Nitric Oxide 75 (2018) 85–94

Contents lists available at ScienceDirect

Nitric Oxide

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

The production of nitric oxide in the coeliac ganglion modulates the effect of cholinergic neurotransmission on the rat ovary during the preovulatory period

María B. Delsouc[a,c], María C. Della Vedova[b,c], Darío Ramírez[b,c], Silvia M. Delgado[a], Marilina Casais[a,c,*]

[a] Laboratorio de Biología de la Reproducción (LABIR), Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, San Luis, Argentina
[b] Laboratorio de Medicina Experimental y Traduccional (LME&T), Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, San Luis, Argentina
[c] Instituto Multidisciplinario de Investigaciones Biológicas de San Luis (IMIBIO-SL), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

ARTICLE INFO

Keywords:
Nitric oxide
First proestrous
Coeliac ganglion
Oxidative stress
Ovarian steroidogenesis
Apoptosis

ABSTRACT

The aim of the present work was to investigate whether the nitric oxide produced by the nitric oxide/nitric oxide synthase (NO/NOS) system present in the coeliac ganglion modulates the effects of cholinergic innervation on oxidative status, steroidogenesis and apoptotic mechanisms that take place in the rat ovary during the first proestrous. An *ex vivo* Coeliac Ganglion- Superior Ovarian Nerve- Ovary (CG-SON-O) system was used. Cholinergic stimulation of the CG was achieved by 10^{-6} M Acetylcholine (Ach). Furthermore, 400 µM Aminoguanidine (AG) – an inhibitor of inducible-NOS was added in the CG compartment in absence and presence of Ach. It was found that Ach in the CG compartment promotes apoptosis in ovarian tissue, probably due to the oxidative stress generated. AG in the CG compartment decreases the release of NO and progesterone, and increases the release of estradiol from the ovary. The CG co-treatment with Ach and AG counteracts the effects of the ganglionic cholinergic agonist on ovarian oxidative stress, increases hormone production and decreases Fas mRNA expression. These results suggest that NO is an endogenous modulator of cholinergic neurotransmission in CG, with implication in ovarian steroidogenesis and the apoptotic mechanisms that take place in the ovary during the preovulatory period in rats.

1. Introduction

Through anatomical descriptions mostly derived from rodent studies, it was demonstrated that the extrinsic innervation of the ovary is primarily provided by sympathetic and sensory nerves, as well as a small contingency of parasympathetic nerves. These nerves reach the ovary via two main routes: (i) the ovarian plexus nerve, which travels along the ovarian artery; and (ii) the superior ovarian nerve (SON), which is associated with the suspensory ligament of the ovary [1,2].

The SON is constituted mainly of catecholaminergic fibers and the neurochemical nature of this projection is mainly noradrenergic. This route provides communication between the coeliac ganglion (CG) and the ovary, with implication in steroidogenesis, follicular maturation, ovulation and luteolysis [2,3].

The CG is a component of the prevertebral ganglionic sympathetic pathway with function of receiving and integrating information from the central nervous system and organizes responses that influence ovarian physiology [4]. This structure has a variety of neurotransmitters (acetylcholine, catecholamines, neuropeptides, and nitric oxide) also specific receptors [5,6].

Acetylcholine (Ach) is the principal preganglionic neurotransmitter of the sympathetic ganglionic pathway [7]. The CG has specific structures to respond to cholinergic stimuli, such as nicotinic and muscarinic receptors in the principal cells as well as muscarinic receptors in other neuronal populations [8,9]. It has been shown that the stimulation of such receptors modifies the release of ovarian steroids in prepubertal [10], cyclic [11], pregnant [12] and peripubertal-pubertal rats [3,13,14].

Respect of nitric oxide (NO), this gasotransmitter is produced by three isoforms of NO synthase (NOS): nNOS (neural), iNOS (inducible), and eNOS (endothelial). The presence of the NO/NOS system in rat prevertebral ganglia has been confirmed [15]. In addition, the three isoforms have been shown to influence autonomic neural function in some manner [16–18]. Unlike conventional neurotransmitters, NO is not stored in synaptic vesicles, its action is not limited to the synaptic regions of the neurons and does not interact with receptor proteins. This

* Corresponding author. Universidad Nacional de San Luis, Ejercito de Los Andes 950, CP D5700HHW San Luis, Argentina.
E-mail address: mcasais@unsl.edu.ar (M. Casais).

https://doi.org/10.1016/j.niox.2018.02.010
Received 19 September 2017; Received in revised form 7 February 2018; Accepted 28 February 2018

gaseous molecule accordingly regulates many functions, such as vascular tone, immune response, synaptic plasticity, neurotransmission, and oxidation-sensitive mechanisms [19,20], suggesting its participation in the physiological and pathological processes.

In a previous report, Casais and her collaborators [10], using the *ex vivo* CG-SON-O system of prepubertal rat and stimulating both CG and ovary with isoform-selective inhibitors of NOS enzymes, corroborated the existence of the NO/NOS system in CG when obtaining relevant results with aminoguanidine (AG, a selective inhibitor of the iNOS). In addition, they highlighted the existence of a contribution of NO from the CG to the ovary through the SON [10]. Afterwards, using the *ex vivo* CG-SON-O system of rats in the first proestrous (PE), Delsouc et al. [13] demonstrated that the addition of 10^{-6} M Ach into the CG compartment increases NO concentration in the incubation media of ovaries and causes oxidative stress in gonadal tissue. Assuming that highest levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) lead to irreversible cell damage, it was also considered interesting to stimulate the ovary with 400 μM AG. Finally, this study demonstrated that the addition of AG in the ovarian compartment counteracts the effects generated by the ganglionic cholinergic agonist, showing a protective action against induced damage in gonadal tissue [13]. Despite the interesting findings, the influence of the ganglion NO/NOS system on the ovary is not known in depth. It is presumed that both NOS and choline acetyltransferase play a vital role in prevertebral ganglia, complementing each other's functions and synergistically modulating the activity of preganglionic neurons [6,21] and, as such, likely to contribute in ovarian physiology.

Given the important role of NO in physiological and pathophysiological processes, several studies have analyzed the expressions and localization patterns of NOS isoforms in reproductive systems in mouse, rat, sheep, and pig [22,23]. Although neuronal pathways are an important factor in the regulation of ovarian function, our understanding of the regulatory factors governing NOS function and the influences of NO on the CG remains limited. The NO generated in the nerve synapses can easily diffuse through short distances and affect one or more cells, influencing the presynaptic and postsynaptic events of both excitatory and inhibitory synapses [17]. Clearly its mechanism of action is complicated and for this reason additional studies are necessary.

Based on the above and without attempting to oversimplify the complex problem of ganglionic functioning and regulation, the aim of this work was to investigate whether the NO/NOS system present in CG modulates the effects of cholinergic innervation on oxidative status, steroidogenesis and apoptotic mechanisms that take place in rat ovary during the first PE.

2. Materials and methods

2.1. Animals

Female 37-day-old virgin Holtzman rats in their first PE and weighing 100 ± 10 g were used in all the experiments. The rats were kept under controlled conditions with light on from 07:00 to 19:00 h and at a temperature of 22 ± 2 °C. Animals had free access to food (Cargill SAIC, Saladillo, Buenos Aires, Argentina) and tap water. Groups of six animals were used for the experimental procedure.

The experiments were performed per duplicate according to the procedures approved in the UFAW Handbook on the Care and Management of Laboratory Animals [24]. The experimental procedures used in this study were approved by the Institutional Animal Care and Use Committees of the National University of San Luis (Protocol# B-96/12, B-200/15).

2.2. Reagents

L-acetylcholine hydrochloride (Ach), aminoguanidine (AG), dextrose, ascorbic acid, bovine serum albumin-fraction V (BSA), sulfanilamide, N-1-naphthylethylenediamine were from Sigma Chemical Co (St. Louis, MO, USA). 1,2,6,7-[^{3}H]-Progesterone (107.0 Ci/mmol) was provided by New England Nuclear Products (Boston, MA, USA). The Estradiol (E_2) DIASource ImmnoAssays kit was purchased from DiagnosMed SRL (Buenos Aires, Argentina). Other reagents and chemicals were of analytical grade.

2.3. Experimental procedure

The animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). The CG-SON-O system was removed by dissecting, as previously described by Delgado et al. [3]. In order to prevent spontaneous depolarization of the nerves, the strip of tissues was carefully dissected avoiding contact between the surgical instruments and the nerve fibers or the ganglion, and the total surgical procedure was completed within 1–2 min (min). The CG-SON-O system was rinsed with incubation medium and immediately placed in a cuvette with two compartments, one for the CG and the other for the ovary, both joined by the SON. The incubation medium was 1 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, with 0.1 mg/ml dextrose and 0.1 mg/ml BSA at 37 °C in a saturated atmosphere of 95% O_2 and 5% CO_2. Incubations were conducted in a Dubnoff metabolic shaking-water bath.

The CG-SON-O system was pre-incubated for 15 min, and the end of this pre-incubation period was considered incubation time 0. After this pre-incubation time, Krebs-Ringer solution (1 ml) was changed in both compartments, and 0.1 mg/ml ascorbic acid was added as an antioxidant agent to the CG compartment. Likewise, the different agents used for each experimental group were added at the same time. To stimulate the CG, Ach was dissolved in 1 ml of Krebs-Ringer solution at a 10^{-6} M final concentration in the CG compartment (Ach group). In addition, to test the role of NO on extrinsic ovarian innervation, AG (a selective inhibitor of the iNOS) was dissolved in 1 ml of Krebs-Ringer solution at a 400 μM final concentration in the CG compartment, with or without 10^{-6} M Ach [10]. The control groups consisted of untreated CG-SON-O systems.

The incubation was performed during 180 min. Periodical extractions of 250 μl were made from the ovary compartment at 30, 120 and 180 min. Liquid samples from the ovary compartment were maintained at −20 °C for further analysis. At the end of the incubation period, the ovaries were collected and frozen at −80 °C for further analysis.

2.4. Nitrite assay

Levels of nitrite, a water-soluble metabolite of NO, were measured spectrophotometrically by Griess reaction [25]. Briefly, 50 μl of each aliquot of incubation medium from the ovaries was mixed with Griess reagent (1% sulfanilamide with 0.1% *N-l*-naphthyl-ethylenediamine/HCl in 1% phosphoric acid). After 10 min incubation at room temperature, the absorbance was read at 540 nm. A solution of nitrite of known concentration was used to prepare a standard curve. The assay sensitivity was < 2.5 nmol/ml. The intra-assay coefficients of variation for all the assays were less than 10%. The results were expressed as nmol of nitrite per milligram of ovarian tissue (nmol/mg ovary).

2.5. Preparation of tissue homogenate

Six ovaries per experimental group were homogenized separately in 150 μl RIPA buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Tissue homogenates were centrifuged at 14,000 × *g* for 15 min at 4 °C to remove nuclei and cell debris. The pellets were discarded whereas the supernatants were collected and used to determine the expression of iNOS, BAX and BCL-2 proteins, the total antioxidant capacity (TAC) and the activity of antioxidant enzymes (catalase and glutathione peroxidase). The concentration of protein carbonyls and thiobarbituric acid reactive

substances (TBARS) were measured as biomarkers of oxidative damage of proteins and lipids, respectively. The total protein concentration in tissue homogenates was measured by the Bradford method.

2.6. Expression of iNOS, BAX and BCL-2 proteins

The expression of iNOS, BAX and BCL-2 proteins was analyzed by an indirect enzyme-linked immunosorbent assay (ELISA). Ten microliters of sample (10 µg of total proteins) were added to 190 µl of 0.1 M bicarbonate buffer pH 9.6, in clear 96-*well* microplates (Corning Incorporated, Corning, NY, USA) and incubated overnight at 4 °C. After washing with PBS/0.05% Tween-20/5% and blocking with PBS/0.05% Tween-20/5% nonfat dry milk for 1 h at 37 °C, the microplates were incubated with 50 µl of rabbit polyclonal anti-iNOS (1:200 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 3 h at 37 °C, rabbit polyclonal anti-Bax (1:500 dilution; Abcam, CA, USA) and rabbit polyclonal anti-Bcl-2 (1:10,000 dilution; Abcam, CA, USA) overnight at 37 °C. After three washes, 50 µl of goat anti-rabbit IgG-HRP conjugate (1:10,000 dilution; Jackson Immuno-Research Labs, West Grove, PA, USA) was added to each well and incubated for 1 h at 37 °C. Finally, immunocomplexes were quantified using 3,3′,5,5′-Tetramethylbenzidine (TMB). The oxidation reaction of the substrate was stopped with 2 M sulfuric acid, and the optical density (OD) at 450 nm was measured using a TECAN microplate reader (Infinite M200 PRO, Research Triangle Park, NC, USA).

2.7. Total antioxidant capacity

The TAC was measured by an improved method of quenching of the 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) by both lipophilic and hydrophilic antioxidants present in the ovary homogenates [26]. The $ABTS^{\bullet+}$ was generated by oxidation of 7 mM ABTS with 2.45 mM potassium persulfate. The TAC was expressed as the percentage of reduction in the absorbance due to the $ABTS^{\bullet+}$, and it was determined as follows: $\mathbf{\%inhibition = [(A_0\text{-}A_f)/A_0]\ x\ 100}$, where A_0 and A_f were the absorbance at 734 nm of the reaction mixtures measured at t = 0 and after 5 min of sample addition, respectively. All measurements were performed in duplicate for each sample.

2.8. Antioxidant enzymes activity

The specific catalase (CAT) and glutathione peroxidase (GSH-Px) enzymatic activities were determined following Aebi's [27] and Flohé and Gunzler's [28] methods, respectively. Briefly, the CAT activity was determined by measuring the decrease in the absorbance at 240 nm when 100 µl of 3 mM H_2O_2 were added to a reaction medium containing a 1/500 dilution of ovary homogenates in 50 mM phosphate buffer, pH 7.3. The decrease of the absorbance at 240 nm was acquired every 5 s during a total time of 30 s. During this time, the decomposition of the H_2O_2 follows a first order reaction kinetic. The GSH-Px activity was determined following NADPH oxidation at 340 nm in a reaction medium containing 0.2 mM GSH, 0.25 IU/ml yeast glutathione reductase and 0.5 mM *tert*-butyl hydroperoxide in 50 mM phosphate buffer, pH 7.2. The results were expressed in international units of enzymatic activity per milligram of total proteins (IU/mg protein).

2.9. Measurement of protein oxidation

As a marker of protein oxidation, protein carbonyls were determined by ELISA following the method of Winterbourn and Buss [29], with some modifications. Briefly, tissue homogenates were derivatized to 2,4-dinitrophenylhydrazone by reaction of carbonyl groups in oxidized proteins with 2,4-dinitrophenylhydrazine in 2 M HCl. Ten microliters of the derivatized or nonderivatized sample were added to 190 µl of 0.1 M bicarbonate buffer, pH 9.6, in clear 96-*well* microplates (Corning Incorporated, Corning, NY, USA) and incubated overnight at 4 °C. After washing with PBS/0.05% Tween-20/5% and blocking with 2.5% cold-water fish skin gelatin (Sigma) in PBS at 37 °C for 1 h, the microplates were incubated with 50 µl of rabbit polyclonal anti-dinitrophenyl antibody (1:2000 dilution; Thermo Fisher Scientific Inc., Waltham, MA, USA) for 1 h at 37 °C. After three washes, 50 µl of goat anti-rabbit IgG-HRP conjugate (1:10,000 dilution; Jackson Immuno Research Laboratories, West Grove, PA, USA), was added to each *well* and incubated for 1 h at 37 °C. Finally, immunocomplexes were quantified using TMB. The oxidation reaction of the substrate was stopped with 2 M sulfuric acid and the absorbance was measured at 450 nm using a TECAN microplate reader (Infinite M200 PRO, Research Triangle Park, NC, USA). The results were expressed as nmol of carbonyl per milligram of total proteins (nmol/mg protein).

2.10. Measurement of lipid peroxidation

TBARS were determined according to the method described by Draper and Hadley [30]. The TBARS assay measures malondialdehyde (MDA) production from lipid hydroperoxides. A calibration curve was performed using 1,1,3,3-tetramethoxypropane as standard. TBARS were determined by the absorbance at 535 nm and were expressed as µmol of MDA per milligram of total proteins (µmol MDA/mg protein).

2.11. Progesterone and estradiol assay

Steroids were measured in the ovarian incubation liquid per duplicate by radioimmunoassay (RIA). The progesterone (P) antiserum, provided by Dr. R. Deis (IMBECU, Mendoza, Argentina), was produced in rabbits against P conjugated to bovine serum albumin at the 11 position. The antiserum was highly specific for P with low cross-reactivities, < 2.0% for 20α-dihidro-progesterone and deoxy-corticosterone and 1.0% for other steroids. The sensitivity was less than 5 ng/ml and the inter- and intra-assay coefficients of variation were less than 10%. This assay has been previously validated [31]. P concentration was expressed as nanogram per milligram of ovarian tissue (ng/mg ovary).

The estradiol (E_2) concentration was determined using a double antibody RIA DiaSource kit (DiagnosMed SRL, Buenos Aires, Argentina) following the manufacturer's instructions. The percentages of cross-reactions were 1.8% for estrone, 1.2% for estriol, 0.0011% for androstenedione and 0.0002% for P. The assay sensitivity was < 2.2 pg E_2/ml. The inter- and intra-assay coefficients of variation in all the assays were < 10.0%. E_2 was expressed as picograms per milligram of ovarian tissue (pg/mg ovary).

2.12. RNA isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis

Total RNA was extracted from three pools of two ovaries each per experimental group. All RNA isolations were performed using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) as suggested by the manufacturers. Purified total RNAs were then quantified and assessed for purity by measurement of the 260/280 ratio using a UV spectrophotometer Beckman DU-640 B (CA, USA). Only samples with 260/280 ratio of 1.8–2.0 were used. The integrity of the total RNAs were checked on a denaturing agarose gel. After GelRed™ (Biotium, Hayward, CA, USA) staining, RNA bands were visualized with a UV transilluminator, and 28S and 18S rRNA band patterns were analyzed. Two micrograms of total RNA were reverse-transcribed at 37 °C with 200 units of M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) using random hexamers in a 26 µl reaction mixture to produce cDNA according to the manufacturer's instructions. For amplification of the cDNAs, the reaction mixture consisted of 1× 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, Madison, WI, USA) in a

Table A
Primers used for PCR amplification.

Gene name	Forward primer 5′-3′	Reverse primer 5′-3′	GenBank accession #	Fragment size
iNOS	GCATGGACCAGTATAAGGCAAGCA	GCTTCTGGTCGATGTCATGAGCAA	S71597	219
eNOS	CGAGATATCTTCAGTCCCAAGC	GTGGATTTGCTGCTCTGTAGG	NM_021838	164
3β-HSD	GTCTTCAGACCAGAAACCAAG	CCTTAAGGCACAAGTATGCAG	NM_001042619	447
20α-HSD	TTCGAGCAGAACTCATGGCTA	CAACCAGGTAGAATGCCATCT	D14424	440
P450arom	TGCACAGGCTCGAGTATTTCC	ATTTCCACAATGGGGCTGTCC	M33986	266
Bax	GATTGCTGACGTGGACACGGACT	TCAGCCCATCTTCTTCCA	AB046392	473
Bcl-2	CACCCCTGGCATCTTCTCCT	GTTGACGCTCCCCACACACA	L14680	349
Fas	TGTCAACCGTGTCAGCCTGGT	GGGTCCGGGTGCAGTTCGTT	NM_139194	190
FasL	GTGCTGGTGGCTCTGGTTGGAA	AGTGGGCCACACTCCTTGGCTT	NM_012908	178
β-actin	CGGAACCGCTCATTGCC	ACCCACACTGTGCCCATCTA	NM_031144	289

final volume of 50 µl. The PCR primers were designed using Primer Express 3.0 software (Applied Biosystems, USA) on the basis of the respective published rat DNA sequences. The primers information is shown in Table A.

PCRs were performed using a thermocycler (My Cycler; BioRad) programmed at 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min by 35 cycles, for iNOS, eNOS, Bax (a pro-apoptotic regulator) and Bcl-2 (an anti-apoptotic regulator). Instead, the reactions were carried out at 95 °C for 1 min, 59 °C for 1 min and 72 °C for 1 min by 35 cycles, for 3β-hydroxysteroid dehydrogenase (3β-HSD, P synthesis enzyme), 20α-hydroxysteroid dehydrogenase (20α-HSD, P degrading enzyme), P450 aromatase (P450arom, E_2 synthesis enzyme), Fas cell surface death receptor (Fas), Fas ligand (FasL) and β-actin (housekeeping gene). All the reactions were terminated with a 5 min extension at 72 °C. The reaction products were electrophoresed on 2% agarose gels, visualized with GelRed (0.05 µl/ml) and examined by ultraviolet transillumination. Band intensities of RT-PCR amplicons were quantified using ImageJ (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij). Relative levels of mRNA were expressed as the ratio between the signal intensity of target genes and that for the housekeeping gene.

2.13. Statistical analysis

All data are presented as means ± SEM for each group of six rats. The differences between the two groups were analyzed with the Student's *t*-test. For multiple comparisons made along the incubation times, a repeated measures analysis of variance followed by Tukey's test was used. For multiple comparisons not involving repeated measures, one-way analysis of variance followed by Tukey's test was used. A difference was considered to be statistically significant when $p < 0.05$.

3. Results

3.1. Effect of ganglionic stimulation with 400 µM AG on the ovarian oxidative status in the ex vivo CG-SON-O system, in the first PE day

The results on NO release and oxidative status in the ovary compartment by ganglionic stimulation with 10^{-6} M Ach were previously published by our research group [13,14].

3.1.1. Release of NO and expression of the NOS isoforms

The addition of 400 µM AG into the CG compartment decreased the ovarian release of NO at 30 (▪$p < 0.001$) and 120 min (*$p < 0.05$), as compared to the control group (Figure A1). Treatment of the CG compartment with AG also decreased iNOS mRNA in the ovarian tissue at 180 min (•$p < 0.01$), as compared to the control group (Figure A2). However, this treatment did not change eNOS mRNA expression (Figure A3) or iNOS protein expression (Figure A4) in ovarian tissue at 180 min.

In addition, the co-treatment of the CG with 10^{-6} M Ach and 400 µM AG decreased the ovarian release of NO at 30 (▪$p < 0.001$), 120 (•$p < 0.01$) and 180 min (▪$p < 0.001$), as compared to the Ach group (Figure A1). The iNOS mRNA expression (•$p < 0.01$; Figure A2) and the iNOS protein expression (*$p < 0.05$; Figure A4) in ovarian tissue at 180 min also decreased. However, there was no change in the eNOS mRNA expression (Figure A3), as compared to the Ach group.

3.1.2. Antioxidant status: TAC and the activity of the enzymes CAT and GSH-Px

The addition of 400 µM AG into the CG compartment did not modify the TAC in ovarian tissue, as compared to the control group. However, the ganglionic co-treatment with 10^{-6} M Ach and 400 µM AG increased the TAC in ovarian tissue at 180 min (•$p < 0.01$), as compared to the Ach group (Figure B1).

No significant effects were observed in CAT and GSH-Px activity in ovarian tissue after CG treatment with 400 µM AG, as compared to the control group. However, the co-treatment of the CG with 10^{-6} M Ach and 400 µM AG increased the CAT activity (▪$p < 0.001$), but decreased the GSH-Px activity (▪$p < 0.001$) in ovarian tissue at 180 min (Figure B, 2 and 3), as compared to the Ach group.

3.1.3. Biomarkers of oxidative stress: protein carbonyl and TBARS levels

The addition of 400 µM AG into the CG compartment did not modify the protein carbonyl content in ovary tissue, as compared to the control group. However, the co-treatment of the CG with 10^{-6} M Ach and 400 µM decreased the protein carbonyl content in ovary tissue at 180 min (▪$p < 0.001$), as compared to the Ach group (Figure C1).

In addition, no significant modification in TBARS concentration in ovary was observed after the ganglionic treatment with AG (Figure C2).

3.2. Effect of ganglionic stimulation with 400 µM AG on the synthesis and release of ovarian steroids in the ex vivo CG-SON-O system, in the first PE day

The results on synthesis and release of ovarian steroids by ganglionic stimulation with 10^{-6} M Ach were previously published by our research group [13,14].

3.2.1. P release and mRNA expression of 3β-HSD and 20α-HSD

The addition of 400 µM AG into the CG compartment decreased the release of ovarian P at 30, 120 (▪$p < 0.001$) and 180 min (*$p < 0.05$) (Figure D1) and increased the 20α-HSD mRNA expression (•$p < 0.01$) in ovarian tissue at 180 min (Figure D3), as compared to the control group.

In addition, the ganglionic co-treatment with 10^{-6} M Ach and 400 µM AG increased the release of ovarian P at 180 min (•$p < 0.01$; Figure D1) without causing changes in the mRNA expression of 3β-HSD and 20α-HSD in ovarian tissue (Figure D, 2 and 3), as compared to the Ach group.

3.2.2. E_2 release and P450arom mRNA expression

The addition of 400 µM AG into the CG compartment of the *ex vivo*

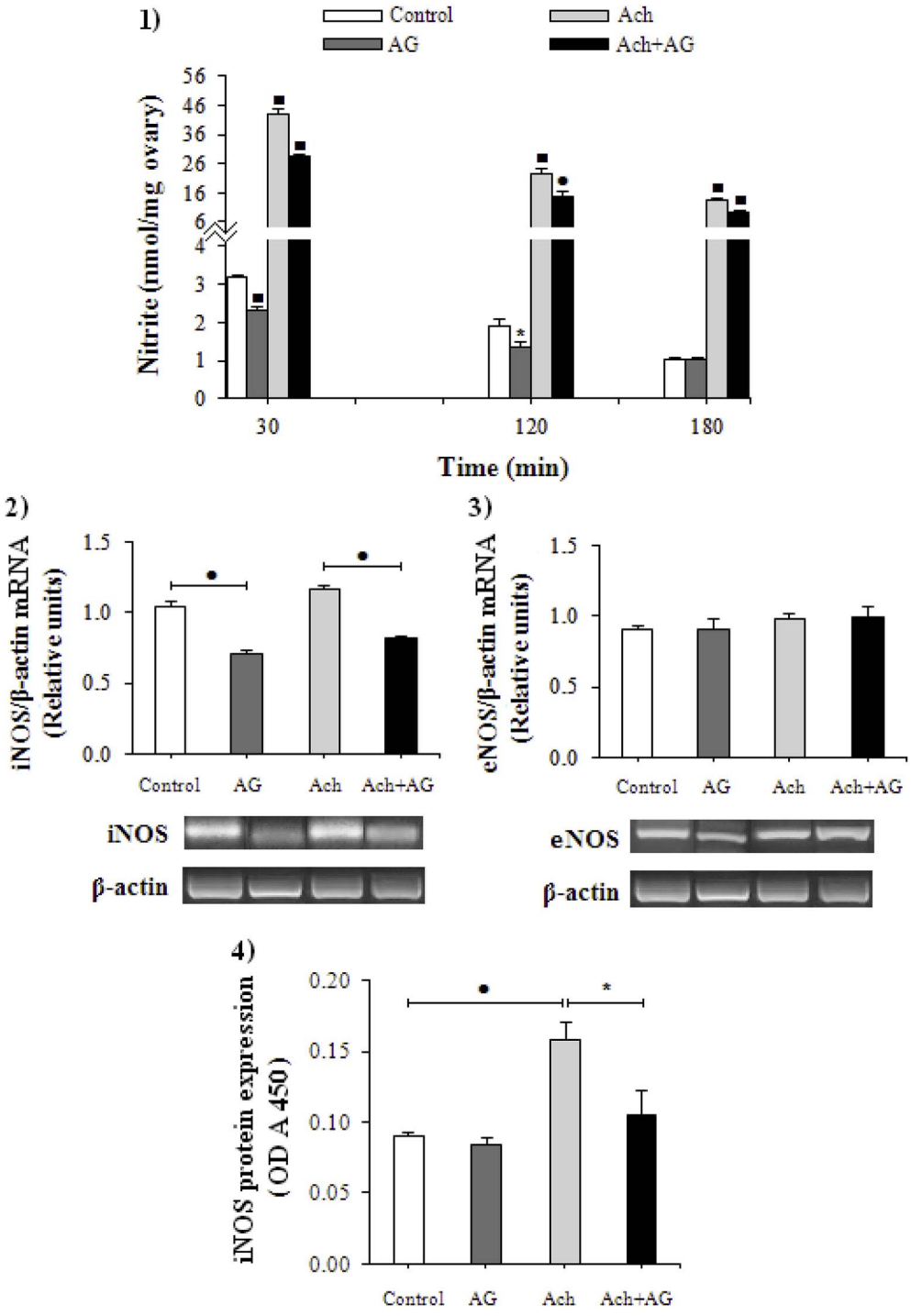

Figure A. Effect of ganglionic stimulation with 10^{-6} M Ach and with 400 μM AG, in the CG-SON-O system of rats in the first PE, on ovarian: 1) nitrite levels (upper panel), expressed as nmol/mg ovary. Results are expressed as mean ± SEM (n = 6). Repeated measures analysis of variance followed by Tukey's test was used; 2) iNOS mRNA expression; 3) eNOS mRNA expression (middle panel). Densitometry analysis of the bands in the gel photographs was performed using the ImageJ software and expressed as arbitrary units. β-actin was used as the housekeeping gene. Results are expressed as mean ± SEM (n = 3 pools of 2 ovaries/group); 4) iNOS protein expression (lower panel). Results are expressed as mean ± SEM (n = 5). One way analysis of variance followed by Tukey's test was used. *p < 0.05; •p < 0.01; ▪p < 0.001; Ach: acetylcholine; AG: aminoguanidine; OD: optical density.

system increased the release of ovarian E_2 at 30 (*p < 0.05), 120 and 180 min (•p < 0.01) (Figure E1), without showing significant changes in the P450arom mRNA expression in ovarian tissue (Figure E2), as compared to the control group.

In addition, the ganglionic co-treatment with 10^{-6} M Ach and 400 μM AG increased the release of ovarian E_2 only at 30 min (*p < 0.05) (Figure E1), without showing significant changes in the P450arom mRNA expression in ovarian tissue at 180 min (Figure E2), as compared to the Ach group.

3.3. Effect of ganglionic stimulation with 10^{-6} M Ach and 400 μM AG on the ovarian expression of pro- and antiapoptotic factors in the ex vivo CG-SON-O system, in the first PE day

Compared with the control group, the addition of 10^{-6} M Ach into the CG compartment did not change the Bax mRNA (Figure F1) and protein expression (0.143 ± 0.017 *vs* 0.147 ± 0.023, n = 5). However, decreased the Bcl-2 mRNA (*p < 0.05; Figure F2) and protein expression (0.149 ± 0.012 *vs* 0.106 ± 0.009, *p < 0.05, n = 5), and increased the ratios of the Bax/Bcl-2 (mRNA and protein, *p < 0.05; Figure F, 3 and 4), in ovarian tissue at 180 min. In addition, the mRNA expression of Fas and FasL (Figure F, 5 and 6) increased in ovarian tissue in this experimental group (*p < 0.05), compared with the

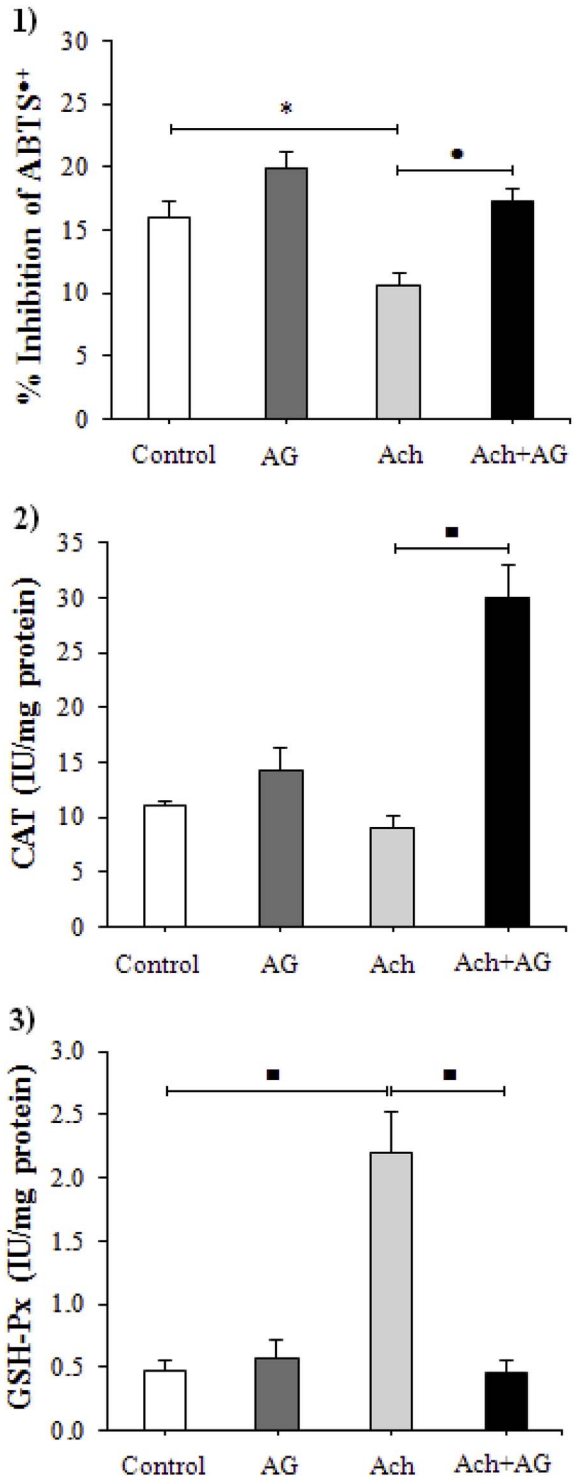

Figure B. Effect of ganglionic stimulation with 10^{-6} M Ach and with 400 μM AG, in the CG-SON-O system of rats in the first PE, on ovarian: 1) TAC, measured as percent bleaching of the ABTS●+ (upper panel); 2) CAT activity, expressed in IU/mg protein; 3) GSH-Px activity, expressed in IU/mg protein (lower panel). Results are expressed as mean ± S.E.M. (n = 6). One way analysis of variance followed by Tukey's test was used to compare groups. *p < 0.05; •p < 0.01; ■p < 0.001; TAC: total antioxidant capacity; ABTS●+: 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; CAT: catalase; GSH-Px: glutathione peroxidase.

control group.

Ganglionic treatment with 400 μM AG did not modify the ovarian mRNA expression of Bax, Bcl-2, Fas or FasL, as compared to the control group. However, the ganglionic co-treatment with 10^{-6} M Ach and 400 μM AG decreased the Fas mRNA expression in ovarian tissue at 180 min (*p < 0.05) (Figure F5), as compared to the Ach group.

4. Discussion

The aim of this work was to investigate whether the NO produced by the NO/NOS system present in CG modulates the effects of cholinergic innervation on oxidative status, steroidogenesis and apoptotic mechanisms that take place in the rat ovary during the first PE.

We study the first PE because on this stage the extrinsic ovarian innervation reaches biochemical and functional maturity [32], which has great relevance on the follicular maturation and the first ovulation.

In the CG-SON-O system, the stimulation of the cholinergic receptors present in CG was carried out through the addition of 10^{-6} M Ach [3,11,13]. In addition, the synthesis of NO in CG was inhibited by adding 400 μM AG in order to analyze the participation of the NO/NOS ganglion system on the ovarian physiology [10]. AG was used for being very effective in animal models as a selective inhibitor of iNOS [33,34], the isoform of the NOS enzyme which is present in CG and in ovary, and has shown to have great participation in the CG-SON-O system of prepubertal [10] and peripubertal rats [13,14].

According to the previously published results [13], 10^{-6} M Ach in the CG compartment causes oxidative stress in ovarian tissue since TAC decreases and the production of NO increases, which probably derives in the formation of ROS and RNS due to the content increase in the carbonyl groups in ovarian tissue, a severe indicator of oxidative damage [35]. In addition, Ach modulates the steroidogenesis since the levels of P decrease and the levels of E_2 increase, highlighting the importance of the cholinergic innervation in the follicular phase of the estral cycle in rats [13,14].

It is well known that a temporary increase in the production of ROS and a decrease in the antioxidant defenses after the preovulatory surge of gonadotropins favor the ovulatory process [36,37]. In addition, higher concentrations of E_2 and NO, essentially synthesized by iNOS, are also considered necessary during the follicular phase of the estral cycle in rats [38,39]. In relation to E_2, it stimulates the follicular grow since it modulates gonadotropins secretion and favors the differentiation of granulosa cells including the induction of receptor systems for FSH, LH and prolactin [40]. NO favors the morphological changes in the microvascularity of the preovulatory follicles which lead to the follicular break, such as vasodilation, increase in the blood flow and hyperpermeability [41,42].

It is also known that high concentrations of NO decrease the cell proliferation and survival through both oxidative and nitrosative stresses [20]. In fact, the present work demonstrates that the cholinergic hyperstimulation of the CG, besides inducing oxidative stress in ovary tissue, may also promote cell death since it increases the BAX/BCL-2 ratio (an indicator of apoptosis) and the gene expression of Fas and FasL. Such effects might contribute to initiate the follicle atresia, characterized by the rapid loss of the granulosa cells, and, to a lesser extent, of the theca cells through apoptosis mechanisms [43], induced by relatively elevated levels of NO [44].

Interestingly, despite the decrease in TAC, the activity of GSH-Px increased in ovary, probably as a compensatory effect in relation to the damage caused in proteins [13]. This effect might eventually explain the lack of susceptibility of the ovary tissue to the lipidic peroxidation since it has been reported that GSH-Px decomposes the lipidic hydroperoxides, thus protecting the cell membranes from peroxidation damages [45].

Considering these results, it is tempting to speculate that, under physiological conditions, the cholinergic innervation of the ovary acts favoring the ovulatory process, probably regulating the production of NO at ovarian level. Given that the majority of choline acetyltransferase-immunoreactive fibers in rat prevertebral ganglia are NOS positive [6], the question arises whether the NO produced in CG modulates the cholinergic neurotransmission altering the ovarian

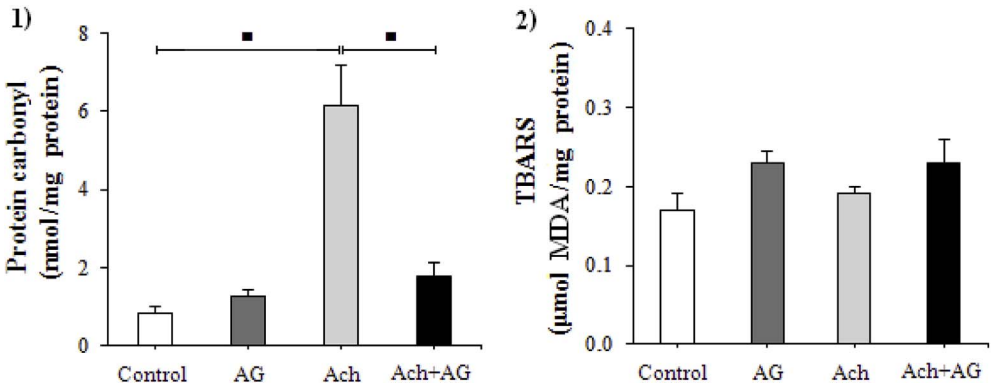

Figure C. Effect of ganglionic stimulation with 10^{-6} M Ach and with 400 µM AG, in the CG-SON-O system of rats in the first PE, on the expression of oxidative stress-related biomarkers in ovarian tissue: 1) protein carbonyl content, expressed as nmol/mg protein; 2) TBARS content, expressed as µmol MDA/mg protein. Results are expressed as mean ± S.E.M. (n = 6). One way analysis of variance followed by Tukey's test was used to compare groups. ▪p < 0.001; TBARS: thiobarbituric acid reactive substances.

function. To answer this question, in the present work, the CG was treated with 400 µM AG.

As a result of the addition of the selective inhibitor of iNOS in the ovary compartment, the gene expression of iNOS and the levels of NO decreased in ovary. These observations expose the NO as an important modulator of the sympathetic tone, as reported by Quinson et al. [9,46], Jordan et al. [47] and Prast and Philippu [48] in other experimental schemes. Remarkably, the co-treatment of the CG with Ach and AG magnified the inhibitory effects on the synthesis and liberation of NO in ovary, in relation to the Ach group. These results allow us to suggest that, in the *ex vivo* CG-SON-O system: i) Ach on CG sensitizes the neural factors participating in the ovarian physiology, and ii) the NO/NOS ganglionic system modulates the cholinergic neurotransmission. In fact, the simultaneous addition of both agents in the CG compartment had a significant impact at ovary level not only because of its effects on NO but also because it increased the TAC and the CAT activity in relation to the Ach group, which possibly prevented the oxidative damage in proteins, an opposite effect to the oxidative stress obtained by cholinergic hyperstimulation. However, the GSH-Px activity decreased in ovary tissue in the same experimental group in comparison with the Ach group.

Under physiological conditions, it has been demonstrated that the

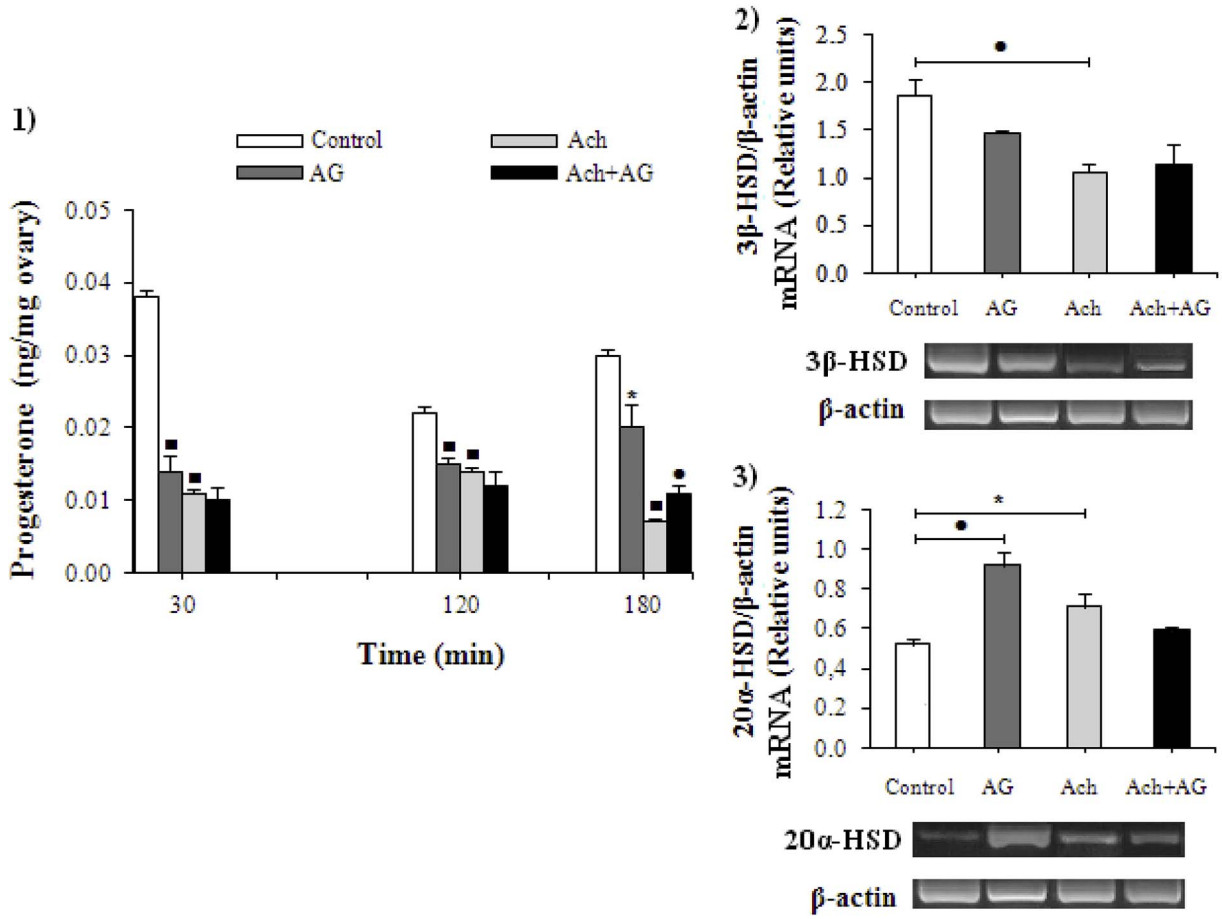

Figure D. Effect of ganglionic stimulation with 10^{-6} M Ach and with 400 µM AG, in the CG-SON-O system of rats in the first PE, on ovarian: 1) Progesterone release, expressed as ng/mg ovary. Results are expressed as mean ± S.E.M. (n = 6). Repeated measures analysis of variance followed by Tukey's test was used; 2) 3β-HSD mRNA expression and 3) 20α-HSD mRNA expression. Densitometry analysis of the bands in the gel photographs was performed using the ImageJ software and expressed as arbitrary units. β-actin was used as the housekeeping gene. Results are expressed as mean ± S.E.M. (n = 3 pools of 2 ovaries/group). One way analysis of variance followed by Tukey's test was used. *p < 0.05; •p < 0.01; ▪p < 0.001; 3β-HSD: 3β-hydroxysteroid dehydrogenase; 20α-HSD: 20α-hydroxysteroid dehydrogenase.

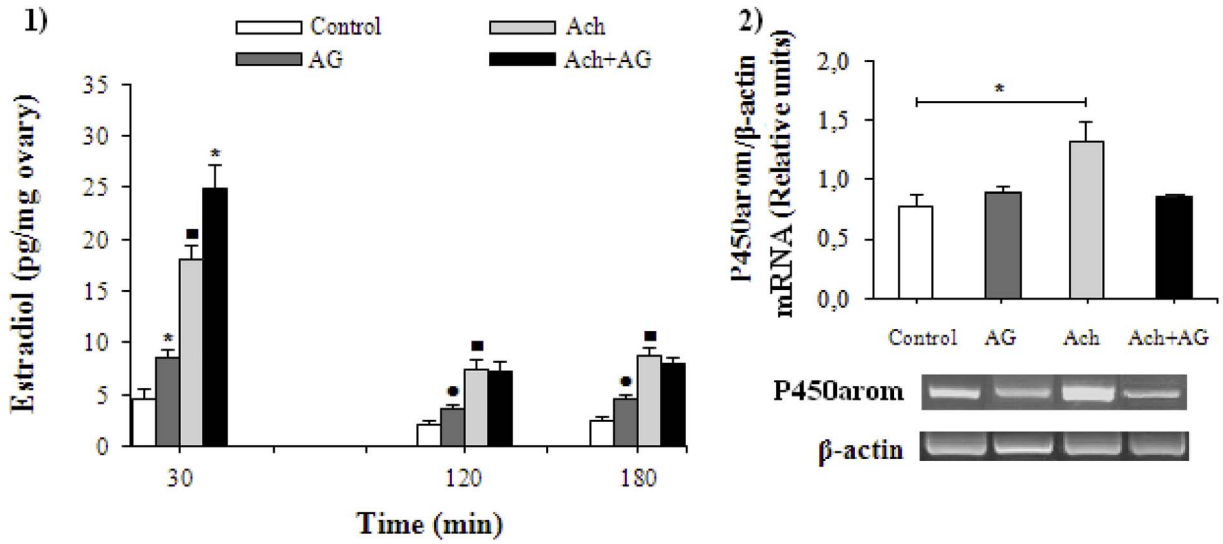

Figure E. Effect of ganglionic stimulation with 10^{-6} M Ach and with 400 μM AG, in the CG-SON-O system of rats in the first PE, on ovarian: 1) Estradiol release, expressed as pg/mg ovary. Results are expressed as mean ± S.E.M. (n = 6). Repeated measures analysis of variance followed by Tukey's test was used; 2) P450arom mRNA expression. Densitometry analysis of the bands in the gel photographs was performed using the ImageJ software and expressed as arbitrary units. β-actin was used as the housekeeping gene. Results are expressed as mean ± S.E.M. (n = 3 pools of 2 ovaries/group). One way analysis of variance followed by Tukey's test was used. *p < 0.05; •p < 0.01; ▪p < 0.001; P450arom: P450 aromatase.

CAT activity and the GSH-Px activity decrease on PE in rats. This occurs because both enzymes can eliminate the hydrogen peroxide (H_2O_2), a ROS that in physiological concentrations acts as second messenger in the hormonal system that regulates the follicular development and ovulation [35,49]. However, H_2O_2 is toxic in elevated concentrations, being CAT its main regulator with the capacity to preserve its low physiological concentration whereas GSH-Px has a limited role in this process [50,51]. Considering this, it might be assumed that the increase in the CAT activity occurs because of an excess in the formation of H_2O_2 due to the CG cholinergic hyperstimulation, which in turn might explain the decrease in the GSH-Px activity since an inverse correlation in the activity of both antioxidant enzymes has been demonstrated [51,52].

Taking into account that the ganglionic NO/NOS system might modulate indirectly the ovarian oxidative status and that the constant production of oxidant agents might increase the risk of an ovarian pathology [53], possible changes on the synthesis and liberation of sexual steroid hormones were analyzed. The only addition of 400 μM AG in the CG compartment modified ovarian steroidogenesis by increasing the P degradation and the E_2 release. In a previous study, using the same experimental model, opposite results were obtained when adding 400 μM AG directly in the ovary compartment [13]. These combined data provide new evidence of the role of NO in CG as modulator of the sympathetic tone. In addition, the co-treatment of the CG with Ach and AG increased the liberation of ovarian P after a long period of incubation (180 min), an effect reverse to the one obtained in absence of the cholinergic agonist. Although P does not exhibit the typical structure of an antioxidant, it has been demonstrated that high levels of this hormone are efficient in preventing oxidative damage [54,55]. This effect of P is likely due to its ability to decrease the generation of free radicals and enhancing endogenous free-radical-scavenging systems [54]. Consequently, the highest ovarian P levels might be contributing to the increase of the TAC and to the decrease of the carbonyl groups' content and of the expression of the death receptor Fas, possibly being one of the protective mechanisms used by the ovary facing a sympathetic hyperstimulation, modulated by NO low levels. This assumption is reinforced by Peluso et al. [56] who demonstrated that P acts as an intraovarian "governor" when inhibiting the rate at which the granulosa cells and the luteal cells undergo apoptosis.

When comparing these results with those previously reported, where 400 μM AG was added in ovary [13], it can be concluded that although the inhibition of NO synthesis in CG seems to protect the ovary from lesions caused by cholinergic hyperstimulation, these effects have less relevance in comparison with the direct action of AG on the NO/NOS ovarian system. These observations are in agreement with previous results which demonstrate that the highest production of NO in the ovary compartment comes from the ovary mainly because of the iNOS action [10].

In relation to the ovarian E_2, the co-treatment of the CG with Ach and AG increased its liberation at a short incubation time (30 min). Curiously, this effect was statistically significant when the ovarian liberation of NO decreased in a higher proportion in comparison with the Ach group. However, in a previous study, it was demonstrated that the ovarian NO/NOS system can regulate positively the synthesis and liberation of E_2 under sympathetic influence [14].

These dissenting results between the levels of the gaseous neurotransmitter and the liberation of steroid hormones in ovary might be suggesting the existence of a double modulation of the synaptic activation mediated by the ganglionic NO/NOS system, a mechanism described by Quinson et al. [46] in rabbit CG.

In summary, the present work demonstrates that the deprivation of NO in CG can modulate the neuronal activity with incidence on the oxidative status, the secretory activity of sexual steroids and the apoptotic mechanisms taking place in the ovary during the preovulatory follicles development. Our findings reveal new aspects of the relation between the sympathetic nervous system and the ovary, which might play a significant role in the development of various pathologies of the female reproductive system, including the polycystic ovary syndrome (PCOS), in which strong evidence of an increase of the ovarian sympathetic innervation is observed [57]. In fact, it has been reported that one of the important factors contributing to the PCOS is the oxidative stress in the ovarian follicles, and another study in human granulosa cells involved norepinephrine in the generation of ROS [57,58]; interestingly, the main neurotransmitter released into the ovary by cholinergic fibers.

It is evident that a better knowledge about the role of the neural factors involved in the ovarian physiology may facilitate the understanding of reproductive diseases.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect

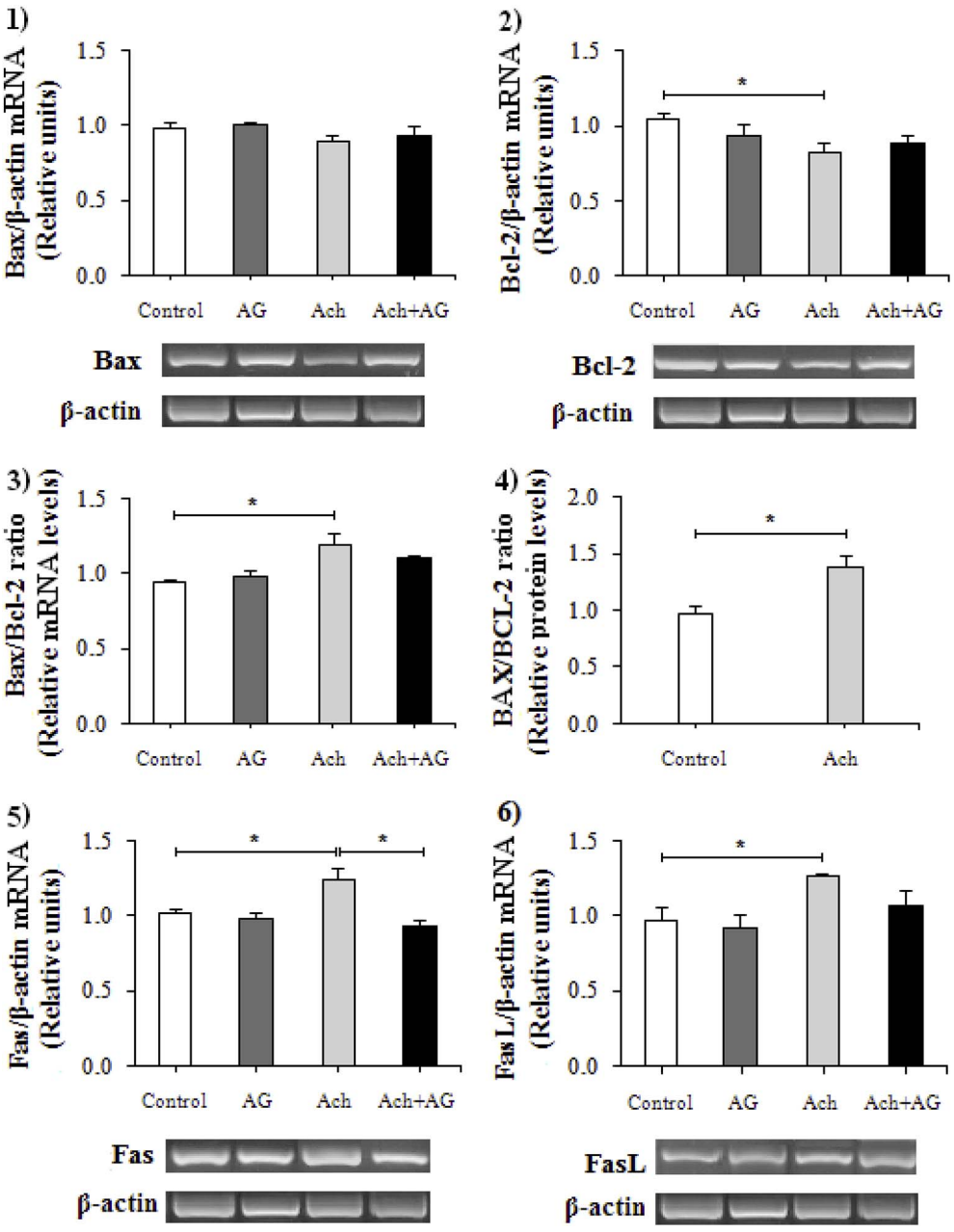

Figure F. Effect of ganglionic stimulation with 10^{-6} M Ach and with 400 μM AG, in the CG-SON-O system of rats in the first PE, on ovarian levels of: 1) Bax mRNA; 2) Bcl-2 mRNA (upper panel); 3) ratio of Bax/Bcl-2 mRNA expression, 4) ratio of BAX/BCL-2 protein expression (middle panel); 5) Fas and 6) FasL mRNA (lower panel). Densitometry analysis of the bands in the gel photographs was performed using the ImageJ software and expressed as arbitrary units. β-actin was used as the housekeeping gene. Results are expressed as mean ± S.E.M. (n = 3 pools of 2 ovaries/group). One way analysis of variance followed by Tukey's test was used. ELISA results (Figure F4) are expressed as mean ± SEM (n = 5). Unpaired Student's t-test was used. *p < 0.05; FasL: Fas ligand.

to the research, authorship, and/or publication of this article.

Acknowledgments

We thank Dr. Ana Maria Rastrilla for her comments and suggestions. This research was supported by Grant 2-2916 from Universidad Nacional de San Luis, Argentina. This research is part of the Ph.D. training of María Belén Delsouc.

Abbreviations

Ach	acetylcholine
AG	aminoguanidine
CAT	catalase
CG	coeliac ganglion
E_2	estradiol
FasL	Fas ligand
GSH-Px	glutathione peroxidase
iNOS	inducible nitric oxide synthase
NO	nitric oxide
P	progesterone
P450arom	P450 aromatase
RNS	reactive nitrogen species
ROS	reactive oxygen species
SON	superior ovarian nerve
TAC	total antioxidant capacity
TBARS	thiobarbituric acid reactive substances
3β-HSD	3β-hydroxysteroid dehydrogenase
20α-HSD	20α-hydroxysteroid dehydrogenase

References

[1] G.A. Dissen, A. Paredes, C. Romero, W. Lee Dees, S.R. Ojeda, Neural and neurotrophic control of ovarian development, in: P.C.K. Leung, E.Y. Adashi (Eds.), The Ovary, Elsevier Academic Press, San Diego, California, 2004, pp. 3–23.

[2] L.I. Aguado, Role of the central and peripheral nervous system in the ovarian function, Microsc. Res. Tech. 59 (2002) 462–473.

[3] S.M. Delgado, C.G. Escudero, M. Casais, M. Gordillo, A.C. Anzulovich, Z. Sosa, A.M. Rastrilla, Ovaric physiology in the first oestral cycle: influence of noradrenergic and cholinergic neural stimuli from coeliac ganglion, Steroids 75 (2010) 685–694.

[4] M.A. De Bortoli, M.H. Garraza, L.I. Aguado, Adrenergic intracerebroventricular stimulation affects progesterone concentration in the ovarian vein of the rat: participation of the superior ovarian nerve, J. Endocrinol. 159 (1998) 61–68.

[5] C.J. Dalsgaard, T. Höldelt, M. Schultzberg, M.J. Lundberg, L. Terenius, G.J. Dockray, Origin of the peptide-containing fibers in the inferior mesenteric ganglion of the Guinea pig: immunohistochemical studies with antisera to substance P, enkephalin, vasoactive intestinal polypeptide, cholecystokinin and bombesin, Neuroscience 9 (1983) 191–211.

[6] M.A. Morales, K. Holmberg, Z.Q. Xu, C. Cozzari, B.K. Hartman, P. Emson, M. Goldstein, L.G. Elfvin, T. Hökfelt, Localization of choline acetyltransferase in rat peripheral sympathetic neurons and coexistence with nitric oxide synthase and neuropeptides, Proc. Natl. Acad. Sci. U.S.A. 92 (25) (1995) 11819–11823.

[7] A. Del Signore, C. Gotti, A. Rizzo, M. Moretti, P. Paggi, Nicotinic acetylcholine receptor subtypes in the rat sympathetic ganglion: pharmacological characterization, subcellular distribution and effect of pre- and postganglionic nerve crush, J. Neuropathol. Exp. Neurol. 63 (2) (2004) 138–150.

[8] 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.

[9] N. Quinson, J.P. Miolan, J.P. Niel, Muscarinic receptor activation is a prerequisite for the endogenous release of nitric oxide modulating nicotinic transmission within the coeliac ganglion in the rabbit, Neuroscience 95 (4) (2000) 1129–1138.

[10] 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, Neurol. Endocrinol. Lett. 28 (2007) 39–44.

[11] Z. Sosa, S. Delgado, M. Casais, L. Aguado, A.M. Rastrilla, Release of ovarian progesterone during the rat oestrous cycle by ganglionic cholinergic influence. The role of norepinephrine, J. Steroid Biochem. Mol. Biol. 91 (2004) 179–184.

[12] M. Casais, S.M. Delgado, Z. Sosa, A.M. Rastrilla, Pregnancy in rats is modulated by ganglionic cholinergic action, Reproduction 131 (2006) 1151–1158.

[13] M.B. Delsouc, M.C. Della Vedova, D. Ramírez, A.C. Anzulovich, S.M. Delgado, M. Casais, Oxidative stress and altered steroidogenesis in the ovary by cholinergic stimulation of coeliac ganglion in the first proestrous in rats. Implication of nitric oxide, Nitric Oxide 53 (2016) 45–53.

[14] M.B. Delsouc, L.D. Morales, S.S. Vallcaneras, D. Bronzi, A.C. Anzulovich, S.M. Delgado, M. Casais, Participation of the extrinsic cholinergic innervation in the action of nitric oxide on the ovarian steroidogenesis in the first proestrous in rats, Gen. Comp. Endocrinol. 236 (2016) 54–62.

[15] T. Domoto, M. Teramoto, K. Tanigawa, K. Tamura, Y. Yasui, Origins of nerve fibers containing nitric oxide synthase in the rat celiac-superior mesenteric ganglion, Cell Tissue Res. 281 (2) (1995) 215–221.

[16] D.S. Bredt, Endogenous nitric oxide synthesis: biological functions and pathophysiology, Free Radic. Res. 31 (6) (1999) 577–596.

[17] H.D. Schultz, Nitric oxide regulation of autonomic function in heart failure, Curr. Heart Fail. Rep. 6 (2) (2009) 71–80.

[18] U. Förstermann, W.C. Sessa, Nitric oxide synthases: regulation and function, Eur. Heart J. 33 (7) (2012) 829–837.

[19] N.A. Simonian, J.T. Coyle, Oxidative stress in neurodegenerative diseases, Annu. Rev. Pharmacol. Toxicol. 36 (1996) 83–106.

[20] C. Napoli, G. Paolisso, A. Casamassimi, M. Al-Omran, M. Barbieri, L. Sommese, T. Infante, L.J. Ignarro, Effects of nitric oxide on cell proliferation: novel insights, J. Am. Coll. Cardiol. 62 (2) (2013) 89–95.

[21] J.K. Juranek, J.A. Wojtkiewicz, Origins and neurochemical complexity of preganglionic neurons supplying the superior cervical ganglion in the domestic pig, J. Mol. Neurosci. 55 (2) (2015) 297–304.

[22] H. Kim, C. Moon, M. Ahn, Y. Lee, S. Kim, T. Ha, Y. Jee, T. Shin, Expression of nitric oxide synthase isoforms in the porcine ovary during follicular development, J. Vet. Sci. 6 (2) (2005) 97–101.

[23] W. Zhang, Q.W. Wei, Z.C. Wang, W. Ding, W. Wang, F.X. Shi, Cell-specific expression and immunolocalization of nitric oxide synthase isoforms and the related nitric oxide/cyclic GMP signaling pathway in the ovaries of neonatal and immature rats, J. Zhejiang Univ. Sci. B 12 (2011) 55–64.

[24] T. Poole, UFAW Hand Book of the Care and Management of Laboratory Animals, seventh ed., Blackwell Publishing, Oxford, 1999.

[25] F. Egami, S. Taniguchi, Nitrite, in: H.U. Bergmeyr (Ed.), Methods of Enzymatic Analysis, second ed., Academic Press, New York, 1974, pp. 2260–2265.

[26] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radic. Biol. Med. 26 (9–10) (1999) 1231–1237.

[27] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1984) 121–126.

[28] L. Flohé, W. Gunzler, Assay of glutathione peroxidase, Methods Enzymol. 105 (1984) 114–121.

[29] C.C. Winterbourn, I.H. Buss, Protein carbonyl measurement by enzyme-linked immunosorbent assay, Methods Enzymol. 300 (1999) 106–111.

[30] H.H. Draper, M. Hadley, Malondialdehyde determination as index of lipid peroxidation, Methods Enzymol. 186 (1990) 421–431.

[31] L.E. Bussmann, R.P. Deis, Studies concerning the hormonal induction of lactogenesis by prostaglandin F 2 α in pregnant rats, J. Steroid Biochem. 11 (4) (1979) 1485–1489.

[32] M. Ricu, A. Paredes, M. Greiner, S.R. Ojeda, H.E. Lara, Functional development of the ovarian noradrenergic innervation, Endocrinology 149 (2008) 50–56.

[33] T.P. Misko, W.M. Moore, T.P. Kasten, G.A. Nickols, J.A. Corbett, R.G. Tilton, M.L. McDaniel, J.R. Williasom, M.G. Currie, Selective inhibition of the inducible nitric oxide synthase by aminoguanidine, Eur. J. Pharmacol. 233 (1993) 119–125.

[34] A.R. Farhad, S.M. Razavi, P.A. Nejad, The use of aminoguanidine, a selective inducible nitric oxide synthase inhibitor, to evaluate the role of nitric oxide on periapical healing, Dent. Res. J. 8 (4) (2011) 197–202.

[35] I. Dalle-Donne, G. Aldini, M. Carini, R. Colombo, R. Rossi, A. Milzani, Protein carbonylation, cellular dysfunction, and disease progression, J. Cell Mol. Med. 10 (2) (2006) 389–406.

[36] M. Laloraya, P.G. Kumar, M.M. Laloraya, Changes in the levels of superoxide anion radical and superoxide dismutase during de estrous cycle of *Rattus norvegicus* and induction of superoxide dismutase in rat ovary by lutropin, Biochem. Biophys. Res. Commun. 157 (1988) 146–153.

[37] K. Shkolnik, A. Tadmor, S. Ben-Dor, N. Nevo, D. Galiani, N. Dekel, Reactive oxygen species are indispensable in ovulation, Proc. Natl. Acad. Sci. U.S.A. 108 (4) (2011) 1462–1467.

[38] A. Faletti, S. Pérez Martínez, C. Perotti, M.A. de Gimeno, Activity of ovarian nitric oxide synthase (NOs) during ovulatory process in the rat: relationship with prostaglandins (PGs) production, Nitric Oxide 3 (4) (1999) 340–347.

[39] N. Bonello, K. McKie, M. Jasper, L. Andrew, N. Ross, E. Braybon, M. Brännström, R.J. Norman, Inhibition of Nitric Oxide: effects on interleukin-lβ-enhanced ovulation rate, steroid hormones, and ovarian leukocyte distribution at ovulation in the rat, Biol. Reprod. 54 (2) (1996) 436–445.

[40] A.E. Drummond, The role of steroids in follicular growth, Reprod. Biol. Endocrinol. 4 (2006) 16.

[41] U. Gerdes, M. Gåfvels, A. Bergh, S. Cajander, Localized increases in ovarian vascular permeability and leucocyte accumulation after induced ovulation in rabbits, J. Reprod. Fertil. 95 (2) (1992) 539–550.

[42] L. Shukovsla, A. Tsafrin, The involvement of NO in the ovulatory process in the rat, Endocrinology 135 (1994) 2287–2290.

[43] G. Maillet, A. Benhaïm, H. Mittre, C. Féral, Involvement of theca cells and steroids in the regulation of granulosa cell apoptosis in rabbit preovulatory follicles, Reproduction 125 (5) (2003) 709–716.

[44] A. Navarro, R. Torrejon, Role of nitric oxide on mitochondrial biogenesis during the ovarian cycle, Front. Biosci. 12 (2007) 1164–1173.

[45] E. Birben, U.M. Sahiner, C. Sackesen, S. Erzurum, O. Kalayci, Oxidative stress and antioxidant defense, World Allergy Organ. J. 5 (2012) 9–19.

[46] N. Quinson, D. Catalin, J.P. Miolan, J.P. Niel, Nerve-induced release of nitric oxide exerts dual effects on nicotinic transmission within the coeliac ganglion in the rabbit, Neuroscience 84 (1998) 229–240.

[47] J. Jordan, J. Tank, M. Stoffels, G. Franke, N.J. Christensen, F.C. Luft, M. Boschmann, Interaction between β-adrenergic receptor stimulation and nitric oxide release on tissue perfusion and metabolism 1, J. Clin. Endocrinol. Metab. 86 (2001) 2803–2810.

[48] H. Prast, A. Philippu, Nitric oxide as modulator of neuronal function, Prog. Neurobiol. 64 (2001) 51–68.

[49] A. Agarwal, S. Gupta, R.K. Sharma, Role of oxidative stress in female reproduction, Reprod. Biol. Endocrinol. 3 (2005) 28.

[50] D. Singh, R.S. Pandey, Changes in catalase activity and hydrogen peroxide level in rat ovary during estrous cycle and induction of catalase in rat ovary by estradiol-17 beta, Indian J. Exp. Biol. 36 (4) (1998) 421–423.

[51] L. Góth, The hydrogen peroxide paradox, Orv. Hetil. 147 (19) (2006) 887–893.

[52] E. Cisneros Prego, J. Pupo Balboa, E. Céspedes Miranda, Enzimas que participan como barreras fisiológicas para eliminar los radicales libres: III. Glutation peroxidasa, Rev. Cubana Invest. Bioméd. 16 (1997) 10–15.

[53] H.R. Behrman, P.H. Kodaman, S.L. Preston, S. Gao, Oxidative stress and the ovary, J. Soc. Gynecol. Invest. 8 (2001) S40–S42.

[54] D.G. Stein, Progesterone exerts neuroprotective effects after brain injury, Brain Res. Rev. 57 (2) (2008) 386–397.

[55] K.M. Webster, D.K. Wright, M. Sun, B.D. Semple, E. Ozturk, D.G. Stein, T.J. O'Brien, S.R. Shultz, Progesterone treatment reduces neuroinflammation, oxidative stress and brain damage and improves long-term outcomes in a rat model of repeated mild traumatic brain injury, J. Neuroinflammation 12 (2015) 238.

[56] J.J. Peluso, A. Pappalardo, R. Losel, M. Wehling, Progesterone membrane receptor component 1 expression in the immature rat ovary and its role in mediating progesterone's antiapoptotic action, Endocrinology 147 (6) (2006) 3133–3140.

[57] S. Saller, J. Merz-Lange, S. Raffael, S. Hecht, R. Pavlik, C. Thaler, D. Berg, U. Berg, L. Kunz, A. Mayerhofer, Norepinephrine, active norepinephrine transporter, and norepinephrine-metabolism are involved in the generation of reactive oxygen species in human ovarian granulosa cells, Endocrinology 153 (3) (2012) 1472–1483.

[58] S. Saller, L. Kunz, D. Berg, U. Berg, H. Lara, J. Urra, S. Hecht, R. Pavlik, C.J. Thaler, A. Mayerhofer, Dopamine in human follicular fluid is associated with cellular uptake and metabolism-dependent generation of reactive oxygen species in granulosa cells: implications for physiology and pathology, Hum. Reprod. 29 (3) (2014) 555–567.