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Oxidative stress and altered steroidogenesis in the ovary by cholinergic stimulation of coeliac ganglion in the first proestrous in rats. Implication of nitric oxide

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ABSTRACT

An *ex-vivo* Coeliac Ganglion-Superior Ovarian Nerve-Ovary (CG–SON–O) system from virgin rats in the first proestrous was used to test whether cholinergic stimulation of CG affects oxidative status and steroidogenesis in the ovary. The CG and the O were placed in separate buffered-compartments, connected by the SON, and the CG was stimulated by acetylcholine (Ach). To test a possible role of nitric oxide (NO) in the ovarian response to cholinergic stimulation of CG, aminoguanidine (AG) – an inhibitor of inducible-NO synthase was added to the O compartment. After 180 min incubation, the oxidative status was assessed in O whereas nitrite and steroidogenesis were assessed at 30, 120 and 180 min. Ach in CG decreased the total antioxidant capacity, but increased NO production and protein carbonization in O. Ach stimulation of CG increased estradiol, but decreased progesterone release in O by reducing the mRNAs related to their synthesis and degradation. The addition of AG to the O compartment caused an opposite effect, which was more pronounced in the presence of Ach in the CG compartment than in its absence. These results show that the stimulation of the extrinsic-cholinergic innervation of the O increases the concentration of NO, causes oxidative stress and modulates steroidogenesis in the first rat proestrous.

1. Introduction

Sympathetic ganglia are aggregates of neurons that innervate several target organs. One example is the coeliac ganglion (CG) that possesses a great variety of specific receptors and neurotransmitters such as catecholamines, acetylcholine (Ach), neuropeptides and nitric oxide (NO) [1–3]. This ganglion constitutes a control center in the pathway of the afferent and efferent fibers between the central nervous system and the ovary [4]. The sympathetic neurons in the CG project their axons through the superior ovarian nerve (SON) to innervate different structural components in the ovary [5,6] including the ovarian theca, which has an important endocrine function [7]. Using the *ex vivo* CG–SON–O system, it has been reported that cholinergic agents in CG modify, through the SON, the release of ovarian steroid hormones in prepubertal [8], pubertal [9], adult [10] and pregnant [11] rats. Delgado et al. [9], using the CG–SON–O system of rats in different stages of the first estral cycle, concluded that this response is related to the specific ovarian structures present in every stage and to their secretion products, on which the NO acts as a modulator. In addition, these authors emphasized that the cholinergic stimulation of the CG caused the highest response on the ovarian release of progesterone (P) and NO during the first proestrous. This pattern exhibited a neurohormonal microenvironment closely similar to the prepubertal stage [9].

Much is known about the mechanism of NO synthesis from

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biochemical studies of the purified NO synthase enzymes (NOS). These enzymes are found in two constitutive isoforms, neuronal (nNOS) and endothelial (eNOS), and one inducible isoform, (iNOS), which is usually expressed in numerous cell types in response to immune or inflammatory stimuli. All three isoforms generate NO from the oxidation of L-arginine, but they have distinctive tissue distribution, functional and structural features [12,13].

When adding selective inhibitors of each isoform of the NOS in the CG and ovary in the CG–SON–O system of prepubertal rats, iNOS, the enzyme responsible for the elevated synthesis of NO [14], was shown to be the isoform with the highest participation in both structures [15]. NO has been involved in various aspects of the ovarian physiology including steroidogenesis, follicular development and ovulation [16]. Studies on rats have demonstrated that the follicular development requires the synthesis of NO [17,18] to enhance the local blood flow and locally increase the concentration of ovulation-inducing factors.

Ovulation, a critical ovarian process, has been compared to an inflammatory reaction [19]. After the pre-ovulatory peak of gonadotropins, inflammatory cells, including macrophages and neutrophils, and vascular endothelial cells residing in the ovarian follicles, produce NO and reactive oxygen species (ROS), such as the radical superoxide anion ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH^-$) [20]. The production of these ROS may be modulated in turn by the ovarian steroid hormones [21–23]. The high levels of ROS and the low levels of antioxidants favor the ovulatory process under determined circumstances; however, the overproduction of ROS (critical oxidative stress) can damage ovarian cells [24–27]. It has been reported, mainly in rodents, that oxidative damage of oocytes may reduce their chance of fertilization due, for example, to mitochondrial damage [28], inhibition of steroidogenic enzymes [29], damage to other proteins involved in the intracellular traffic of cholesterol [30,31], damage of lipids of the cellular membrane, and DNA damage which may lead to cell death by apoptosis or necrosis [32–35]. In turn, these oxidative damages may be worsen by the action of the reactive nitrogen species (RNS), such as the peroxynitrite (ONOO–), a potent oxidant derived from the reaction between NO and $^{\bullet}O_2^-$ [36,37]. The antioxidant enzymes, such as catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD), and the non-enzymatic antioxidants, such as vitamin E, vitamin C and albumin, present in the ovarian follicles [38–40], help to avoid oxidative damage.

A reduction in the antioxidant enzymes levels has been reported in the follicular fluids of women with unexplained infertility [41]. This study suggests that the oxidative stress, a state characterized by an imbalance between the pro-oxidant production and antioxidant defenses, plays a key role in the pathophysiology of infertility in females. However, the mechanisms involved are still unclear.

In the present work, we aim at using the *ex-vivo* CG–SON–O system to investigate how cholinergic stimulation of the CG affects oxidative status, NO production and steroidogenesis in the ovary of the first rat proestrous.

2. Materials and methods

2.1. Animals

Female 37-day-old virgin Holtzman rats in their first proestrous and weighing 100 ± 10 g were used in all the experiments. The rats were kept under controlled conditions with lights on from 07:00 to 19:00 h and at a temperature of 24 ± 2 °C. Animals had free access to food (Cargill 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 [42]. 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).

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). The Estradiol (E_2) DIASource ImmnoAssays kit was purchased from DiagnosMed SRL (Buenos Aires, Argentina). 1,2,6,7-[^{3}H]-Progesterone (107.0 Ci/mmol) was provided by New England Nuclear Products (Boston, MA, USA). Other reagents and chemicals were of analytical grade.

2.3. Experimental procedure

The surgical procedures for removing the *ex-vivo* CG–SON–O system from rats were performed as previously described [8,9]. Briefly, a piece of tissue containing the left O, the fibers constituting the SON and the CG was removed. We worked with the left CG–SON–O system because Vega Orozco et al. [43] previously showed that the left ovary is more sensitive to sympathetic stimuli than the right ovary in every estrous stage. Similarly, Morán et al. [44] found an apparent asymmetry in the behavior and activity of the neural connections between the ovaries and the prevertebral coeliac-mesenteric ganglia. In order to prevent spontaneous depolarization of nerve fibers, the strip of tissues was carefully dissected avoiding contact between the surgical instruments and the nerve or the ganglion, and the total surgical procedure was completed within 1–2 min. The CG–SON–O system was rinsed with an incubation medium and immediately placed in a cuvette with two compartments, one for the CG and the other for the O, 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 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, the Krebs–Ringer solution (1 ml) was changed in both compartments and 0.1 mg/ml ascorbic acid was added as an antioxidant agent in the CG compartment.

To stimulate the CG, Ach was dissolved in 1 ml of Krebs–Ringer solution plus ascorbic acid at a 10^{-6} M final concentration in the CG compartment. In addition, to test the role of NO synthesis in ovary, AG was dissolved in 1 ml of Krebs–Ringer solution at a 400 μM final concentration in the O compartment with or without 10^{-6} M Ach in the CG compartment. The control groups consisted of untreated CG–SON–O systems.

The incubation was performed during 180 min. At 30, 120 and 180 min, aliquots (250 μl) of the incubation medium in the O compartment were removed and frozen at −20 °C for further analysis. At the end of the incubation (180 min), the ovary was collected and frozen at −80 °C for further analysis.

2.4. Preparation of tissue homogenate

Six ovaries per experimental group were homogenized separately in 150 μl RIPA buffer (Thermo Fisher Scientific Inc, 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 total antioxidant capacity (TAC) and activity of antioxidant enzymes (CAT and GSH-Px). The concentration of protein carbonyls and TBARS were measured as markers of oxidative damage of proteins and lipids, respectively. The total protein concentration in tissue homogenates was measured by the Bradford method [45].

2.5. 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 [46]. 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: $\mathbf{\%\ inhibition = [(A_0 - A_f)/A_0] \times 100}$, 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.

2.6. Antioxidant enzymes activity

The CAT and GSH-Px specific enzymatic activities were determined following Aebi's [47] and Flohe and Gunzler's [48] 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 prot).

2.7. Measurement of protein oxidation

As a marker of protein oxidation, protein carbonyls were determined as previously reported using an Enzymo-linked immune-sorbent assay (ELISA) [49], 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) and incubated overnight at 4 °C. After washing with 0.05% Tween 20 in PBS and blocking with 2.5% cold-water fish skin gelatin (Sigma) in PBS at 37 °C for 1 h, the microplates were incubated for 1 h at 37 °C with the rabbit polyclonal anti-dinitrophenyl antibody (1:2000 dilution in washing buffer). The immunocomplexes were quantified using a goat anti-rabbit IgG-HRP conjugate (1/10,000 in washing buffer), and the oxidation of the HRP substrate 3,3′,5,5′-Tetramethylbenzidine (TMB) was read at 450 nm using a TECAN microplate reader (Infinite M200 PRO, Research Triangle Park, NC). The results were expressed as nmol of carbonyl per milligram of total proteins (nmol/mg prot).

2.8. Measurement of lipid peroxidation

Lipid peroxidation was measured spectrophotometrically by determining MDA concentration as thiobarbituric acid reactive substances (TBARS) at 535 nm [50]. The results were expressed as μmol of MDA per milligram of total proteins (μmol MDA/mg prot).

2.9. Nitrite assay

The concentration of nitrite in the incubation medium from the O compartment was measured spectrophotometrically [51]. Briefly, 50 μl of sample were 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). Appropriate corrections were made in all cases according to the volume of liquid extracted in each tested period from the O compartment.

2.10. Steroids assay

Steroids were measured in the ovarian incubation liquid per duplicate by radioimmunoassay (RIA). The estradiol concentration was determined using a double antibody RIA Diasource kit (DiagnosMed SRL) 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 progesterone. The assay sensitivity was <2.2 pg estradiol/ml. The inter- and intra-assay coefficients of variation in all the assays were <10.0%. Estradiol was expressed as picograms per milligram of ovarian tissue (pg/mg ovary). The progesterone antiserum, provided by Dr R. Deis (IMBECU, Mendoza, Argentina), was produced in rabbits against progesterone conjugated to bovine serum albumin at the 11 position. The antiserum was highly specific for progesterone with low cross-reactivities, <2.0% for 20α-dihidro-progesterone and deoxycorticosterone 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 [52]. Progesterone concentration was expressed as nanogram per milligram of ovarian tissue (ng/mg ovary). Appropriate corrections were made in all cases according to the volume of liquid extracted in each tested period from the O compartment.

2.11. RNA isolation and reverse transcriptase reaction

Total RNA was extracted from three pools of two ovaries each, per experimental group. Every RNA isolation was performed using Trizol reagent (Invitrogen, Carlsbad, CA, USA) as suggested by the manufacturers. Gel electrophoresis and GelRed™ nucleic acid gel stain (Biotium Inc., San Francisco, CA, USA) confirmed the integrity of the samples. The concentration of RNA was assessed based on its absorbance 260 nm. 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 by following the manufacturer's instructions.

2.12. Polymerase chain reaction amplification

Transcript concentration of iNOS, P450 aromatase (P450arom, E_2 synthesis enzyme), 3β-hydroxysteroid dehydrogenase (3β-HSD, P synthesis enzyme) and 20α-hydroxysteroid dehydrogenase (20α-

HSD, P degrading enzyme) were determined by reverse transcriptase polymerase chain reaction (RT-PCR) and normalized to β-actin as housekeeping gene. Fragments coding for these genes were amplified by PCR in a 50 μl reaction-solution mix containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.25 IU of Taq polymerase, and 50 pmol of each rat specific oligonucleotide primer and RT-generated cDNA (1/10 of RT reaction). Nucleotide sequences of the specific primers are shown in Table 1.

cDNA amplification was performed using a thermocycler (MyCycler; BioRad Laboratories, Inc, Hercules, California). 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 signal intensity of target genes and that for the housekeeping gene β-actin.

2.13. Statistical analysis

All data are shown as means ± S.E.M. in each group. 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 utilized. A difference was considered to be statistically significant when $p < 0.05$.

3. Results

3.1. Effect of ganglionic cholinergic stimulation with 10^{-6} M Ach and ovary treatment with 400 μM AG on the ovarian oxidative status in the ex vivo CG–SON–O system, in the first proestrous day

3.1.1. Total antioxidant capacity

The addition of 10^{-6} M Ach into the CG compartment of the *ex vivo* system decreased the TAC in ovary (*$p < 0.05$), as compared to the control group (Fig. 1. 1). Conversely, the addition of 400 μM AG into the O compartment of the *ex vivo* system increased the TAC in ovary (*$p < 0.05$) also as compared to the control group (Fig. 1. 1).

In addition, the joint effect of 10^{-6} M Ach into the CG compartment and 400 μM AG into the O compartment increased the TAC in ovary (•$p < 0.01$), as compared to the Ach group (Fig. 1. 1).

3.1.2. Enzymatic activities of CAT and GSH-Px

No significant effect was observed in the CAT activity after the cholinergic stimulation of the CG and/or ovarian treatment with AG (Fig. 1. 2).

The addition of 10^{-6} M Ach into the CG compartment of the *ex vivo* system increased the GSH-Px activity in ovary (▪$p < 0.001$), as compared to the control group (Fig. 1. 3). However, no significant effects were observed on GSH-Px activity after the ovarian stimulation with 400 μM AG as compared to the control group, or the joint addition of 10^{-6} M Ach into the CG compartment and 400 μM AG into the O compartment as compared to the Ach group (Fig. 1. 3).

3.1.3. Protein oxidation

The addition of 10^{-6} M Ach into the CG compartment of the *ex vivo* system increased the carbonyl formation in ovary (▪$p < 0.001$), as compared to the control group (Fig. 1. 4). No significant effect was observed on carbonyl formation when 400 μM AG was added to the O compartment, as compared to the control group.

Adding 10^{-6} M Ach into the CG compartment and 400 μM AG into the O compartment decreased the carbonyl formation in ovary (▪$p < 0.001$), as compared to the Ach group (Fig. 1. 4).

3.1.4. Lipid peroxidation

No significant modification in TBARS concentration in ovary was observed after the cholinergic stimulation of the CG and/or ovarian treatment with AG (Fig. 1. 5).

3.2. Effect of ganglionic cholinergic stimulation with 10^{-6} M Ach and ovary treatment with 400 μM AG on the ovarian release of nitrites and iNOS mRNA expression in the ex vivo CG–SON–O system, in the first proestrous day

The addition of 10^{-6} M Ach into the CG compartment of the *ex vivo* system increased the ovarian release of nitrites at 30, 120 and 180 min (▪$p < 0.001$), without showing significant changes in the iNOS mRNA expression in the ovary tissue at 180 min, as compared to the control group (Fig. 2. 1 and 2).

The addition of 400 μM AG into the O compartment of the *ex vivo* system decreased the ovarian release of nitrites at 30 (▪$p < 0.001$), 120 (•$p < 0.01$) and 180 min (▪$p < 0.001$) as well as iNOS mRNA expression at 180 min (*$p < 0.05$), as compared to the control group (Fig. 2. 1 and 2).

The addition of 10^{-6} M Ach into the CG compartment and 400 μM AG into the O compartment decreased the ovarian release of nitrites at 30 (▪$p < 0.001$), 120 (•$p < 0.01$) and 180 min (▪$p < 0.001$) as well as iNOS mRNA expression in the ovary tissue at 180 min (*$p < 0.05$), as compared to the Ach group (Fig. 2. 1 and 2).

3.3. Effect of ganglionic cholinergic stimulation with 10^{-6} M Ach and ovary treatment with 400 μM AG on the synthesis and release of ovarian steroids in the ex vivo CG–SON–O system, in the first proestrous day

3.3.1. E_2 release and P450arom mRNA expression

The addition of 10^{-6} M Ach into the CG compartment of the *ex vivo* system increased E_2 ovarian release of at 30, 120 and 180 min (▪$p < 0.001$) and the P450arom mRNA expression in the ovary tissue at 180 min (*$p < 0.05$), as compared to the control group (Fig. 3. 1 and 3).

No effects were observed on the ovarian release of E_2 and the P450arom mRNA expression in the ovary tissue when AG was added into the O compartment or when Ach was added into the CG compartment and AG was added into the O compartment (Fig. 3. 1 and 3).

Table 1
Primer pairs used for RT-PCR.

Gene name	Forward primer 5′-**3**′	Reverse primer 5′-**3**′	Fragment size
iNOS	GCATGGACCAGTATAAGGCAAGCA	GCTTCTGGTCGATGTCATGAGCAA	219
3β-HSD	GTCTTCAGACCAGAAACCAAG	CCTTAAGGCACAAGTATGCAG	447
20α-HSD	TTCGAGCAGAACTCATGGCTA	CAACCAGGTAGAATGCCATCT	440
P450arom	TGCACAGGCTCGAGTATTTCC	ATTTCCACAATGGGGCTGTCC	266
β-actin	CGGAACCGCTCATTGCC	ACCCACACTGTGCCCATCTA	289

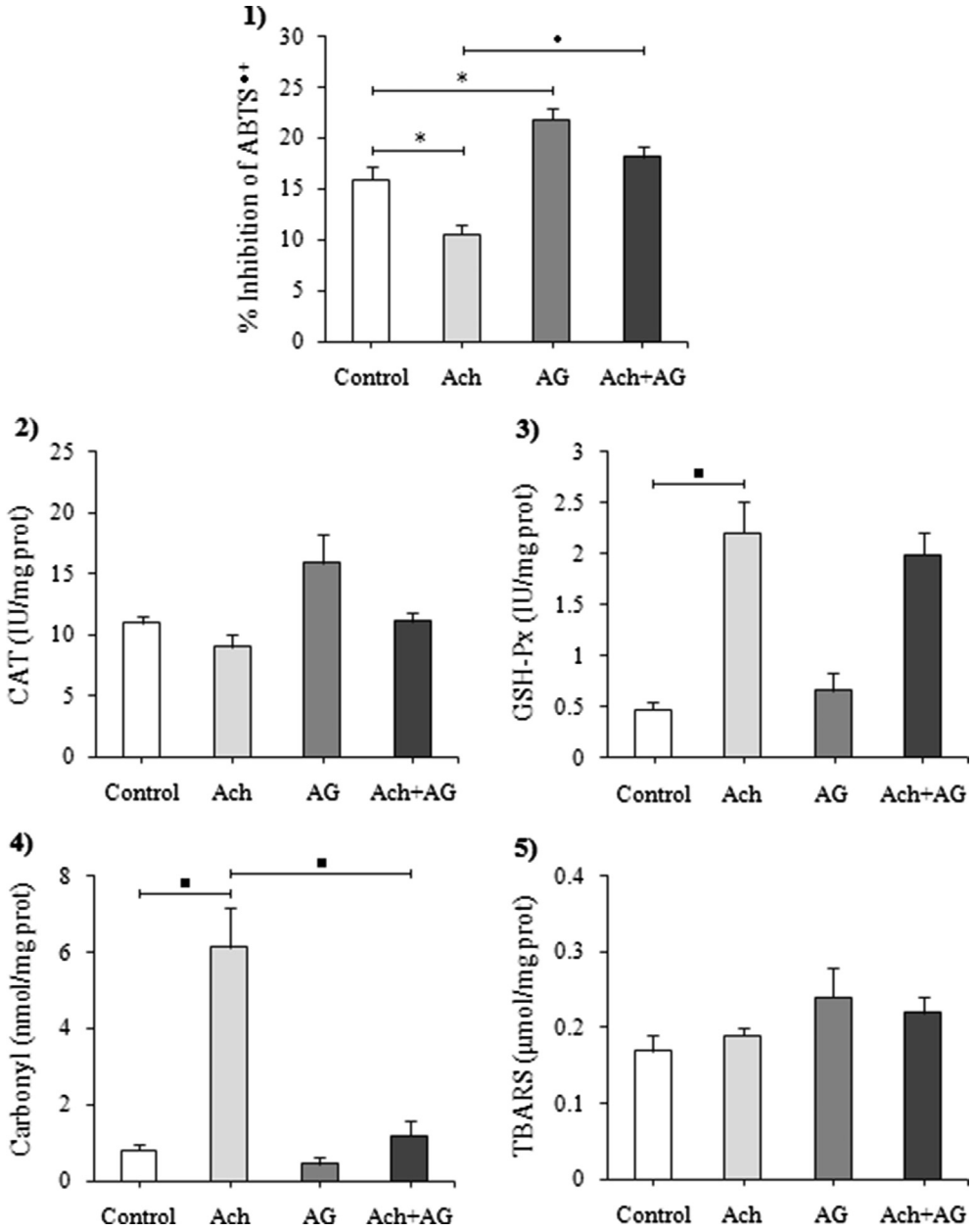

Fig. 1. Effect of 10^{-6} M Ach ganglionic stimulation and 400 µM AG ovarian stimulation in the *ex vivo* CG–SON–O system in the first proestrous in rats, on: **1)** total antioxidant capacity, measured as percent bleaching of the $ABTS^{\bullet+}$; **2)** CAT activity, expressed in IU/mg prot; **3)** GSH-Px activity, expressed in IU/mg prot; **4)** protein carbonyl content, expressed as nmol/mg prot; **5)** TBARS content, expressed as µmol/mg prot. 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; Ach: acetylcholine; AG: aminoguanidine; $ABTS^{\bullet+}$: 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; CAT: catalase; GSH-Px: glutathione peroxidase; TBARS: thiobarbituric acid reactive substances.

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

The addition of 10^{-6} M Ach into the CG compartment of the *ex vivo* system decreased P ovarian release at 30, 120 and 180 min (▪p < 0.001) and the 3β-HSD mRNA expression in the ovary tissue at 180 min (•p < 0.01), whereas it increased the 20α-HSD mRNA expression at 180 min (*p < 0.05), as compared to the control group (Fig. 3. 2, 4 and 5). Conversely, the addition of 400 µM AG into the O compartment of the *ex vivo* system increased P ovarian release at 30 (*p < 0.05), 120 (▪p < 0.001) and 180 min (*p < 0.05) and decreased the 20α-HSD mRNA expression in the ovary tissue at 180 min (•p < 0.01), as compared to the control group (Fig. 3. 2, 4 and 5).

The joint effect of adding 10^{-6} M Ach into the CG compartment and 400 µM AG into the O compartment increased P ovarian release at 30 (*p < 0.05), 120 (*p < 0.05) and 180 min (•p < 0.01) and decreased the 20α-HSD mRNA expression in the ovary tissue at 180 min (•p < 0.01), as compared to the Ach group (Fig. 3. 2, 4 and 5).

4. Discussion

A number of recent reports highlighted a functional neuronal connection between the peripheral nervous system and the modulation of the ovary's physiology [8,11,53–55]. However, there is little information about the effect of the ovarian innervation in rats during the first estral cycle [9].

In the present work, we focus on the first proestrous since

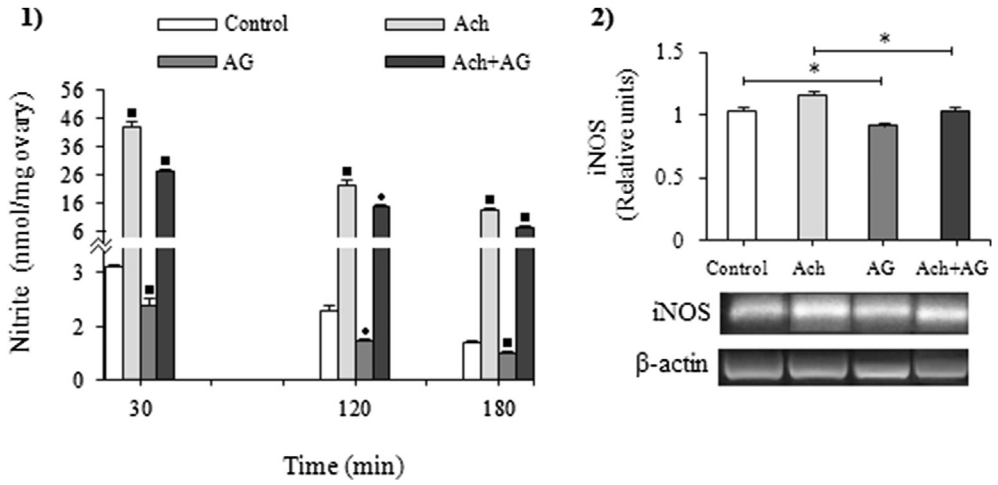

Fig. 2. Effect of 10^{-6} M Ach ganglionic stimulation and 400 μM AG ovarian stimulation in the *ex vivo* CG–SON–O system in the first proestrous in rats, on: **1)** ovarian nitrites release, expressed as nmol/mg ovary. Results are expressed as mean ± S.E.M. (n = 6). Repeated measures analysis of variance followed by Tukey's test was used; **2)** ovarian mRNA expression of enzyme iNOS. 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). One way analysis of variance followed by Tukey's test was used to compare between groups. *p < 0.05; •p < 0.01; ▪p < 0.001; Ach: acetylcholine; AG: aminoguanidine; iNOS: inducible nitric oxide syntase.

during this stage the extrinsic ovarian innervation reaches biochemical and functional maturity [56], thus favoring the development of the follicles which will give place to the first ovulation.

The CG–SON–O system allows simulating *in vivo* conditions using an *ex-vivo* model, thus preserving the paracrine and autocrine regulation mechanisms which take place in the ovary, without any humoral influence [53]. The use of this system showed that cholinergic stimulation of the CG modifies, throughout the SON, the steroids secretory activity and NO production in the ovary through the SON [8,9,15].

Among the various signaling molecules induced during different ovarian processes, NO and other reactive species have been considered as modulators of steroidogenesis, consequently affecting ovulation in mammals [57,58].

We aimed at studying whether the cholinergic stimulation of the CG modulates the ovary oxidative status affecting steroidogenesis in the first proestrous in rats. To this end, we analyzed possible modifications in the TAC, in the activity of the antioxidant enzymes (CAT and GSH-Px), and in the markers of oxidative stress (protein carbonyls and TBARS), in the rat ovary. In addition, we analyzed possible modifications in the ovarian production and release of NO, E_2 and P in the incubation medium as well as changes in the iNOS, P450arom, 3β-HSD and 20α-HSD mRNA expression.

Ach was used as a cholinergic agonist because it is considered to be a typical preganglionic neurotransmitter of the sympathetic ganglionic pathway [59,60].

The results on nitrites, E_2 and P release in the O compartment by ganglionic stimulation with 10^{-6} M Ach for 120 min of incubation were previously published by our research group [9]. In the present work, ganglionic stimulation with 10^{-6} M Ach was performed for 180 min of incubation, simultaneously with the other experimental groups, thus strengthening the results previously mentioned.

CG stimulation with Ach decreased the TAC, but increased the GSH-Px activity in the ovary. This effect may be due to a compensatory antioxidant effect in the ovary to challenge oxidant production caused by cholinergic stimulation of the CG.

It has been shown that a temporary increase of the ROS production and, thus a decrease of antioxidants after the gonadotropins pre-ovulatory peak triggers the ovulation [27]. In addition, NO plays an important role in vasodilatation during the pre-ovulatory period in rats by increasing blood flow, blood volume, and plasma exudation, which enhances follicle breakdown and subsequent ovulation [61]. Ganglionic cholinergic stimulation may increase ROS and NO synthesis in the ovary, thus favoring the ovulatory process. However, NO in high levels reacts with ROS to produce peroxynitrite, thus promoting macromolecule oxidation. CG stimulation increased protein oxidation in the ovary tissue accompanied by altered steroidogenesis. Consequently, our data emphasizes the importance of keeping a delicate balance between pro-oxidant molecules and antioxidant defenses because the oxidative stress may affect ovulation [58].

In relation to steroidogenesis, CG stimulation with Ach increased the release of E_2, but decreased the ovarian release of P. These results are in agreement with those results previously reported by Delgado et al. [9]. The increase in the E_2 production, caused by stimulation of the CG with Ach in the CG–SON–O system, might be related to the increased NO production, since both increase the vasodilation and blood flow during the follicular development [43,62].

In addition, it has been reported that P release was also inhibited by stimulation of the CG with Ach in prepubertal rats using the same system [8] and that NO inhibits the P production [63,64]. Thus these studies are in agreement with our results, indicating that the neurohormonal microenvironment in the ovary in the first proestrous exhibits similarities with the prepuberal stage [9].

The effects caused by the ganglionic cholinergic stimulus on the ovarian steroidogenesis might be due to an increase in the P450arom and of 20α-HSD expression and a decrease in the of 3β-HSD mRNA production.

The generation of ROS by the P450 cytochrome is important during the metabolic process of steroid-hormones synthesis from cholesterol in endocrine organs, such as the ovary [58]. However, although the cholinergic agonist decreased TAC and increased the expression of P450arom, no oxidative damage was observed in the lipids.

In addition, iNOS has been widely studied in reproductive organs [58] since the ovulation process has been compared with an inflammatory reaction [19], and this isoform produces high quantities of NO in response to inflammatory stimuli [65].

After investigating the ganglionic cholinergic influence on the oxidative status and the steroidogenesis in ovary, we analyzed iNOS

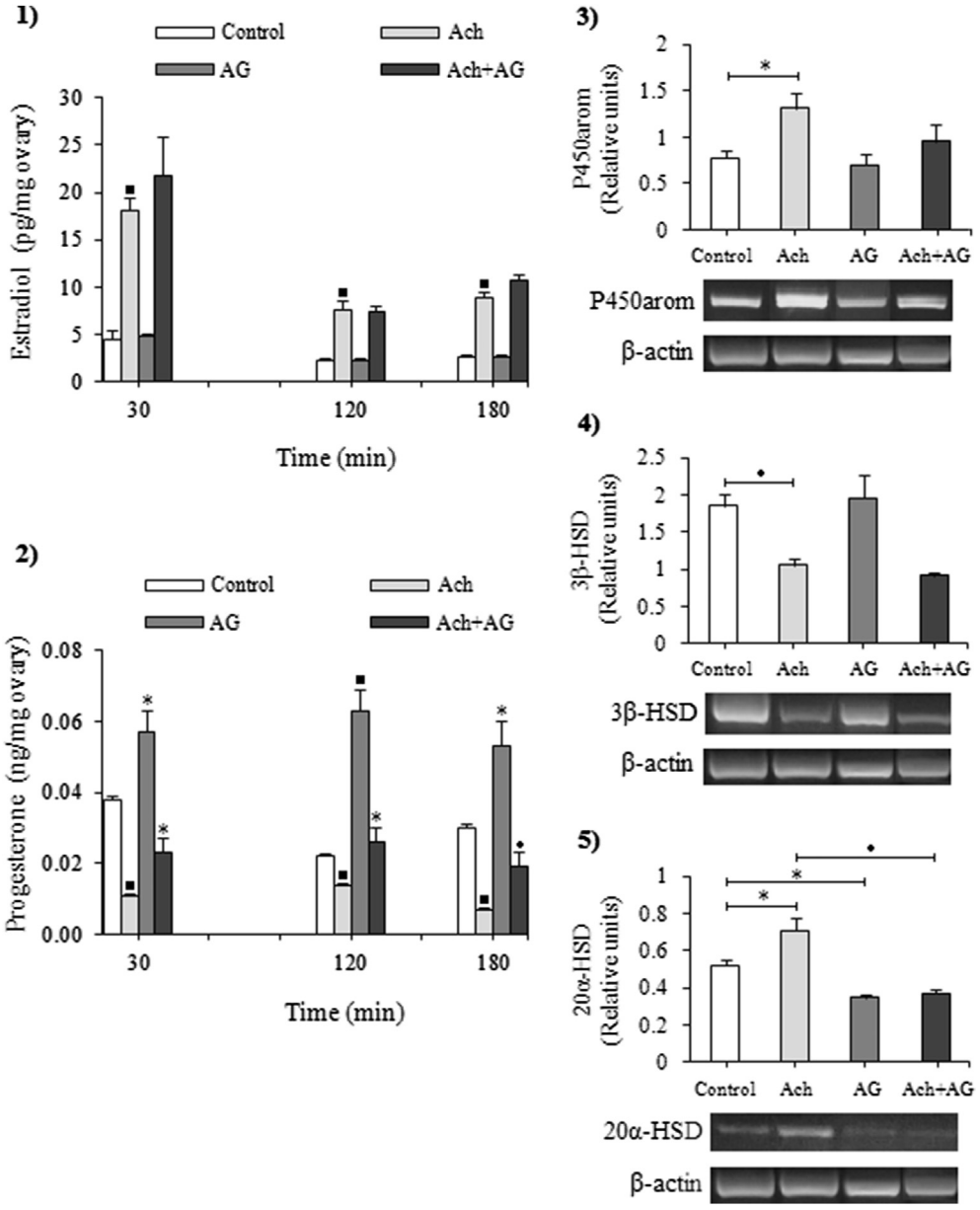

Fig. 3. Effect of 10^{-6} M Ach ganglionic stimulation and 400 μM AG ovarian stimulation in the *ex vivo* CG–SON–O system in the first proestrous in rats, on: **1)** ovarian E_2 release, expressed as pg/mg ovary; **2)** ovarian P release, expressed as ng/mg ovary. Results are expressed as mean ± S.E.M. (n = 6). Repeated measures analysis of variance followed by Tukey's test was used; **3)** ovarian mRNA expression of enzyme P450arom; **4)** ovarian mRNA expression of enzyme 3β-HSD; **5)** ovarian mRNA expression of enzyme 20α-HSD. 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). One way analysis of variance followed by Tukey's test was used to compare between groups. *$p < 0.05$; •$p < 0.01$; ▪$p < 0.001$; Ach: acetylcholine; AG: aminoguanidine; E_2: estradiol; P: progesterone; P450arom: P450 aromatase; 3β-HSD: 3β-hydroxysteroid dehydrogenase; 20α-HSD: 20α-hydroxysteroid dehydrogenase.

participation in these processes. To this end, since it has been demonstrated that AG is an effective and fairly selective iNOS's inhibitor in animal models [15,66,67], we added this compound into the O compartment in order to investigate the role of NO in the oxidative status and steroidogenesis in the ovary caused by cholinergic stimulation of the CG.

In the present work, we added 400 μM AG because this is the lowest concentration needed to inhibit NO production in the ovary of Holtzman rats [15]. Moreover, the addition of AG into the O compartment and Ach in the CG compartment counteracted in part the effects on ovary caused by stimulation of the CG with Ach in the CG–SON–O system. AG decreased the expression of iNOS, NO release and carbonyls content, whereas TAC was increased. AG may have pro- and anti-oxidant effects, depending on the concentration added [68]. Previous studies have reported that 400 μM AG cause antioxidant effects diminishing ROS production, enhancing anti-oxidant–enzyme activity and preventing cellular damage [69–71]. These antioxidant properties of AG, in association with its inhibitory effect on NO production, may explain our data showing the protective effects of AG against oxidative stress and altered metabolism of steroid-hormones caused in the ovary when the CG is stimulated with Ach in the CG–SON–O system from rats in the first proestrous. Furthermore, AG increased the release of P and decreased the expression of 20α-HSD, but it did not modify the

release of E_2. 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 [72–76]. This effect of P is likely due to its ability to decrease the generation of free radicals and enhancing endogenous free-radical-scavenging systems [76]. Accordingly, the increase of P might be contributing to the increase of TAC and the decrease of carbonyls content in ovary treated with AG, as opposed to the results obtained with the addition of Ach in the CG.

Finally, since the ovary is a metabolically and inflammatory active organ, which is continually exposed to a variety of microenvironmental stresses, different mechanisms might likely exist to keep a healthy balance between oxidants and antioxidants. This balance is needed to ensure efficient steroidogenesis and to favor ovulation.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Abbreviations

Ach	acetylcholine
AG	aminoguanidine
CAT	catalase
CG:	coeliac ganglion
E_2	estradiol
GSH-Px	glutathione peroxidase
iNOS	inducible nitric oxide syntase
NO	nitric oxide
O	ovary
P	progesterone
P450arom	P450 aromatase
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

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