzymes of the catecholamine synthesis pathway, tyrosine β-hydroxylase and dopamine β-hydroxylase, as well as with cholineacetyltransferase, the enzyme for the acetylcholine synthesis pathway [18]. NOS has also been observed in ovary in its constituting and inducible type. Endothelial NOS is expressed in the stroma and theca cells, in the granulosa cells of mature follicles and in steroidogenic cells of the theca–luteal region of the corpus luteum [19]. NO is involved as a luteolytic and/or luteotrophic factor on the corpus luteum, depending on the oestrous cycle stage [20,21].

Our research group has demonstrated a relation between the neural cholinergic action and nitric oxide on ovarian steroidogenesis in prepubertal rats [22,23] and in the oestrus stage during the oestrous cycle [9]. Although the stimulus for the synthesis of steroid hormones is mainly of endocrine nature, there is at present strong evidence of the participation of the nervous system in the ovary response.

There is evidence that NO decreases the steroidogenesis in different experimental scheme [20]. NO causes an inhibition of the enzymatic activities [24] and gene expression of steroid synthesis—limiting enzymes such as cytochrome P450scc, steroidogenic acute regulatory (StAR) protein and 3-beta-hydroxysteroid dehydrogenase (3β-HSD) in bovine luteal cells [25].

Steroidogenesis at ovarian level results in a series of successive steps involving enzymes such as 3β-HSD, a progesterone synthesis enzyme, and the degradation enzyme of P_4, 20α-hydroxysteroid dehydrogenase (20α-HSD). 3β-HSD has been identified in interstitial and luteal cells [26,27]. Teerds and Dorrington [28] have demonstrated that the immunoreactivity for 3β-HSD in corpus luteum is independent of the development stage. In addition, the 20α-HSD which has been determined, cloned and sequenced in CL of rats is hormonally regulated and plays an important role in the luteolysis reducing P_4 to an inactive metabolite, 20-α-hidroxipregn-4-ene-3-one [29].

With these antecedents in mind and in order to elucidate the effects of the innervation from the ovarian nervous plexus on the corpus luteum functioning, the aims of this work are (a) to determine whether acetylcholine (Ach) in the superior mesenteric ganglion (SMG) modifies the release of steroids and ovarian nitrites in an *ex vivo* superior mesenteric ganglion–ovarian nervous plexus (ONP)–ovary integrated system (SMG–ONP–O) on dioestrus (D) I and II, and (b) to demonstrate if the activities and gene expression of the steroidogenic enzymes 3β-HSD and 20α-HSD are modified by cholinergic stimulus at 120 min of incubation.

2. Materials and methods

2.1. Animals

Virgin Holtzman strain adult female rats on DI and DII weighing 250 ± 50 g were used in all the experiments. The rats were kept in a light (lights on from 07:00 to 19:00 h) and temperature-controlled 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 were used for the experimental procedure. The experiments were performed per duplicate and according to the procedures approved in Ref. [30]. The experimental protocol was approved by the University of San Luis Animal Care and Use Committee (protocol number B17/04, ordinance CD 006/02).

2.2. Reagents

The following drugs: L-acetylcholine hydrochloride (Ach), ascorbic acid, bovine serum albumin fraction V (BSA), sulphanilamide and N-1-naphthyl-ethylenediamine were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Other reagents and chemicals were of analytical grade. 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).

2.3. Surgical procedures

2.3.1. Extraction of superior mesenteric ganglion–ovarian nervous plexus–ovary system

The procedure was carried out between 15:00 and 16:00 h taking into account previous descriptions of the anatomical trajectory of this neural pathway as a guide [8,10]. Rats were anaesthetized with ether under bell, and the system was immediately removed by dissection. Each system was conformed by the ovary, the fibres constituting the ovarian nervous plexus, parallel to the ovarian artery, and the superior mesenteric ganglion, surrounded by some small ganglia. 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 in order to prevent spontaneous depolarisations of the nerves, which might be caused by inappropriate contact. The extracted systems were washed with incubation solution and immediately placed in one cuvette with two compartments.

2.4. Experimental procedure

Once extracted, the systems were immediately placed in a cuvette with two separate compartments, each cuvette containing 2 ml of work solution, Krebs–Ringer bicarbonate buffer, pH 7.4, in the presence of dextrose (0.1 mg/ml) and BSA (0.1 mg/ml) as described in previous works [3,9].

The ganglion was placed in one compartment and the ovary in the other one, both joined by the ONP, which was maintained humid with the work solution. The system was immediately preincubated in a metabolic bath at 37 °C in a 95% O_2–5% CO_2 atmosphere for 15 min in order to achieve the stabilization of the system as described in a previous work [9].

After 15 min of preincubation (time 0 of incubation), 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 [31] in the ganglion compartment whereas Krebs–Ringer solution was added in the ovary compartment.

Progesterone, androstenedione and nitrites values released under these conditions were considered as control value (control group). For the experimental group, acetylcholine was used which was dissolved in Krebs–Ringer solution plus ascorbic acid at a 10^{-6} M final concentration in the ganglion compartment [9]. The ganglionic cholinergic effect on the steroids and nitric oxide release in the ovary compartment was evaluated in relation to the control group.

Liquid samples from the ovary compartment (250 μl) were collected at 15, 30, 60 and 120 min and kept at −20 °C until the determination of progesterone (P_4) and androstenedione (A_2) by RIA and nitrites (NO) by Griess method.

The results were expressed as nanogram of progesterone and picogram of androstenedione per milligram of ovarian tissue per ml and nitrites as nanomol of nitrites per milligram of ovarian tissue per millilitres (nmol/mg ovary/ml) all against incubation time. The respective corrections were made in all cases considering the volume extracted in each tested period.

When the system incubation was finished (120 min), the ovaries were kept at −80 °C until determination of 3β-HSD and 20α-HSD activities and gene expression.

2.5. Progesterone and androstenedione assay

The steroids contents were measured in duplicate by radioimmunoassay (RIA). Antisera were kindly provided by IMBECU (Instituto de Medicina y Biología Experimental de Cuyo). Progesterone was measured in ng/mg ovary/ml, and assay sensitivity was less than 5 ng progesterone/ml. Androstenedione was expressed as picogram per milligram of ovarian tissue per ml (pg/mg ovary/ml), all against incubation time. The assay sensitivity was less than 10 pg androstenedione/ml. The inter- and intraassay coefficient variations in all the assays were less than 10.0%.

2.6. Nitrite assay

Levels of nitrites, a water-soluble metabolite of nitric oxide, were measured spectrophotometrically by the Griess method and were expressed in nanomol of nitrites per milligram of ovarian tissue per ml (nmol/mg ovary/ml) [32]. The samples (50 μl) were immediately mixed with Griess reagent (sulphanilamide with N-1-naphthyl-ethylenediamine/HCl). After a 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 less than 2.5 nmol/ml. The intraassay coefficients variation for all the assays was less than 10.0%.

2.7. Enzymatic activity

The activities of 3β-HSD and 20α-HSD were measured according to Kawano et al. [33] with a slight modification. The ovary from each animal was homogenized in 0.7 ml of 0.1 M Tris–HCl, 1 mM EDTA (pH 8) at 0 °C with a glass homogenizer. The homogenates were centrifuged at 105 000 × *g* for 60 min. The supernatant fluids were used for the assay of 20α-HSD activity. The precipitates were rehomogenized with 0.7 ml of 0.25 M sucrose and centrifuged at 800 × *g* for 5 min. The supernatants were used as the enzyme solution for the assay of 3β-HSD activity. The substrates for 3β-HSD and 20α-HSD were pregnenolone (5 μg) and 20α-hydroxypregn-4-en-3-one (12.5 μg), respectively. Both enzyme activities were assayed spectrophotometrically, depending on the increase in NADH or NADPH in 1 min at 37 °C, and the values were expressed as mU/mg protein/min. The method of Lowry et al. [34] was used for protein determination with BSA as the standard.

2.8. RNA isolation and RT-PCR analysis

Once the ovaries were defrosted, the total ribonucleic acid (RNA) was extracted using the TRIZOL-Reagent method (Invitrogen Life Technologies), following the manufacturer's instructions for the RNA extraction [35].

Two μg of total RNA were reverse transcribed using a Moloney murine leukemia virus (MMLV) reverse transcriptase (RT) and random primers. First, a pre-reversetranscription (pre-RT) was carried out, using random primers, by incubating 10 min at environmental temperature and then, 45 min at 42 °C. The buffer RT, the enzyme MMLV-RT (Invitrogen/Life Technologies) and a mixture of deoxynucleoside triphosphates (dNTPs) were added to the total pre-RT product, and this was taken to a final volume of 50 μl. The mixture was incubated for 60 min at 42 °C, then, 15 min at 70 °C, and finally the reverse transcriptase activity was inactivated through the tubes incubation ice bath.

For amplification of the reverse transcription (RT) products, the reaction mixture consisted of 10 μl Green Go Taq reaction buffer, 0.2 mM deoxynucleoside triphosphates, 0.5 μM specific oligonucleotide primers and 1.25 U Go Taq DNA polymerase (Promega Inc.) in a final volume of 50 μl. Amplification was carried out for 30 cycles using 93 °C for denaturing (1 min), 59 °C for annealing (1 min), and 72 °C for extension (15 min) in Eppendorf Cycler thermal cycler.

Specific primers for 3β-HSD were (5′-CTGCCTGGTGACAGGAGCAGG-3′ and 5′-GCCAGCACTGCCTTCTCGGCC-3′) and for 20α-HSD (5′-TTCGAGCAGAACTCATGGCTA-3′ and 5′-CAACCAGGTAGAATGCCATCT-3′). Each reaction also included primers (5′-CAAGACTGAGTGGCTGGATGG-3′ and 5′-ACTTGAAGGGGAATGAGGAAAA-3′) to amplify protein cyclophilin A as an internal control. The predicted sizes of the PCR-amplified products were 489 pb for 3β-HSD, 440 pb for 20α-HSD and 293 pb for cyclophilin A. Reaction products were electrophoresed on 1.5% agarose gels, visualized with ethidium bromide (5.5 mg/ml), and examined by ultra-violet transillumination. Band intensities of RT-PCR products were quantified using ImageJ (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/) and expressed as arbitrary units.

2.9. Statistical analysis

All data are presented as means ± S.E.M. in each group of six rats. Differences between two groups were analyzed with Student's *t*-test. Analysis of variance (ANOVA I) followed by Duncan's multiple range test was used for several comparisons. A value of $p < 0.05$ was considered statistically significant [36].

3. Results

3.1. Effect of acetylcholine in ganglion on progesterone and androstenedione release on DI and DII

3.1.1. Progesterone

In order to assess whether the presence of Ach in the ganglion compartment modulated P_4 release in the ovarian compartment by the neural way from the SMG, and considering that P_4 has proved to be the most sensitive steroid to neural influence in *in vitro* studies, Ach (10^{-6} M) was added in the superior mesenteric ganglion. The ovary incubation liquids were obtained at different times and P_4 levels were measured by RIA. The results of each experiment are expressed with respect to the corresponding control group.

On DI and DII, a significant increase in the release of P_4 at all the studied times was observed ($^{*}p < 0.001$) (Fig. 1). When the control values in both stages were compared, the values on DI were significantly higher.

3.2. Androstenedione

Considering that A_2 is synthesized by the ovarian interstitial cells and that ONP innervated these structures, A_2 release in the ovarian compartment by stimulation with Ach (10^{-6} M) at ganglionic level was assessed. The results show that the release of A_2 in the ovarian compartment increased significantly on DI at 15 min (4.345 ± 0.0249 *vs.* 6.89 ± 0.88) ($^{o}p < 0.01$) and at 30 min (4.62 ± 0.0283 *vs.* 6.5 ± 1.23) ($^{\bullet}p < 0.05$), and on D II, only at 30 min (4.07 ± 0.004 *vs.* 33 ± 4.02) ($^{*}p < 0.001$) (Fig. 2).

Under control conditions, the level of A_2 in the ovarian incubation liquid was significantly lower on DI than on DII in all the incubation times studied.

3.3. Effect of acetylcholine in ganglion on the enzymatic activity and gene expression of ovarian 3β-HSD on DI and DII

With the purpose of knowing whether the addition of Ach in the mesenteric ganglion modified the activity and expression of the P_4 synthesis enzyme on DI and DII, the ovaries of the system were obtained after 120 min of incubation. On DI and DII, a significant increase in the activity of 3β-HSD (DI: 0.60 ± 0.005 *vs.* 1.23 ± 0.041; DII: 0.45 ± 0.014 *vs.* 0.68 ± 0.020) was observed in relation to the control group ($^{*}p < 0.001$, respectively) (Fig. 3).

In order to determine if Ach action at ganglionic level also affects the expression of the enzymes involved in P_4 synthesis, 3β-HSD was measured. On DI and DII, Ach at ganglionic level caused an increase in the gene expression of the P_4 synthesis enzyme in relation to the control at 120 min ($^{o}p < 0.01$, respectively) (Figs. 4 and 5).

3.4. Effect of acetylcholine in ganglion on the enzymatic activity and gene expression of ovarian 20α-HSD on DI and DII

In order to determine if Ach action at ganglionic level also affects the activity and gene expression of the enzyme involved in P_4 degradation 20α-HSD were measured. In the DI and DII stage, Ach at ganglionic level caused a decrease in the activity of 20α-HSD (DI: 0.41 ± 0.005 *vs.* 0.20 ± 0.009; DII: 0.11 ± 0.004 *vs.* 0.073 ± 0.004) ($^{*}p < 0.001$, respectively) in relation to the control group (Fig. 6).

On the other hand, the ovarian gene expression of 20α-HSD diminished only on DII relation to the control at 120 min ($^{\bullet}p < 0.05$). Do not changes was observed in the 20α-HSD gene expression in DI (Figs. 7 and 8).

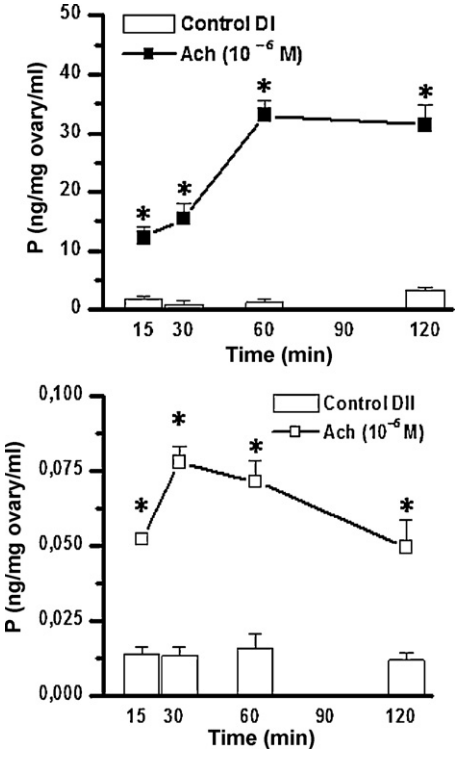

Fig. 1. Effect of cholinergic agonist in ganglion compartment on ovarian progesterone release in the superior mesenteric ganglion–ONP–ovary system removed from rats on DI (top) and DII (bottom). Each bar represents the mean ± S.E.M. of six animals per experimental group. P: progesterone; Ach: acetylcholine; $^{*}p < 0.001$ compared with the control group (Student's *t*-test. ANOVA-Duncan).

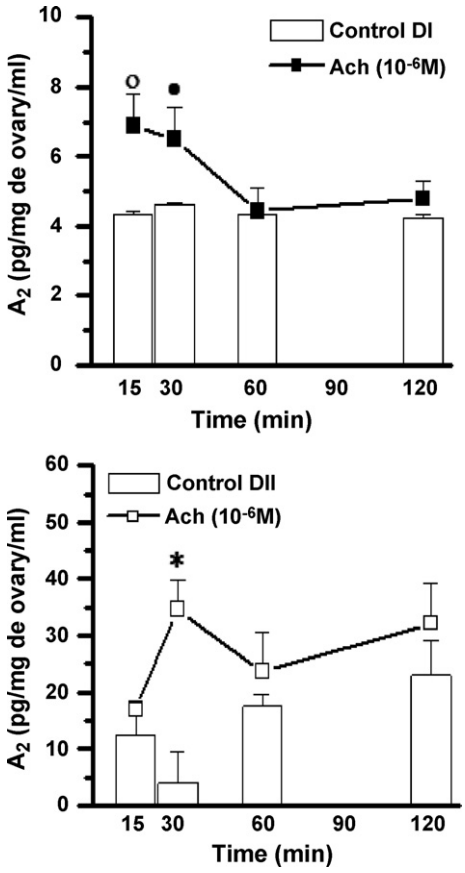

Fig. 2. Effect of cholinergic agonist in ganglion compartment on ovarian androstenedione release in the superior mesenteric ganglion–ONP–ovary system removed from rats on DI (top) and DII (bottom). Each bar represents the mean ± S.E.M. of six animals per experimental group. A_2: androstenedione; Ach: acetylcholine; $^{*}p < 0.001$; $^{o}p < 0.01$; $^{\bullet}p < 0.05$ compared with the control group (Student's *t*-test; ANOVA-Duncan).

3.5. Effect of acetylcholine in ganglion on nitrites release on DI and DII

Since the modulation of nitrites release by neural stimulus and its importance in ovarian steroidogenesis have been demonstrated in our research group, we attempted to determine whether the addition of Ach in ganglion compartment modifies nitrites release on the ovarian compartment during the luteal stage. A significant increase was observed in the release of nitrites in the ovarian compartment on DI at 30 min (5.17 ± 0.26 *vs.* 8.78 ± 0.88) ($^{*}p < 0.001$), while on DII at 15 min (6.53 ± 0.99 *vs.* 11.3 ± 0.96) ($^{\circ}p < 0.01$) and at 30 min (16.29 ± 0.14 *vs.* 24.4 ± 0.70) ($^{*}p < 0.001$) in relation to the control group (Fig. 9).

4. Discussion

In this work we have attempted to integrate the knowledge obtained in previous works with the contribution of a new sympathetic pathway, the ovarian nervous plexus in the luteal phase. The secretions of the corpus luteum (CL) are modified by a variety of molecules encompassing hormones, growth factors, NO, cytokines and neurotransmitters such as noradrenaline (NA) [37], SP or NPY [38,39]. Our general purpose was to evaluate the steroidogenic response in the *ex vivo* superior mesenteric ganglion–ONP–ovary integrated system, in which the paracrine and autocrine relations are preserved without the humoral influence, and the different neuroendocrine interrelations may be integrated [9]. For this reason,

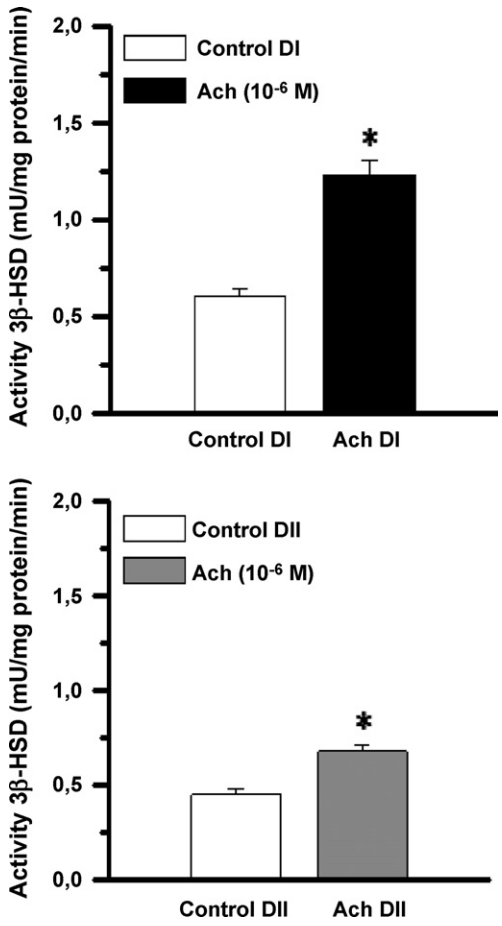

Fig. 3. Effect of cholinergic agonist in ganglion compartment on the enzymatic activity of 3β-HSD in ovaries extracted from the superior mesenteric ganglion–ONP–ovary system with and without Ach stimulus (10^{-6} M) in the mesenteric ganglion on DI (top) and DII (bottom). Each bar represents the mean ± S.E.M. of six animals per experimental group. Ach: acetylcholine. $^{*}p < 0.001$ (Student's *t*-test; ANOVA-Duncan).

this neural pathway might be an adequate *in vitro* model to study these relations.

The different reactivity of the system and the basal tone permitted to investigate if the ovary physiology was affected by the influence of a cholinergic agent on the ganglionic neurons [9].

The results of the present study revealed an enhanced release of P_4 and A_2 in both stages at all the incubation times, although with different release profiles. In addition, P_4 control values were significantly higher on DI than on DII, and it is evident that this effect is related to the high activity of the recently formed corpora lutea on DI. On the other hand, A_2 showed a stimulating effect at short times on DI and DII. It is important to emphasize that the control values observed on DII are significantly higher than on DI since the ovaries at this stage are preparing to complete a new cycle and ensure the continuation of the oestrous cycle.

Considering that P_4 is the principal secretion product of the CL, the activity and gene expression of the enzymes participating in its synthesis and degradation on both stages were also analyzed.

The ganglionic cholinergic stimulation increased the activity and gene expression of 3β-HSD on DI and DII at 120 min of incubation, in agreement with the increase observed on both stages in the release of P_4 in relation to the control group. The determination of the enzymatic activities was carried out at 120 min of incubation since no modifications were observed between the different incubation times by neural stimulus, which is a surprising fact since there are no reports of enzymatic activity modulation at such short times as observed in this work. Variations in luteal cell cultures occurring at 36 h without neural influence and without the paracrine relations present in our *ex vivo* system have been reported [40,41], demonstrating the importance of cell–cell interactions.

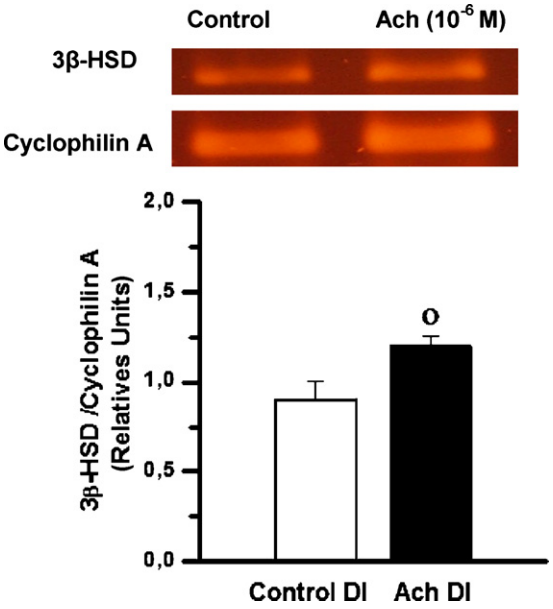

Fig. 4. Effect of cholinergic agonist on the gene expression of 3β-HSD in ovaries extracted from the superior mesenteric ganglion–ONP–ovary system with and without Ach stimulus in the mesenteric ganglion on DI. Measurement by RT-PCR of expression of 3β-HSD (489 pb) and cyclophilin A (293 pb). Ethidium bromide fluorescent photograph of the gel electrophoresis of the amplification products (top). Expression of 3β-HSD relative to cyclophilin A (bottom). Results are expressed as a mean ± S.E.M. ($n = 3$). $^{o}p < 0.01$ (Student's *t*-test; ANOVA-Duncan).

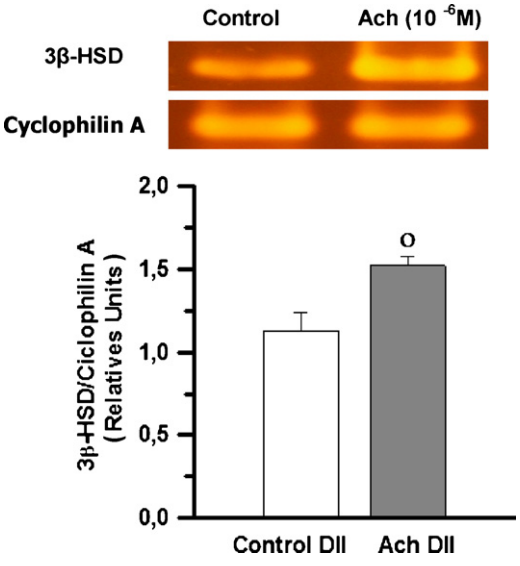

Fig. 5. Effect of cholinergic agonist on the gene expression of 3β-HSD in ovaries extracted from the superior mesenteric ganglion–ONP–ovary system with and without Ach stimulus in the mesenteric ganglion on DII. Measurement by RT-PCR of expression of 3β-HSD (489 pb) and cyclophilin A (293 pb). Ethidium bromide fluorescent photograph of the gel electrophoresis of the amplification products (top). Expression of 3β-HSD relative to cyclophilin A (bottom). Results are expressed as a mean ± S.E.M. ($n = 3$). $^{o}p < 0.01$ (Student's *t*-test; ANOVA-Duncan).

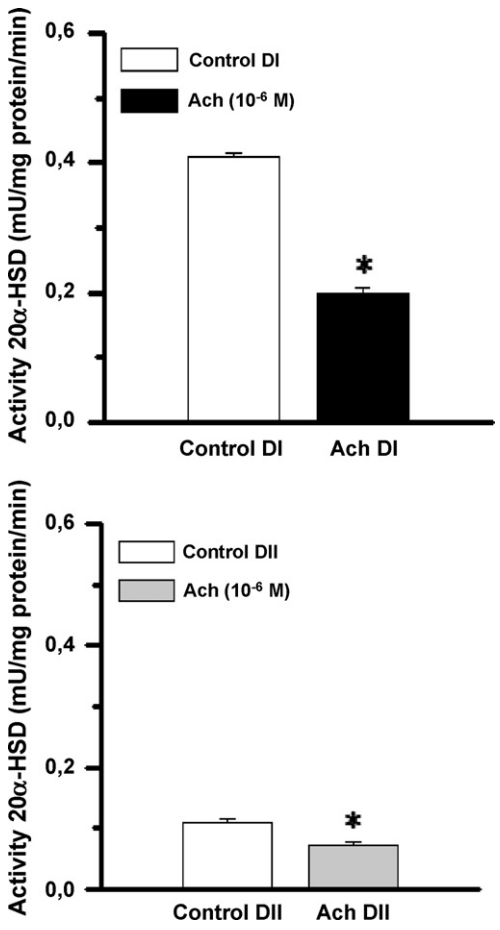

Fig. 6. Effect of cholinergic agonist on the enzymatic activity of 20α-HSD in ovaries extracted from the superior mesenteric ganglion–ONP–ovary system with and without Ach stimulus in the mesenteric ganglion on DI (top) and DII (bottom). Each bar represents the mean ± S.E.M. of six animals per experimental group. Ach: acetylcholine. $^{*}p < 0.001$ (Student's *t*-test; ANOVA-Duncan).

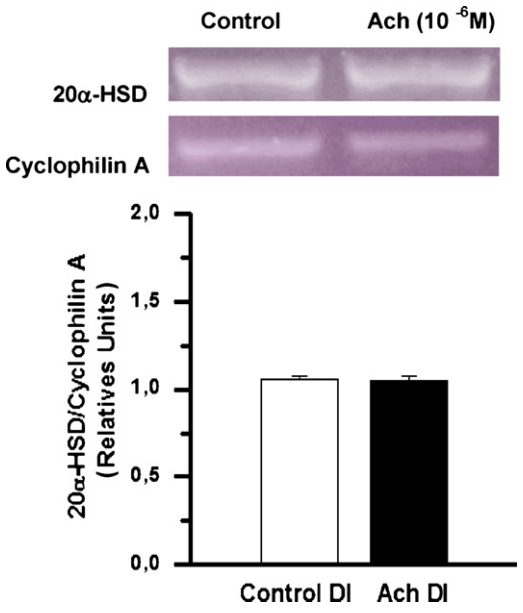

Fig. 7. Effect of cholinergic on the gene expression of 20α-HSD in ovaries extracted from the superior mesenteric ganglion–ONP–ovary system with and without Ach stimulus (10^{-6} M) in the mesenteric ganglion on DI. Measurement by RT-PCR of expression of 20α-HSD (440 pb) and cyclophilin A (293 pb). Ethidium bromide fluorescent photograph of the gel electrophoresis of the amplification products (top). Expression of 20α-HSD relative to cyclophilin A (bottom). Results are expressed as a mean ± S.E.M. ($n = 3$) (Student's *t*-test; ANOVA-Duncan).

On the other hand, the accumulation of P_4 in steroidogenic cells is also influenced by the participation of the enzyme 20α-HSD. A significant decrease in the activity of this enzyme was observed in both stages, with the gene expression depending on the cycle stage, in agreement with the P4 increase.

These results might be accounted for by the presence of catecholamines at ovarian level, since a mechanism has been reported with similar effects in luteal cells cultured with catecholamines [42]. It has been postulated that the site of action of the β-agonists may be 3β-HSD [43]. Miszkiel et al. [44] have reported that NA stimulated the activity of 3β-HSD. At the same time, P_4 produced by NA stimulus reduces the activity of aminooxidase (MAO) and catechol-o-methyl-transferase (COMT), the enzymes primarily responsible for an intracellular degradation of catecholamines [45]. Thus, in this way, P_4 prolongs the half-life of NA and the duration of its stimulatory influence on P_4 synthesis. However, Rekawiecki et al. [46] have proposed that NA affects neither StAR, cytochrome P450scc or 3β-HSD gene expression nor the level of functional proteins encoding these genes. Recent studies using this experimental scheme (unpublished data) have determined the release of catecholamines by ganglionic stimulus. Therefore, we assumed that in the short times employed with stimulation of Ach in ganglion, there is possible release of NA and/or other neurotransmitters that stimulate P_4 secretion from the releasable pool at short times, but not its synthesis. Also, taking into account that Kotwika et al. [47] and Rekawiecki et al. [48] have postulated the autorregulation of P_4 synthesis in the corpus luteum, which affects the transcription of the genes encoding the steroidogenic enzymes, it can be assumed that the values of P_4 at 120 min can be due to the increase in the activity of 3β-HSD caused by the same P_4 released throughout time, or by the decrease in the activity of 20α-HSD or by action of other neurotransmitters [11]. Thus, NO might be involved in the modulation of output and synthesis of NA, as already reported by Yamamoto et al. [49] for other tissues.

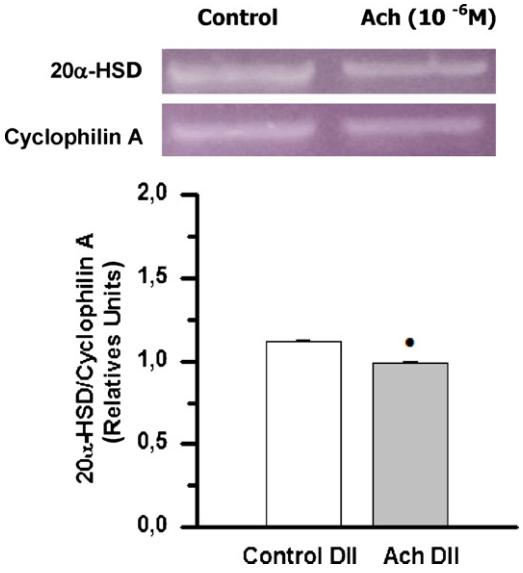

Fig. 8. Effect of cholinergic agonist on the gene expression of 20α-HSD in ovaries extracted from the superior mesenteric ganglion–ONP–ovary system on DII. Measurement by RT-PCR of expression of 20α-HSD (440 pb) and cyclophilin A (293 pb). Ethidium bromide fluorescent photograph of the gel electrophoresis of the amplification products (top). Expression of 20α-HSD relative to cyclophilin A (bottom). Results are expressed as a mean ± S.E.M. ($n = 3$). $^{\bullet}p < 0.05$ (Student's *t*-test; ANOVA-Duncan).

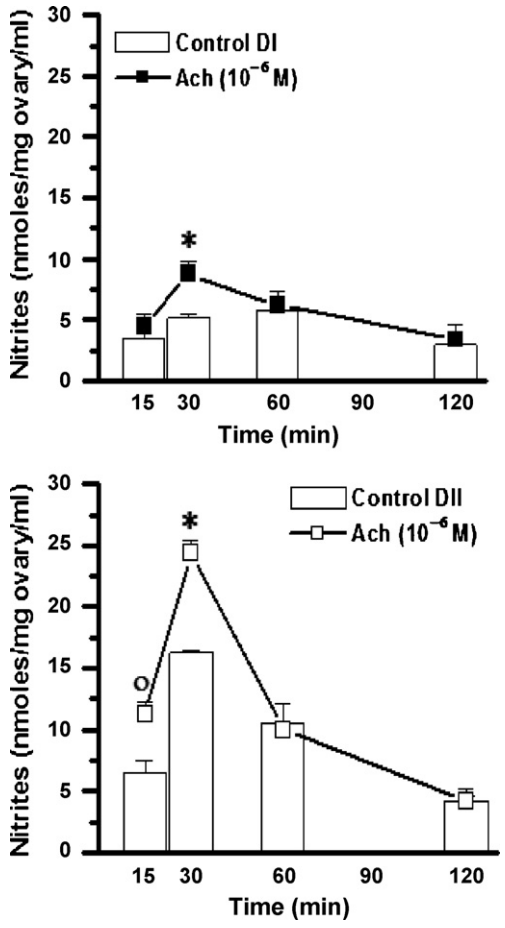

Fig. 9. Effect of cholinergic agonist in ganglion compartment on nitrites release in the incubation liquid of the ovarian compartment in the superior mesenteric ganglion–ONP–ovary system of rats on DI (top) and DII (bottom). Each bar represents the mean ± S.E.M. of six animals per experimental group. Ach: $^{*}p < 0.001$; $^{o}p < 0.01$ (Student's *t*-test; ANOVA-Duncan).

The presence in the ovary of NO and of the isoforms of the synthesis enzyme has been demonstrated [50,51]. In our case, both on DI and DII, neural stimulus provoked an increase of NO at short times, which could also explain the increase of the activity and gene expression of 3β-HSD at long times, in coincidence with the P_4 increase.

Fridén et al. [21] demonstrated that the endothelial NOS is found in the blood vessels near the functional CL. The ONP is known to be involved in the regulation of the vascular bed and favors the irrigation of the CL. In our system, although there is no blood flow or intravascular fluid movement, it must be taken into account that numerous neurotransmitters are released from the ONP, among them NO. These factors diffuse from their site of synthesis and affect the function of steroidogenic cells. It has been reported that NO decreases P_4 and A_2 release in *in vitro* cellular cultures [20,21,52] and it is considered a luteolitic and/or luteotrophic factor [21]. However, in this work this decrease was not observed either on DI or DII by ganglionic stimulus via the ONP. Motta et al. [53] demonstrated in rat that endogenous NO increased the production of glutathione and P_4 in corpora lutea in the middle stage of the development, thus showing its dual, protective or pro-oxidizing effect according to the stage of the oestrous cycle during which the tissue was obtained [54]. This effect may partly explain the fact that the activity of 20α-HSD decreases in both stages and the gene expression decreases only on DII. On the other hand, the different results might be due to the presence or absence of different subpopulations of steroidogenic and non-steroidogenic cells, and also to the cell–cell contact already described by other authors [55–57]. Further studies are required to elucidate the possible mechanisms involved.

In conclusion, this work shows that the superior mesenteric ganglion–nervous plexus–ovary neural pathway not only regulates the blood flow in the ovary, as reported by other authors, but also participates, through the ganglionic cholinergic receptors, in the maintenance of the corpus luteum during the oestrous cycle.

Acknowledgements

This research was supported by Grant 9302 from Universidad Nacional de San Luis, Argentina. We thank Dr. R.P. Deis for providing the progesterone antiserum. We also acknowledge Luis Villegas for his technical assistance. This work is part of the doctoral thesis of Biochemist Adriana Vega Orozco by Secretaria de Ciencia y Técnica, Universidad Nacional de San Luis.

References

[1] L.I. Aguado, S.R. Ojeda, Prepubertal ovarian function is finely regulated by direct adrenergic influences: role of noradrenergic innervation, Endocrinology 114 (1984) 1845–1853.
[2] L.I. Aguado, Role of the central and peripherical nervous systems in the ovarian function, Microsc. Res. Tech. 59 (2002) 462–473.
[3] Z.Y. Sosa, M. Casais, A.M. Rastrilla, L.I. Aguado, Adrenergic influences on coeliac ganglion affect the release of progesterone from cycling ovaries. Characterisation of an in vitro system, J. Endocrinol. 166 (2000) 307–318.
[4] M. Casais, S.M. Delgado, Z.Y. Sosa, C.M. Telleria, A.M. Rastrilla, The celiac ganglion modulates LH-induced inhibition of androstenedione release in late pregnant rat ovaries, Reprod. Biol. Endocrinol. 4 (2006) 66–72.
[5] M.J. Prud Homme, E. Houdeau, R. Serghini, Y. Tillet, M. Schemann, J.P. Rousseau, Small intensely fluorescent cells of the rat paracervical ganglion synthesize adrenaline, receive afferent innervation from postganglionic cholinergic neurones, and contain muscarinic receptors, Brain Res. 821 (1999) 141–149.
[6] R. Jarvi, Localization of bombesin, neuropeptide Y, enkephalin, and tyrosine hydroxylase-like immunoreactivities in rats coeliac-superior mesenteric ganglion, Histochemistry 92 (1989) 231–236.
[7] H.W. Burden, I.E. Lawrence Jr., Experimental studies on the acetyl cholinesterase positive nerves in the ovary of the rat, Anat. Rec. 190 (1978) 233–238.
[8] I.E. Lawrence Jr., H.W. Burden, The origin of the extrinsic adrenergic innervation to the ovary, Anat. Rec. 196 (1980) 51–59.
[9] A. Vega Orozco, Z. Sosa, V. Fillipa, F. Mohamed, A.M. Rastrilla, 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, J. Endocrinol. 191 (2006) 587–598.
[10] C.M. Klein, H.W. Burden, Anatomical localization of afferent and postganglionic sympathetic neurons innervating the rat ovary, Neurosci. Lett. 85 (1988 a) 217–223.
[11] S.R. Ojeda, L.I. Aguado, S. Smith, Neuroendocrine mechanisms controlling the onset of female puberty: the rat as a model, Neuroendocrinology 37 (1983) 306–313.
[12] E.Y. Adashi, A.J. Hsueh, Stimulation of β_2-adrenergic responsiveness by follicle stimulating hormone in rat granulosa cells *in vitro* and *in vivo*, Endocrinology 108 (1981) 2170–2178.
[13] L.I. Aguado, S.L. Petrovic, S.R. Ojeda, Ovarian β-adrenergic receptors during the onset of puberty: characterization, distribution, and coupling to steroidogennic responses, Endocrinology 110 (1982) 1124–1132.
[14] G.F. Erickson, D.A. Magofin, C.A. Dyer, C. Hofeditz, The ovarian androgen-producing cells: a review of structure/function relationships, Endocr. Rev. 6 (1985) 371–399.
[15] C.C. Felder, Muscarinic acetylcholine receptors: signal transduction thorough multiple effectors, FASEB J. 9 (1995) 619–625.
[16] S. Batra, L.D. Popper, C.S. Iosif, Characterisation of muscarinic cholinergic receptors in human ovaries, ovarian tumours and tumour cell lines, Eur. J. Cancer 29 (1993) 1302–1306.
[17] A. Mayerhofer, S. Fritz, Ovarian acetylcholine and muscarinic receptors: hints of a novel intrinsec ovarian regulatory system, Microsc. Res. Tech. 59 (2002) 503–508.
[18] E. Lars-Gösta, K. Holmberg, P. Emson, M. Schemmann, T. Hokfelt, Nitric oxide synthase, choline acetyltransferase, catecholamine enzymes and neuropeptides and their colocalization in the anterior pelvic ganglion and hypogastric nerve of the male guinea pig, Anat. Rec. 35 (1997) 68–76.
[19] A. Jablonka-Shariff, A.A. Basuray, L.M. Olson, Inhibitors of nitric oxide synthase influence oocyte maturation in rats, J. Soc. Gynecol. Invest. 6 (1999) 95–101.

[20] L. Olson, B.Ch. Jone, A. Jablonka-Shariff, Nitric oxide decreases estradiol synthesis of rats luteinized ovarian cells: possible role for nitric oxide in functional luteal regression, Endocrinology 137 (1996) 3531–3536.
[21] B. Fridén, E. Runesson, M. Hahlin, M. Brannstrom, Evidence for nitric oxide acting as a luteolytic factor in the human corpus luteum, Mol. Hum. Reprod. 6 (2000) 397–405.
[22] S.M. Delgado, Z. Sosa, N.S. Dominguez, M. Casais, L. Aguado, A.M. Rastrilla, Effect of the relation between neural cholinergic action and nitric oxide on ovarian steroidogenesis in prepubertal rats, J. Steroid Biochem. Mol. Biol. (2004) 139–145.
[23] M. Casais, S.M. Delgado, S. Vallcaneras, Z. Sosa, A.M. Rastrilla, Nitric oxide in prepubertal rat ovary contribution of the ganglionic nitric oxide synthase system via superior ovarian nerve, Neuro Endocrinol. Lett. 28 (2007) 39–44.
[24] M. Vega, L. Urrutia, G. Iñiguez, F. Gabier, L. Devoto, M.C. Johnson, Nitric oxide induces apoptosis in the human corpus luteum *in vitro*, Mol. Hum. Reprod. 6 (2000) 681–687.
[25] R. Rekawiecki, M. Nowik, J. Kotwica, Stimulatory effect of LH, PGE2 and progesterone on StARprotein, cytochrome P450 cholesterol side chain cleavage and 3β-hydroxysteroid dehydrogenase gene expression in bovine luteal cells, Prostaglandins Other Lipid Mediat. 78 (2005) 169–184.
[26] J.C. Chapman, J.R. Polanco, S. Min, S.D. Michael, Mitochondrial 3beta-hydroxysteroid dehydrogenase (HSD) is essential for the synthesis of progesterone by corpora lutea: an hypothesis, Reprod. Biol. Endocrinol. 3 (2005) 11–18.
[27] G. Pelletier, S. Li, V. Luu-The, Y. Tremblay, A. Bélanger, F. Labrie, Immunoelectron microscopic localization of three key steroidogenic enzymes (cytochrome P450(scc), 3beta-hydroxysteroid dehydrogenase and cytochrome P450(c17)) in rat adrenal cortex and gonads, J. Endocrinol. 171 (2001) 373–383.
[28] K.J. Teerds, J.H. Dorrington, Immunohistochemical localization of 3beta-hydroxysteroid dehydrogenase in the rat ovary during follicular development and atresia, Biol. Reprod. 49 (1993) 989–996.
[29] J. Mao, R.W. Duan, L. Zhong, G. Gibori, S. Azhar, Expression, purification and characterization of the rat luteal 20α-hydroxysteroid dehydrogenase, Endocrinology 1 (1997) 182–190.
[30] T. Poole, UFAW Hand Book of the Care and Management of Laboratory Animals, vol 1. Terrestrial vertebrates, Blackwell Publishing, 1999.
[31] D.S. Koh, B. Hille, Modulation by neurotransmitters of catecholamine secretion from sympathetic ganglion neurons detected by amperometry, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 1506–1511.
[32] F. Egami, S. Taniguchi, Nitrate, in: H.U. Vergmeyer (Ed.), Method of Enzymatic Analysis, 2nd ed., Academic Press, New York, 1974, pp. 2260–2266.
[33] T. Kawano, H. Okamura, C. Tajima, K. Fukuma, H. Katabuchi, Effect of RU 486 on luteal function in the early pregnant rat, J. Reprod. Fertil. 83 (1988) 279–285.
[34] O.H. Lowry, H.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[35] P. Chomczynski, A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples, Biotechniques 15 (1993) 532–534, 536–537.
[36] W.G. Snedecor, W.G. Cochram, Statistical Methods, The Iowa State University Press, Ames, IA, 1976.
[37] L. Devoto, P. Kohen, M. Vega, O. Castro, R.R. Gonzalez, I. Retamales, P. Carvallo, L.K. Christenson, J.F. Strauss, Control of human luteal steroidogenesis, Mol. Cell. Endocrinol. 186 (2002) 137–141.
[38] M.I. Garraza, L.I. Aguado, M.A. De Bortoli, *In vitro* effect of neuropeptides on ovary or celiac ganglion affects the release of progesterone from ovarios in the rat, Med. Sci. Monit. 10 (2004) 440–446.
[39] M.H. Garraza, M. Forneris, L. Virginia Gatica, L.B. Oliveros, Norepinephrine modulates the effect of neuropeptides in coeliac ganglion on ovarian hormones release: Its relationship with ovarian nitric oxide and nerve growth factor, Neuro. Endocrinol. Lett. 31 (2010) 19–25.
[40] C.M. Tellería, C.O. Stocco, A.O. Stati, A.M. Rastrilla, D.G. Carrizo, L.I. Aguado, R.P. Deis, Dual regulation of luteal progesterone production by androstenedione during spontaneous and RU486-induced luteolysis in pregnant rats, J. Steroid Biochem. Mol. Biol. 55 (1995) 385–393.
[41] C.O. Stocco, R.P. Deis, Luteolytic effect of LH: inhibition of 3β-hydroxysteroid dehydrogenase and stimulation of 20α-hydroxysteroid dehydrogenase luteal activities in late pregnant rats, J. Endocrinol. 150 (1996) 423–429.
[42] L.I. Aguado, S.R. Ojeda, Ovarian adrenergic nerves play a role in maintaining preovulatory steroid secretion, Endocrinology 114 (1984 b) 1944–1946.
[43] A.J. Hsueh, E.Y. Adashi, P.B.C. Jones, T.H. Welsh Jr., Hormonal regulation of the differentiation of cultured ovarian granulosa cells, Endocr. Rev. 5 (1984) 76–127.
[44] G. Miszkiel, D. Skarzynski, M. Bogacki, J. Kotwica, Concentrations of catecholamines, ascorbic acid, progesterone and oxytocin in the corpora lutea of cyclic and pregnant cattle, Reprod. Nutr. Dev. 39 (1999) 509–516.
[45] S. Kalsner, Steroid potentiation of responses to sympathomimetic amines in aortic strips, Br. J. Pharmacol. 36 (1969) 582–593.
[46] R. Rekawiecki, M. Nowik, J. Kotwica, Stimulatory effect of LH, PGE2 and progesterone on StAR protein, cytochrome P450 cholesterol side chain cleavage and 3β-hydroxysteroiddehydrogenase gene expression in bovine luteal cells, Prostaglandins Other Lipid Mediat. 78 (2005) 169–184.
[47] J. Kotwica, R. Rekawiecki, M. Duras, Stimulatory influence of progesterone on its own synthesis in bovine corpus luteum, Bull. Vet. Inst. Pulawy 48 (2004) 139–145.
[48] R. Rekawiecki, M.K. Kowalik, D. Slonina, J. Kotwica, Regulation of progesterone synthesis and action in bovine corpus luteum, J. Physiol. Pharmacol. 59 (2008) 75–89.
[49] R. Yamamoto, A. Wada, Y. Asada, H. Niina, A. Sumiyoshi, N omega-nitro-L-arginine, an inhibitor of nitric oxide synthesis, decreases noradrenaline outflow in rat isolated perfused mesenteric vasculature, Naunyn Schmiedebergs Arch. Pharmacol. 347 (1993) 238–240.
[50] S. Moncada, R.M. Palmer, E. Higgs, Nitric oxide; physiology, pathophysiology and pharmacology, Pharmacol. Rev. 43 (1991) 109–142.
[51] D.W. Brann, G.K. Bhat, C.A. Lamar, V.B. Mahesh, Gaseous transmitters and neuroendocrine regulation, Neuroendocrinology 65 (1997) 385–395.
[52] R.C. Dunnam, M.J. Hill, D.M. Lawson, J.C. Dunbar, Ovarian hormone secretory response to gonadotropins and nitric oxide following chronic oxide deficiency in the rat, Biol. Reprod. 60 (1999) 959–963.
[53] A.B. Motta, A. Estevez, T. Tognetti, M.A. Gimeno, A.M. Franchi, Dual effects of nitric oxide in functional and regressing rat corpus luteum, Mol. Hum. Reprod. 7 (2001) 43–47.
[54] Y.L. Dong, P.R. Gangula, L. Fan, C. Yallampalli, Nitric oxide reverses prostangladin-induce inhibition in ovarian progesterone in rats, Mol. Hum. Reprod. 14 (1999) 27–32.
[55] A. Jablonka-Shariff, A.T. Grazul-Bilska, D.A. Redmer, L.P. Reynolds, Growth and cellular proliferation of ovine corpora lutea throughout the estrous cycle, Endocrinology 133 (1993) 1871–1879.
[56] R.P. Del Vecchio, J.K. Thibodeaux, W. Hansel, Contact-associated interactions between large and small bovine luteal cells during the estrous cycle, Domest. Anim. Endocrinol. 133 (1995) 1871–1879.
[57] J.L. Pate, Intercellular communication in the bovine corpus luteum, Theriogenology 45 (1996) 1381–1397.