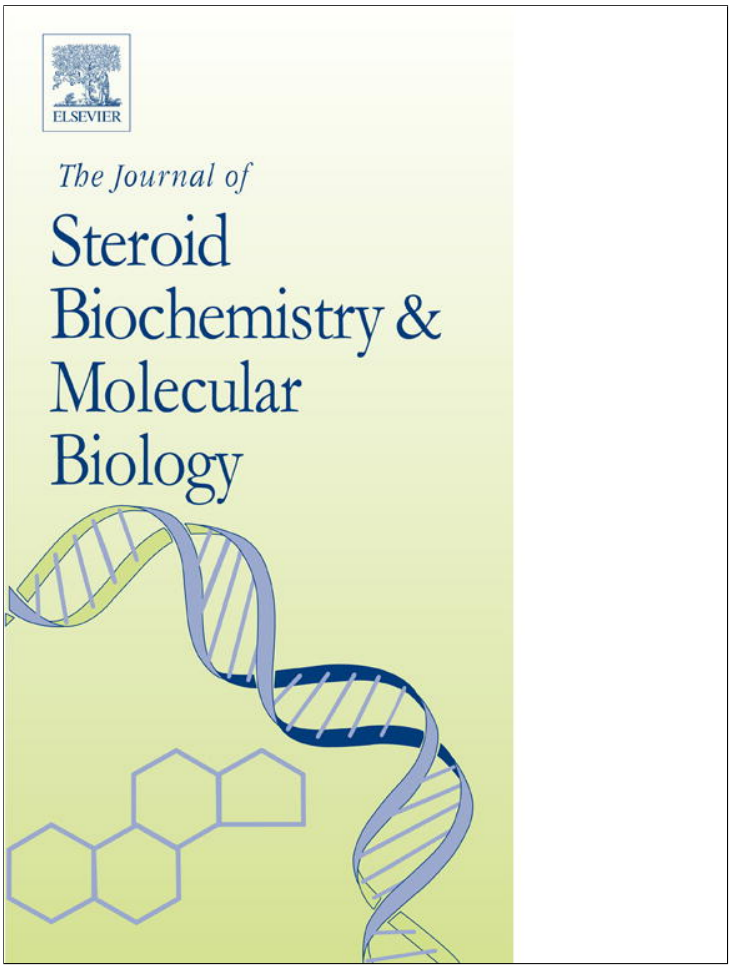

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Protective effect of oestradiol in the coeliac ganglion against ovarian apoptotic mechanism on dioestrus

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ABSTRACT

The aims of this work were to investigate if oestradiol 10^{-8} M in the incubation media of either the ovary alone (OV) or the ganglion compartment of an ex vivo coeliac ganglion–superior ovarian nerve–ovary system (a) modifies the release of ovarian progesterone (P_4) and oestradiol (E_2) on dioestrus II, and (b) modifies the ovarian gene expression of 3β-HSD and 20α-HSD enzymes and markers of apoptosis. The concentration of ovarian P_4 release was measured in both experimental schemes, and ovarian P_4 and E_2 in the ex vivo system by RIA at different times. The expression of 3β-hydroxysteroid dehydrogenase, 20α-hydroxysteroid dehydrogenase and antiapoptotic bcl-2 and proapoptotic bax by RT-PCR were determined. E_2 added in the coeliac ganglion caused an increase in the ovarian release of the P_4, E_2 and 3β-HSD, while in the ovary incubation alone it decreased P_4 and 3β-HSD but increased and 20α-HSD and bax/bcl-2 ratio. It is concluded that through a direct effect on the ovary, E_2 promotes luteal regression in DII rats, but the addition of E_2 in the coeliac ganglion does not have the same effect. The peripheral nervous system, through the superior ovarian nerve, has a protective effect against the apoptotic mechanism on DII.

1. Introduction

Cell communication is the ability of every cell to exchange information with the physical–chemical environment and other cells through chemical mediators that react with specific receptors [1].

Our group has shown that the ovary is an optimal model of the interaction between the peripheral nervous system and the endocrine system [2–7]. The rat ovary is innervated by two principal pathways, the superior ovarian nerve (SON) and the ovarian nervous plexus (ONP). Both are mainly constituted by sympathetic postganglionic fibers whose neuronal somas are located in the coeliac ganglion (CG) and superior mesenteric ganglion (SMG), respectively [8–10]. The SON, constituted by ganglionic neuron axons, innervates interstitial and theca cells, which are responsible for androgen synthesis [11–13]. This indicates that the peripheral nervous system and prevertebral ganglia, such as the CG, constitute a fast information pathway between the central nervous system and the ovary [3,4]. It has been demonstrated that sympathetic ganglia exhibit high density and variety of neurotransmitter receptors [14–16]. This highlights the complexity of the ganglia structure and the large number of factors that may be simultaneously involved in a particular effect. Anesetti et al. [17] found that neurons located in the coeliac ganglion contribute to ovarian innervation and that the sympathetic coeliac neurons increase their size and modify the expression of neurotrophin p75 receptor by estrogen exposure. This action affects mainly small and medium size neurons and is mediated through two different intracellular receptors, estrogen receptor α (ERα) and β (ERβ), which bind to specific deoxyribonucleic acid (DNA) sequences and regulate transcription [18].

Estrogen receptor knockout mice exhibit alterations in the reproductive functions, such as modified ovarian folliculogenesis and failure of ovulation and of corpus luteum (CL) formation [18]. E_2 receptors are regulated in a tissue and/or cell type specific manner and E_2 itself seems to play an important role in this regulation [19]. In addition, they play crucial roles in the pituitary gonad axis development, ovarian folliculogenesis, ovulation and implantation. A simple dose of oestradiol valerate can modify the levels of expression of the tyrosine hydroxylase enzyme, which participate in catecholamine biosynthesis. Catecholamines induce changes in the sympathetic nervous activity in the ovary of mammals [20,21], provoking alterations in the follicular growth, which leads to polycystic ovaries development [22,23]. At the end of oestrous cycle in the rat, it is necessary that the corpus luteum (CL), which is the dominant steroidogenic structure in the ovaries, undergo

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functional regression, which is evidenced by the decrease in the ability to produce progesterone (P_4) in order to continue the cycle. The structural involution of the CL, however, is clearly evident only at proestrus stage between 7.00 and 10.00 p.m., and it is shown by the ovary reduction in size and weight as a consequence of an apoptotic phenomenon [24]. There are several apoptotic genes that are associated to luteal regression and are up-regulated before the next cycle, as a consequence of serum P_4 level decrease [25]. The molecular mechanisms that originate luteal regression are, however, not yet entirely comprehended. Previous studies have suggested that in rat E_2 is a key hormone regulating luteal apoptosis in reproductive processes, such as is pregnancy [26]. In order to clarify the effects of E_2 in coeliac ganglion through the SON on the functioning of the CL, the aims of this work were (a) to investigate if $E_2 10^{-8}$ M modifies the release of ovarian P_4 and E_2 on dioestrus II (DII) stage, acting either directly on the ovarian tissue or indirectly via the SON from the coeliac ganglion and (b) to demonstrate if this concentration of E_2 modifies the ovarian gene expression of P_4 synthesis and catabolism enzymes and of apoptosis markers in two experimental schemes.

2. Materials and methods

2.1. Animals

The animals used for this study were similar to those used by Sosa et al. [4]. Virgin Holtzman strain adult female rats on DII stage weighing 250 ± 50 g were used in all the experiments. The rats were kept in a light (lights on from 07:00 h to 19:00 h) and temperature controlled room (24 ± 2 °C). Animals had free access to food (Cargill, SACI, Saladillo, Buenos Aires, Argentina), and tap water was available ad libitum. Vaginal smears were taken daily, and only the rats exhibiting at least two-day consecutive dioestrus stage were used. Six animals were used in each group of the experimental procedure. The experiments were performed per triplicate according to the procedures approved in the UFAW Handbook on the Care and Management of Laboratory Animals (Trevor Poole, 1999) [27]. The experimental protocol was approved by the University of San Luis Animal Care and Use Committee (number protocol: 23/09-CS, ordinance CD 12/08).

2.2. Reagents

The following drugs: oestradiol, ascorbic acid, bovine serum albumin fraction V (BSA), were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Other reagents and chemicals were of analytical grade. 1,2,6,7-[^{3}H] progesterone (107.0 Ci/mmol) and17-β-2,4,6,7-[^{3}H] estradiol (102.0 Ci/mmol) were provided by New England Nuclear Products (Boston, MA, USA).

2.3. Extraction of coeliac ganglion–superior ovarian nerve–ovary system

2.3.1. Experimental procedure

The procedure was carried out between 15.00 and 16.00 h taking into account previous descriptions of the anatomical trajectory of this neural pathway as a guide [4,8,10]. The rats were anaesthetized, and the system was immediately removed by dissection. Each system was conformed by the ovary, the fibers constituting the SON and the coeliac ganglion. The total surgical procedure was completed in 1–2 min. The strip of tissue was carefully dissected, avoiding contact between the surgical instruments and the nerve fibers and the ganglia in order to prevent spontaneous depolarisations of the nerves. The extracted systems were washed with incubation solution and immediately placed in one cuvette with two separate compartments, each containing 3 ml of work solution, Krebs–Ringer bicarbonate buffer, pH 7.4, in the presence of dextrose (0.1 mg/ml) and BSA (0.1 mg/ml) as described in other works [4–7].

After 15 min of preincubation (time 0 of incubation), the Krebs–Ringer solution was changed in both compartments, and ascorbic acid (0.1 mg/ml in Krebs–Ringer) was added as an antioxidant agent in the ganglion compartment whereas Krebs–Ringer solution was added in the ovary compartment.

Ovary P_4 and E_2 values released under these conditions were considered as control level (control group). For the experimental group, E_2 was dissolved in Krebs–Ringer solution plus ascorbic acid at a 10^{-8} M final concentration in the ganglion compartment. The ganglionic effect of E_2 on the steroid release in the ovary compartment was evaluated in relation to the control group.

In the second experimental scheme, whole ovaries were removed from the rats and incubated under the same conditions that in the ex vivo system. The effects on luteal regression of E_2 directly added on the ovary (OV) incubation media was analyzed.

Liquid samples from the OV compartment (250 μl) in both experimental schemes were collected at 60, 120 and 180 min for P_4 and 60, 120 min for E_2 and kept at −20 °C until the determination by RIA. When the incubation time was finished, the ovaries were kept at −80 °C until determination of 3β-hydroxysteroid dehydrogenase (3β-HSD), 20α-hydroxysteroid dehydrogenase (20α-HSD), bax and bcl-2 gene expressions.

2.4. Steroids assay

Progesterone contents were measured in duplicate by radioimmunoassay (RIA). The results were expressed as nanogram of P_4 per mg of ovarian tissue per ml (ng P_4/mg ovary/ml), all against incubation time and assay sensitivity was less than 5 ng progesterone/ml. E_2 was expressed as picograms per milligrams of ovarian tissue per ml (pg E_2/mg ovary/ml). Assay sensitivity was <2.2 pg E_2/ml in all incubation times in the experimental scheme. The respective corrections were made in all cases considering the volume extracted in each tested period.

2.5. RNA isolation and RT-PCR analysis

Once the ovaries were defrosted, the total ribonucleic acid (RNA) was extracted using the TRIZOL-Reagent method (Invitrogen Life Technologies), following the manufacturer's instructions for the RNA extraction [28]. Two micrograms of total RNA were reverse transcribed at 37 °C using random primers and M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) in a 26 μl reaction mixture. Fragments coding for 3β-HSD, 20α-HSD, bcl-2 and bax were amplified by PCR in 50 μl of reaction solution containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.25 U of Taq polymerase, 50 pmol of each rat specific oligonucleotide primer and RT-generated cDNA (1/5 of RT reaction). The sequences of the specific primers are shown in Casais et al. [26]. The amplification of the cDNA was performed using a thermal cycler (My Cycler, BioRad Laboratories, Inc., Hercules, CA, USA). Reaction products were electrophoresed on 2% agarose gels, visualized with ethidium bromide, and examined by ultraviolet transillumination. Band intensities of RT-PCR products 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.

3. Statistical analysis

All data are presented as means ± S.E.M. in each group. For comparisons made throughout the time of incubation, variance analysis of repeated measures 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 value of $p < 0.05$ was considered statistically significant [29].

4. Results

4.1. Effect of E_2 in coeliac ganglion on P_4 and E_2 release and the gene expression of 3β-HSD, 20α-HSD on DII day

E_2 (10^{-8} M) was added in the coeliac ganglion to evaluate whether it modulates the release of ovarian P_4, E_2 and the gene expression of the P_4 metabolism enzymes on DII. Progesterone has proved to be the most sensitive steroid to neural influence in *in vitro* studies [3,4]. E_2 in the coeliac ganglion increased P_4 release at all times compared to control (*$p < 0.001$; Fig. 1a). The ovarian 3β-HSD gene expression was increased by E_2 in ganglion (*$p < 0.001$; Fig. 1b) while 20α-HSD was decreased (*$p < 0.001$; Fig. 1c) compared to the control.

4.2. Effect of E_2 in coeliac ganglion on the gene expression of bax and bcl-2 on DII day

Proapoptotic bax and antiapoptotic bcl-2 were measured at the end of the incubation period. No variations were observed either in the gene expression of bax and bcl-2 or in the bax/bcl-2 ratio (Fig. 2a–c) as compared to the control.

4.3. Effect of E_2 in coeliac ganglion on the E_2 release on DII day

Oestradiol 10^{-8} M in coeliac ganglion increased the ovarian E_2 release at 60 min (2.60 ± 0.0001 vs. 10.51 ± 0.01), 120 min (924.45 ± 0.01 vs. 2812.05 ± 0.01) (*$p < 0.001$; Fig. 3).

4.4. Effect of E_2 in ovarian incubations on P_4 release and the gene expression of 3β- and 20α-HSD on DII day

E_2 in the ovarian compartment decreased the P_4 release at 60 min (0.004 ± 0.001 vs. 0.00036 ± 0.001)(*$p < 0.001$), 120 min (0.003 ± 0.001 vs. 0.00283 ± 0.001) (**$p < 0.05$) and 180 min (0.0023 ± 0.001 vs. 0.0017 ± 0.001) (*$p < 0.001$; Fig. 4a).

In order to evaluate if the addition of E_2 in the ovarian incubation modifies the gene expression of P_4 synthesis and degradation enzymes in DII stage, the ovaries were separated after 180 min of incubation. 3β-HSD decreased (*$p < 0.001$; Fig. 4b). 20α-HSD showed a slight, although non-significant, tendency to increase (Fig. 4c).

4.5. Effect of E_2 in ovarian incubations, on genes bax and bcl-2 on DII day

The bax was increased (*$p < 0.001$) (Fig. 5a) by E_2 in ganglion, while the bcl-2 was decreased (*$p < 0.001$; Fig. 5b). The relationship between bax/bcl2 increased (*$p < 0.001$; Fig. 5c) compared to the control.

5. Discussion

We have previously demonstrated that, in the oestrus stage, E_2 regulates ovarian physiology through the sympathetic neurons of the superior mesenteric ganglion, which is a component of the sympathetic prevertebral ganglia pathway [7]. The objective of this study was to investigate the effect of E_2 on ovarian apoptosis on DII, either directly on the ovarian tissue or indirectly via the SON from the coeliac ganglion.

It should be noted that in both cases the ovary autocrine and paracrine relationships remain intact and without humoral influence. A dose–response curve (data not shown) showed that E_2 10^{-8} M provoked a higher P_4 release response at all the incubation time in the *ex vivo* system. The presence of E_2 in CG increased the release of P_4 and E_2 in the ovarian compartment in the system, while in the ovary alone P_4 decreased. The gene expression of the 3β-HSD and 20α-HSD enzymes, which are involved in synthesis and catabolism of P_4, respectively, are related with the effects observed in its release.

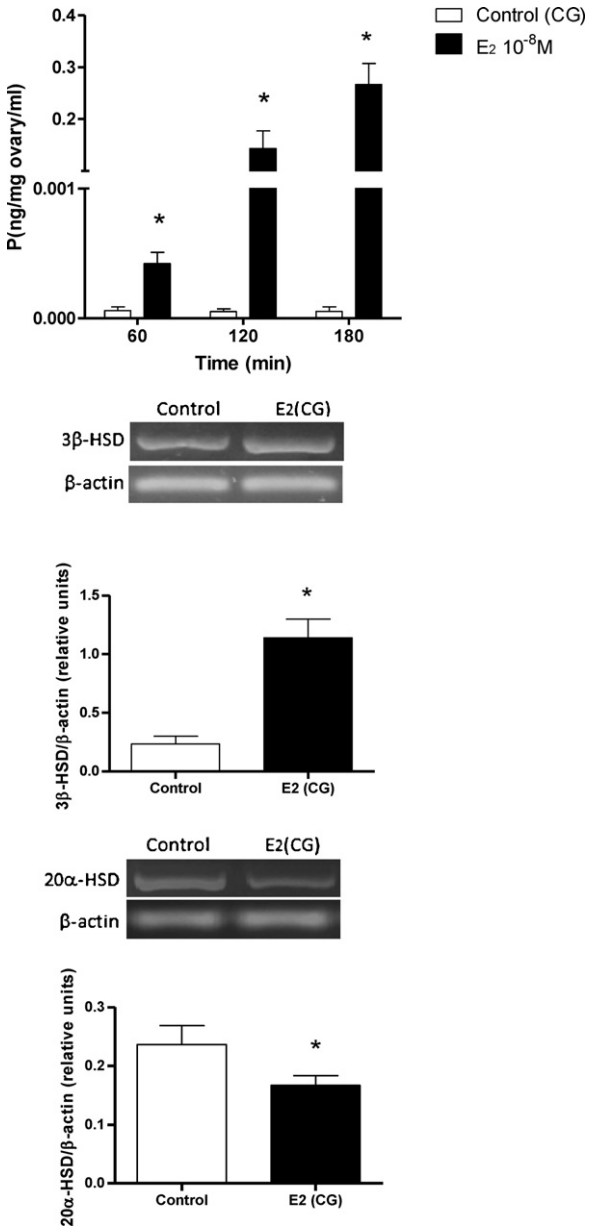

Fig. 1. Effect of E_2 in the coeliac ganglion on ovarian P_4 release (a), mRNA expression of the 3β-HSD (b), mRNA expression of the 20α-HSD (c) in ovary extracted of the coeliac ganglion–SON–ovary systems on DII day. The gel photographs were quantified using Image J software and expressed as relatives units. β-actin was used as housekeeping gene. Results are expressed as mean ± S.E.M. ($n = 3$) *$p < 0.001$. E_2: oestradiol; P_4: progesterone; 3β-HSD: 3β-hydroxysteroid dehydrogenase; 20α-HSD: 20α-hydroxysteroid dehydrogenase; CG: coeliac ganglion.

These results are consistent with those obtained by Anesetti et al. [17], who found estrogen receptor (ER) α and β in the coeliac ganglion neurons, which would indicate that this steroid may act through these receptors on peripheral organs and sympathetic neurons associated with related to reproduction functions. ERα are also localized in the plasma membrane and may help to elucidate the mechanisms through which rapid, "nongenomic" estrogen

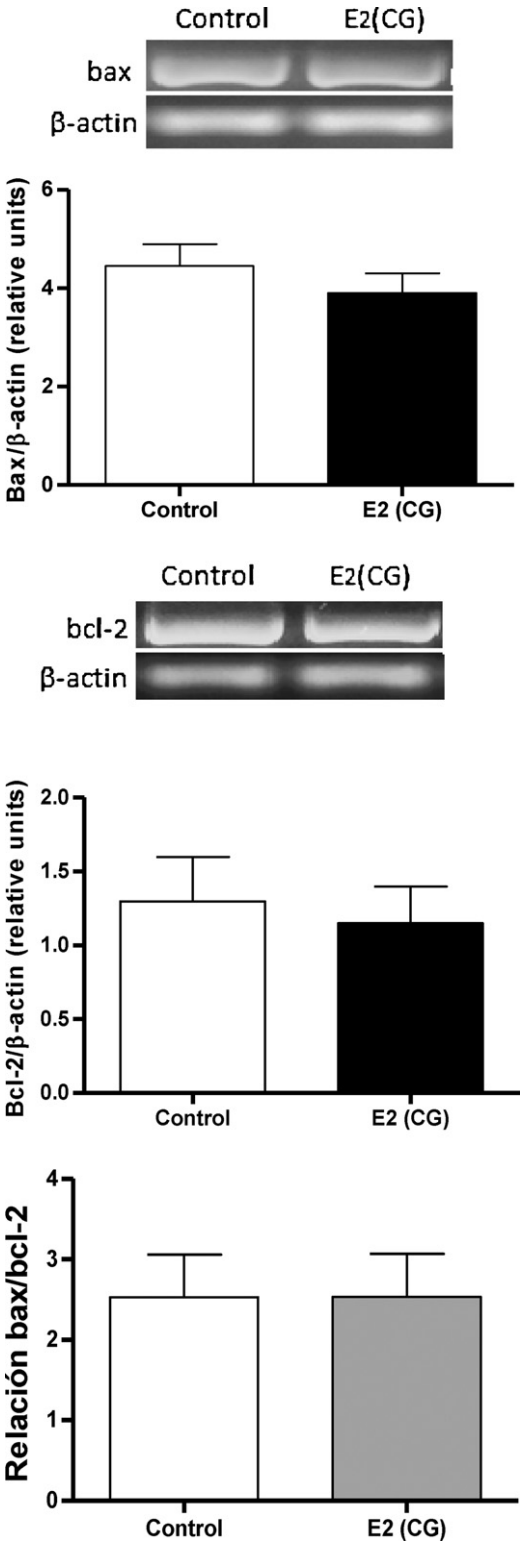

Fig. 2. Ganglionic effect of E_2 on ovarian mRNA gene expression of proapoptotic bax (a), antiapoptotic bcl-2 (b). Ratio of bax/bcl-2 (c) on DII day. β-actin was used as housekeeping gene. Results are expressed the mean ± S.E.M. ($n = 3$). The gel photographs were quantified using Image J and expressed as relatives units. E_2: oestradiol; CG: coeliac ganglion.

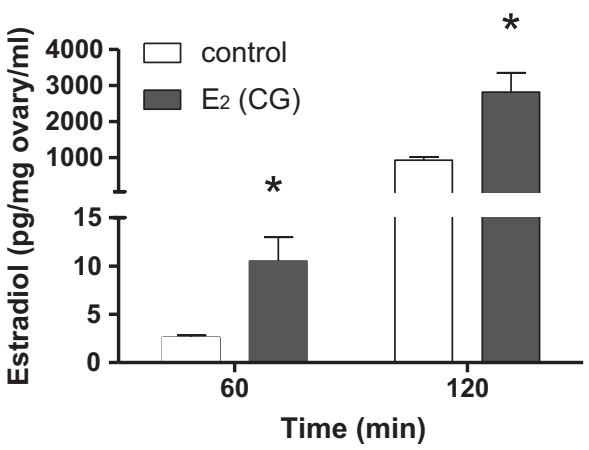

Fig. 3. Effect of E_2 in the CG on the ovarian oestradiol release in the coeliac ganglion–SON–ovary system on DII day. Results are expressed as mean ± S.E.M. ($n = 5$) $^*p < 0.001$; E_2: oestradiol; SON: superior ovarian nerve.

signaling occurs [30–32]. Mani et al. [33] in recent studies indicate that the nonclassical, rapid effect that involves extranuclear ER located at the membrane, which interacts with cytoplasmic kinase signaling molecules and G-proteins [33]. Sosa et al. [4] and Bronzi et al. [34] have previously determined the increase of catecholamines by neural stimulus; and Lara et al. [20] found that a simple dose of oestradiol valerate can modify the levels of expression of the tyrosine hydroxylase enzyme, the rate-limiting enzyme in catecholamine biosynthesis [22]. This enzyme, together with dopamine β-hydroxylase, which also participates in the biosynthesis pathway of catecholamines, co-localizes with immunoreactive neuropeptides and with cholineacetyl transferase, the enzyme for the acetylcholine synthesis pathway [35]. This is important because the observed P_4 and 3β-HSD increase in this work could be due to ganglionic input generated by increasing the release of catecholamines at the ovarian level. Thus, Anesetti et al. [17] observed that the effects of estrogens on innervations of coeliac ganglion in target organs were heterogeneous, inducing a significant increase in catecholaminergic innervations of the ovary. These findings support the role of estrogen as a neuronal plasticity modulator. Koszykowska et al. [36] have suggested that estrogen could modify some features involved in the relationship between sympathetic immature peripheral neurons and their target organs through a neurotrophin-dependent mechanism. All these antecedents could explain the increase of P_4 observed in this study. On the other hand, Sosa et al. [4] and Bronzi et al. [34] demonstrated that catecholamines reach the ovary through the SON by neural stimulus. The fact that the fibers constituting the SON are mostly catecholaminergic suggests that they might act through α or β-adrenergic receptors in the ovary [4,5]. In this regard, it is well known that there exists a stimulating effect on the release of P_4 caused by catecholamines, when the NA increases and dopamine (DA) decreases on DII, by catecholaminergic stimulus in ganglion, without changes in their catabolites [34]. This effect on DII may be related to the conversion of DA into NA at this stage. This is in agreement with Kotwica et al. [37] who postulated that DA is the precursor in the biosynthetic pathway of NA. Therefore, the DA concentration in the corpus luteum, is related with those of NA during the estrous cycle, are higher in the newly formed of DI than in the developed corpus luteum of DII or the corpus luteum in regression.

Although from our results it cannot be determined which neurotransmitter is released by the neural stimulus with E_2, it can be hypothesized that NA or DA are responsible for the antiapoptotic effect on the corpus luteum. Rekawiecki et al. [38] found that bovine corpus luteum can synthesize de novo NA from DA as a precursor.

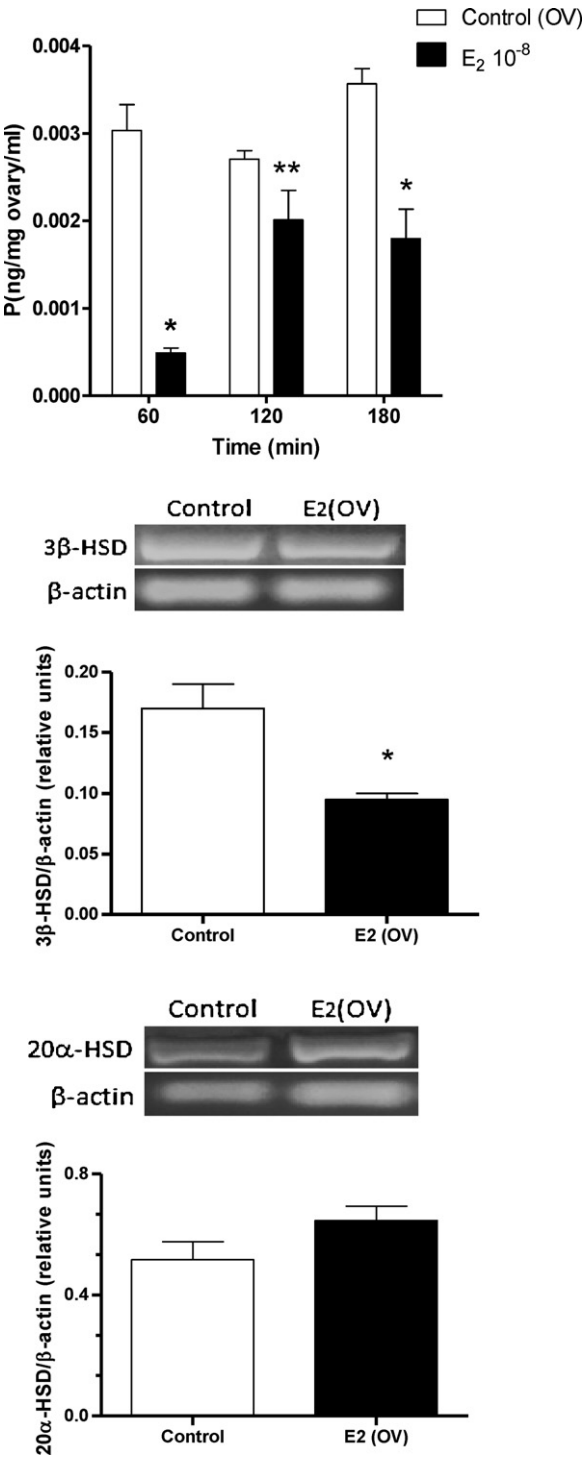

Fig. 4. Effect of E_2 on the ovary on the ovarian P_4 release (a), mRNA expression of the 3β-HSD (b), mRNA expression of the 20α-HSD (c) on DII day. The gel photographs were quantified using Image J software and expressed as relative units. β-actin was used as housekeeping gene. Results are expressed as mean ± S.E.M. ($n = 3$) $^{*}p < 0.001$, $^{**}p < 0.05$. E_2: oestradiol; P_4: progesterone; OV: ovary; 3β-HSD: 3β-hydroxysteroid dehydrogenase; 20α-HSD: 20α-hydroxysteroid dehydrogenase.

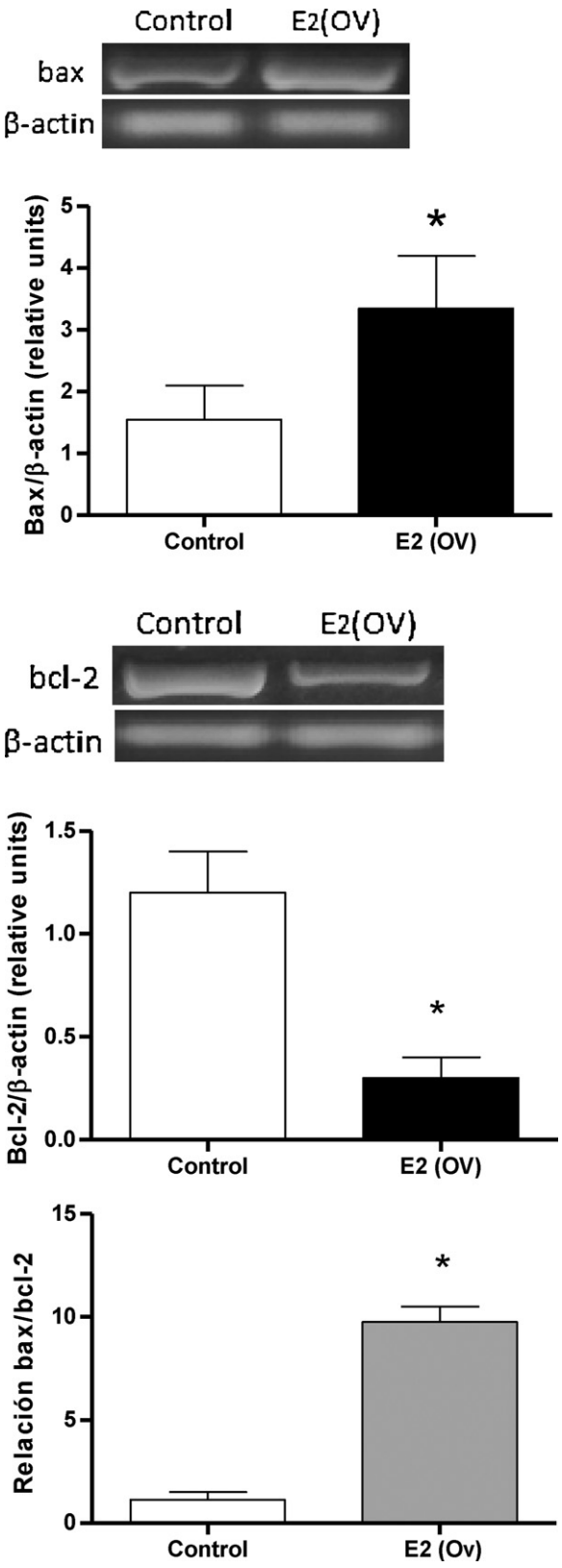

Fig. 5. Effect of E_2 in the ovary on the ovarian mRNA gene expression of proapoptotic bax (a), mRNA gene expression of antiapoptotic bcl-2 (b). Ratio of bax/bcl-2 (c) on DII day. β-actin was used as housekeeping gene. Results are expressed the mean ± S.E.M. ($n = 3$) $^{*}p < 0.001$. The gel photographs were quantified using Image J and expressed as relative unites. E_2: oestradiol; OV: ovary.

Their data indicate that noradrenergic stimulation can be an important part of the supporting mechanism for the good corpus luteum functioning.

In agreement with these observations, Rosa et al. [23] reported a remarkable increase in the ovarian content of P_4 and androstenedione (A_2) in oestradiol-valerate treated rats. On the other hand, when the ovary was stimulated with isoproterenol, a β-adrenergic agonist, no changes were observed in response to E_2.

The addition of E_2 in coeliac ganglion in the system produced no changes in either bax or bcl-2. These results suggest that E_2 acts by inhibition of the mechanisms that trigger apoptosis, and thus, protects the corpus luteum during the regression. This is in agreement with reports of Lara et al. [22] that the injection of oestradiol valerate increases catecholamines and lead to polycystic ovary.

Unlike the results observed when it was placed in the ganglion of the *ex vivo* system, E_2 placed directly in the ovary incubation provoked an increase in the bax/bcl2 ratio, which leads us to postulate that E_2 favors luteal regression on DII through a direct effect on the ovary. This effect is very important and is in agreement with results reported by Casais et al. [39], who found that at the end of pregnancy E_2 added directly to the ovary incubation or to the coeliac ganglion of the CG–SON–OV system caused a decline in the concentration of P_4 accumulated in the incubation media. They concluded that through a direct effect on the ovary, E_2 favors luteal regression at the end of pregnancy in rats, in association with neural modulation from the coeliac ganglion via the SON. On the other hand, Rosa et al. [23] found that oestradiol valerate administration during juvenile period suppressed circulating LH, FSH, and A_2, increased ovarian NA, E_2, and 3β-HSD activity, disrupted ovarian dynamics evidenced as absent corpus luteum, and presence of ovarian cysts and culminated in anovulation. When the SON is sectioned, cyclicity in these animals is restored; LH and E_2 levels, as well as ovarian 3β-HSD activity, are normalized. This recovered ovarian dynamics is evidenced by the disappearance of ovarian cysts and the appearance of CL with its restored function. These findings provide evidence that oestradiol valerate exposure during juvenile life lead to long-lasting deleterious reproductive consequences via sympathetic ovarian nerve activation.

This work shows that E_2, through a direct effect on the ovary, promotes regression of the corpus luteum in rats in DII. However, the addition of steroid in the coeliac ganglion does not have the same effect but rather protects against ovarian apoptotic mechanisms and thus facilitates the continuity of the estrous cycle.

In conclusion, this study demonstrates that E_2 has a crucial role in the regulation of apoptotic mechanisms in ovarian, and that the peripheral nervous system, through the superior ovarian nerve, could be a regulator of these mechanisms and has protective effect against the apoptosis in DII.

This work may be useful for further understanding of the mechanisms that lead to pathologies such as polycystic ovary syndrome.

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