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Apoptosis induced by a GnRH analogue in a granulosa cell line from bovine ovary (BGC-1)

Apoptosis inducida por un análogo de GnRH en una línea celular de granulosa de ovario bovino (BGC-1)

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SUMMARY

Gonadotrophin releasing hormone receptors (GnRHR) have been identified in ovarian granulosa cells (GC) and luteal cells (CL). It has also been observed that the activation of GnRHR in GC regulates gonadal function. Studies using GnRH agonists (GnRHa) in rat ovaries, *in vivo* and *in vitro*, showed an increase in follicular atresia. Our objective was to study the regulation of apoptosis inan established line of bovine granulosa cells (BGC-1) usin gleuprolide acetate (LA) as GnRHa and ANTIDE as competitive antagonist.

Our results demonstrate a significant increase in the proportion of apoptotic cells induced for 24 and 48 hs with 100 nM LA by morphological techniques (DAPI staining and Hematoxilin), membrane markers expression (Annexin V-FITC and PI detected by flow cytometry) and biochemical techniques such as determining caspase 3 activity. Apoptosis was partially inhibited by ANTIDE, showing *per se* activity when caspasa 3 activity was evaluated.

The analysis of the intracellular pathways regulating the apoptotic process revealed the existence of a mechanism that involves the activation of the intrinsic pathway, throughthe analysis of protein expression of Bcl-2 family (Bax and Bcl-2) andtheinhibition of the extrinsic pathway by inhibiting the activity of the enzyme phospholipase D (PLD). It is proposed that ANTIDE works as antagonist of the intrinsic pathway but as extrinsic pathway agonist as inhibiting the activity of PLD.

Key words: (apoptosis), (BGC-1), (leuprolide acetate), (GnRH)

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RESUMEN

Se han identificado receptores de la hormona liberadora de gonadotrofina (GnRHR) en células de granulosa ovárica (CG) y en células luteales (CL). Asimismo, se ha observado que la activación de los GnRHR en CG regula la función gonadal. Estudios realizados utilizando agonistas de GnRH (GnRHa) en ovarios de ratas, *in vivo* e *in vitro*, demostraron un incremento de la atresia folicular. Nuestro objetivo fue estudiar la regulación de la apoptosis en una línea establecida de células de granulosa de ovario bovino (BGC-1) utilizando el acetato de leuprolide (LA) como GnRHa y el ANTIDE como antagonista competitivo.

Nuestros resultados demuestran un aumento significativo de la proporción de células apoptóticas inducidas durante 24 y 48 h con 100 nM LA, confirmado mediante técnicas morfológicas (tinción con DAPI y Hematoxilina), técnicas de expresión de marcadores en membrana (Anexina V-FITC y PI detectada por citometría de flujo) y técnicas bioquímicas como la determinación de la actividad de caspasa 3. La apoptosis fue parcialmente inhibida por el ANTIDE, registrándose una actividad *per se* del mismo cuando se evaluó actividad de caspasa 3.

El análisis de las vías intracelulares de regulación del proceso apoptótico reveló la existencia de un mecanismo que involucra la activación por la vía intrínseca, a través del análisis de la expresión de proteínas de la familia Bcl-2 (Bax y Bcl-2) y la inhibición de la vía extrínseca, a través de la inhibición de la actividad de la enzima fosfolipasa D (PLD). Proponemos que el ANTIDE actúa como antagonista de la vía intrínseca pero como agonista de la vía extrínseca inhibiendo la actividad de PLD.

Palabras clave: (apoptosis), (BGC-1), (acetato de leuprolide), (GnRH).

INTRODUCTION

The mammalian ovary is composed of interstitial tissue and follicles in various stages of development. These stages can find antral follicles that have a wall composed of several layers of granulosa cells (GC) and a central cavity, the follicular antrum developed from follicular fluid production by these same cells.

Apoptosis and cell death pattern which was originally identified by their morphological criterion is now identified and defined by the result of activation of a common molecular mechanism and relevant both as pathological and physiological circumstances[32].

Apoptosis is programmed cell death which involves an orderly process genetically regulated by the synthesis of proteins such as Bcl-2 family, which includes pro and anti-apoptotic genes, caspases, and initiator and effectors proteins that culminate with the orderly DNA fragmentation.

Several publications detected GnRH receptor (GnRHR) in human and rat granulose and luteal cells[7, 11, 25, 14, 22, 15, 31]. It was also demonstrated the expression of messenger RNA (mRNA) for GnRHR in reproductive tissue and human cell lines[9, 10] and likewise in rat[19] gonads. The technique of in situ hybridization for detecting mRNA allowed GnRHR in granulose cells from primary, secondary and tertiary follicles from rat ovary[6, 31]. It is also possible to detect the presence of GnRHR mRNA corresponding to the bovine ovary[24] but not performed GnRHR protein detection in cattle. These authors utilized the technique of RT-PCR to detect the presence of mRNA in these follicles and corpora lutea. The small follicles present greater GnRHR mRNA expression than medium and large size. Results from our laboratory demonstrated by immunocytochemistry (ICQ) the presence of such receptors in BGC-1[5].

In all species studied, the initiation of apoptosis in granulose cells is one of the first signs of follicular atresia [28, 35]. The presence of apoptosis in granulose cells from atretic follicles has been documented based on morphological[20, 33, 26] and biochemical[13, 18, 17, 34] criteria.

Evidence suggests that GnRH plays a role in the induction of follicular atresia[12, 23, 2]. During

the follicular phase, the rat atretic follicle showed increased expression of mRNA for GnRHR[31]. *In vitro* studies showed the inhibited synthesis of ADN[27] and induced apoptosis in granulose cell cultures of rat[2] treated with GnRH. It was also observed that a GnRH agonist (GnRHa) induced increased formation of apoptotic corps (AC) in human granulose cells[36]. More recently, in an *in vivo* assay demonstrated that the GnRH induced apoptosis in rat granulosa cells[21].

Our laboratory conducted a series of initial experiments in an established line of bovine granulosa cells (BGC-1), with high density, to determine the optimal experimental conditions for the study of apoptosis induced by GnRHa, leuprolide acetate (LA). The effect of 100 nM LA concentration at 6, 12 and 24 h of incubation in the presence or absence of 5% fetal bovine serum (FBS). In these experiments, morphologically assessed by staining with DAPI (fluorophor which binds to the DNA minor curvature), there was an increase in apoptosis induced by LA after 24 h incubation in the presence of 5% FBS[3].

In other previous experiments analyzed with hematoxylin, TUNEL and Annexin V, showed a significant increase of 50% in the proportion of apoptotic cells induced for 24 and 48 h with 100 nM LA in the presence of 5% FBS. Analyzing the results by ICQ against Bax evidenced again a significant increase in apoptosis induced with 100 nM LA[4].

The LA is widely used in insemination protocols. Inhibition of local apoptosis-inducing effect of LA, would significantly improve the quality of oocytes obtained by follicular puncture in animals induced to superovulate with GnRH agonist (GnRHa).

The overall goal of this work is to study the regulation of apoptosis in an established line of bovine granulosa cells (BGC-1) using the LA as GnRHa and competitive antagonist Antide.

MATERIALS AND METHODS

Cell culture:

Bovine Granulosa Cell Line 1 – (BGC-1): The BGC-1 was obtained through spontaneous immortalization of primary cultures of granulosa cells removed by aspiration of 2 to 8 mm diameter follicles from adult females of this species[1, 16].

Sowing: 310 cells/mm^2 were seeded (50,000 cells/ml) for 24 h, until induction. Cells were grown in DMEM + F12 supplemented with 5% fetal bovine serum (FBS), 2 mM Glutamine, Gentamicin 50 mg/l, 5% CO_2, saturated humidity and 38.5 °C.

Experimental design and treatments:

Test 1: concentration curve at 24 and 48 h of incubation.

Treatments:

FBS 5%.
FBS 5% + 1 nM LA.
FBS 5% + 10 nM LA.
FBS 5% + 100 nM LA.

Test 2: Incubation for 24 h

Treatments:

FBS 5%.
FBS 5% + 100 nM LA.
FBS 5% + 100 nM LA + 100 nM ANT.
FBS 5% + 100 nM ANT.

Test 3: Incubation for 24 h

Treatments:

FBS 5%.
FBS 5% + 1 nM LA.
FBS 5% + 10 nM LA.
FBS 5% + 100 nM LA.
FBS 5% + 100 nM LA + 100 nM ANT.
FBS 5% + 100 nM ANT.

Incubations with Antide were performed 3 hours before stimulation with LA.

Methodology for apoptosis evaluation:

Morphological Analysis of apoptosis by staining with hematoxylin: Cells were seeded on 18 x 18 mm coverslips with a density of 310 cells per mm^3. They were cultured for 24 h prior to the hormonal induction. After incubation with the treatment, 24 or 48 hours depending on the case, the reaction was stopped by fixation with

pure methanol for 5 minutes and air dried. They were then stained for 15 minutes with hematoxylin and tacking with warm water for 5 minutes. The assembly was done with DPX mounting medium (Sigma - Aldrich).

Counting the images of apoptosis and mitosis was performed in a bright field microscope, consigning further images for necrosis and phagocytosis. Approximately 500 cells were counted per coverslip. Images were captured using an imaging system consisting of a DMLS model trinocular microscope (Leica Inc.) and fluorescence sensitivity camera DC-180 model (Leica Inc.). The system is supported by IM50 capture software (Leica Inc.).

Caspase 3 activity: The caspase family proteins are serine proteases that mediate cell death and are important in the process of apoptosis. Caspase 3 is a member of this family and is one of the critical enzymes in this process. Also plays a central role in mediating nuclear apoptosis including chromatin condensation, contraction of the cytoskeleton and DNA fragmentation. Cells were seeded on 60 mm plates and allowed to grow for 24 h until the hormonal induction. Cells were incubated for 24 or 48 h, the reaction was stopped by lifting rake. Kit was used for colorimetric detection of caspase 3 activity CASP-3-C Sigma-Aldrich. The cell pellets were resuspended in lysis buffer (LB) for caspase in a ratio of 1 million cells in 10 ul of LB keeping at - 80 ° C until the reaction. Prior to sample incubation with the substrate, acetyl-Asp-Glu-Val-Asp p-nitroaniline (Ac-DEVD-pNA) centrifuging the cell lysate at 15,000 rpm for 15 minutes at 4 ° C was performed. Incubating the sample with the substrate was performed in 96 well ELISA plate wells in a moist chamber at 37° C for 20 h. The reading was performed on ELISA reader with a 405 nm absorbance. Along with the reaction of the treatments were performed a positive control (purified caspase 3), a negative (or positive control sample more caspase 3 inhibitor) and a calibration curve using p-nitroaniline (product). Reading at time 0 was used as blank.

Three experiments were assayed in triplicate for trials 1 and 2, yielding 9 repeat treatments for Antide 100 nM, 1 and 10 nM LA but for the treatments100 nM LA and control there were 15 replicates. The 6 treatments were analyzed in the same two-way ANOVA.

Analysis of intracellular pathways for regulating apoptosis: Intrinsic pathway: Protein expression of Bcl-2 family (Bax and Bcl-2) by Western blotting (WB). A representing of each treatment was planted in polyacrylamide gel 12% in an amount of 40 μg of protein per seed. Samples were run for 2 h at 40 mA. The transfer of proteins to nitrocellulose membranes with 0.2 μm pore (Bio-Rad 162-0147) was performed at 40V, over night (ON) at 4°C. Anti-Bax antibody: Monoclonal anti-Bax (mouse origin) of Sigma (B8449) ON working dilution 1:800 at 4° C. Bcl-2 antibody: Bcl-2 (C 21): sc-783, Santa Cruz, polyclonal (rabbit origin), working dilution 1:200, ON at 4° C. The secondary antibodies against rabbit and mouse were used 1:10000 and incubated for 2 h at room temperature with 2% skim milk in the event of Bax. The membranes were treated with 2N NaOH for 5 minutes to lift before blocking antibodies and incubating them with anti β-actin as the reference basal protein expression. The bands were identified by calculating "rf" and analyzed with Image J program, expressed as values relative band intensity with the intensity of β-actin.

Extrinsic pathway: PLD Activity. Cells were seeded on 100 mm^2 plates. Samples were centrifuged in lysis buffer (the same used to assay for protein activity of caspase 3) at 4° C (20,000 g). It took between 300 to 800 μg proteins per sample for performing the analysis. Reaction mix: 360 μl Vf (25 mM HEPES, pH 7.2), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM phosphatidyl choline (PC), 6 mM oleic acid, 1.6 mM $(NH_4)SO_4$. Incubation was performed in heat bath at 37° C for 60 minutes in the first test and for 2 h in the latter. The tube was placed in boiling water for 10 minutes, cooled to room temperature and added 360 μl of chloroform vortexing for

1 minute at 2000 rpm. Centrifuged at 4000 g for 10 minutes, taking 200 µl of the supernatant (aqueous phase) for testing the concentration of choline liberated by the PLD from the PC.

Determination of choline: 200 µl of standard or choline SN + 800 µl Color reagent: 45 mM TRIS-HCl (pH 8), 5 U peroxidase, 0.3 mg 4 - aminoantipyrina, 0.2 mg phenol and 1 U choline oxidase. Samples were incubated at 37° C in thermal bath in dark for 90 minutes. The reaction was stopped with 1 ml of 50 mM TRIS-HCl (pH: 8) chill on ice. Absorbance was measured in a spectrophotometer at 505 nm. Choline standard curve: 10-120 nmol.

Statistical analysis: Statistical comparisons were made by two-way ANOVA, using the Bonferroni test as a means comparison analysis and α = 10%. In the case of PLD activity assay and caspase 3 was analyzed by Dunnett's mean comparison against control. Three experiments were performed in triplicate in all cases unless otherwise specified. For analysis Statistic 8 software was used.

RESULTS

Morphological analysis by hematoxylin staining: Test 2 - BGC-1 treated for 24 h incubation with LA and ANT 100 nM. The bright field quantification showed a significant increase in apoptotic index (AI) with respect to control incubation for 24 h with 100 nM LA yielding a 2.05 (AI) and a 2.4% basal apoptosis. This effect was partially inhibited by preincubation with the antagonist (p = 0.0017). The Antide *per se* didn't reveal significant differences compared to control. No significant differences were observed for any of the treatments based on the percentage of mitosis (Figure 1).

Caspase activity: tests 1 and 2 were analysated. Three experiments were performed in triplicate. Upon incubation of 24 h showed a significant increase (AI = 1.47; p = 0.0156) of the caspase 3 activity in the presence of 100 nM LA. This increase was partially inhibited by the presence of Antide, confirming what was observed at the morphological and cytometric analysis (FACS) of fosfatidilserinas exposure[5]. Regarding induction was performed for 48 h ANOVA analysis separately for each test due to the variations observed by the treatment with Antide observed an increased caspase activity at 100 nM LA in both assays (trial 1: p = 0, 0017, trial 2: p = 0.0007). The ANOVA test 2, which examines the effect of Antide was analyzed by the Dunnett's multiple test against the control revealing a significant increase in caspase 3 activity with 100 nM Antide, indicating a possible agonist effect (possible effect per se of the antagonist in these conditions) (Figure 2).

Analysis of intracellular pathways:

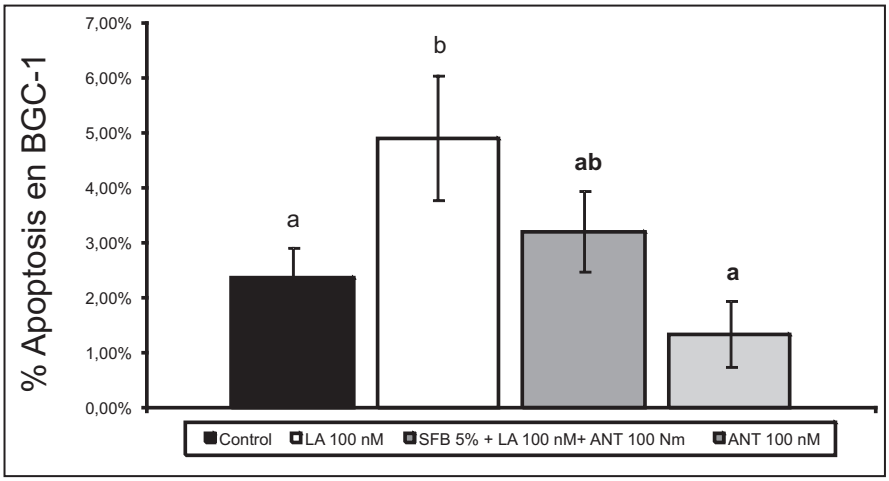
a) Analysis of protein expression of Bax and Bcl-2 by Western blotting (WB). Test 3 was performed with an incubation of 48 h for the different concentrations. Data from 5 membranes yielded significant results for Bax (p = 0.067, n = 5) and the proportion of Bax/Bcl-2 (p = 0.1, n = 5). Treatment with 100 nM LA showed a higher level of Bax expression compared to control. There was no significant treatment effect for expression levels of Bcl-2. This effect was inhibited by pre-incubation with 100 nM Antide (Figure 3).

b) PLD activity. For the analysis of PLD activity test 3 was evaluated with 24 h incubation. There was a significant decrease (p = 0.0079, n = 6) of PLD activity in all treatments respect to the control (Figure 4).

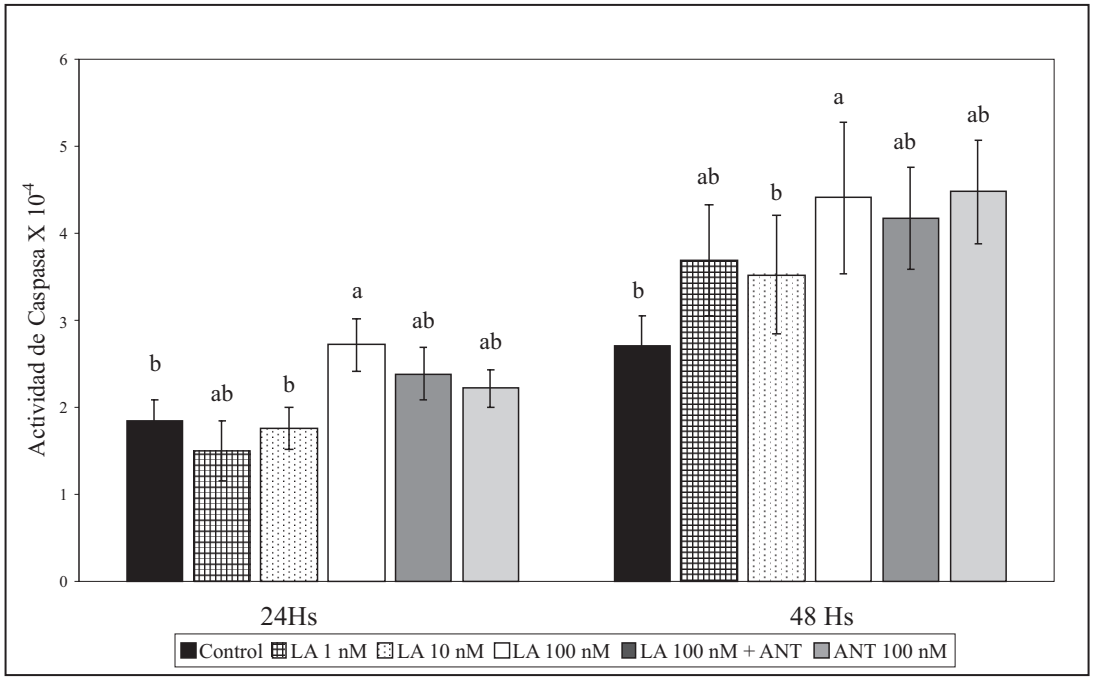
DISCUSSION

Given that, in the bovine ovary as in other species, granulosa cells express GnRHR[11, 22, 15, 31, 24], it results interesting that BGC-1 cells also express and maintain an agonist response to this hormone.

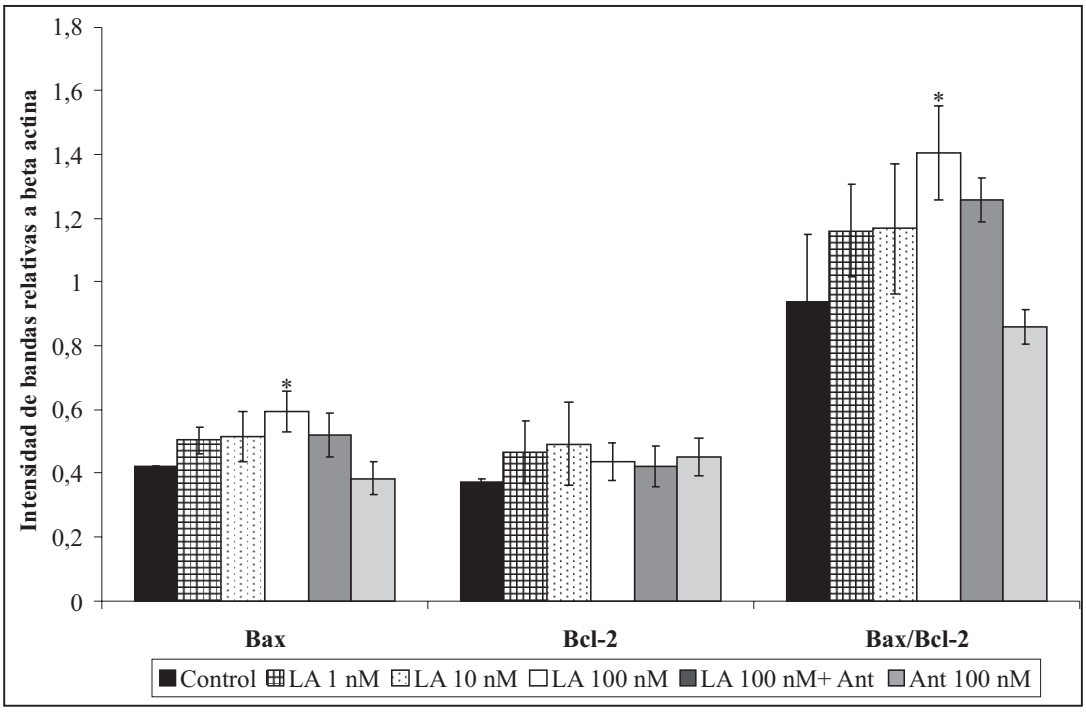
Other researchers have shown that GnRH agonists induce apoptosis in granulosa cells (GC) from eCG-treated pre-pubertal rats *in vivo* [21], and rat, human and pig *in vitro* [26, 36, 8, 30] but has not been reported until the point on the *in vitro* behavior of GnRH agonists on GC from line established as BGC-1. In this work it was shown that under the experimental



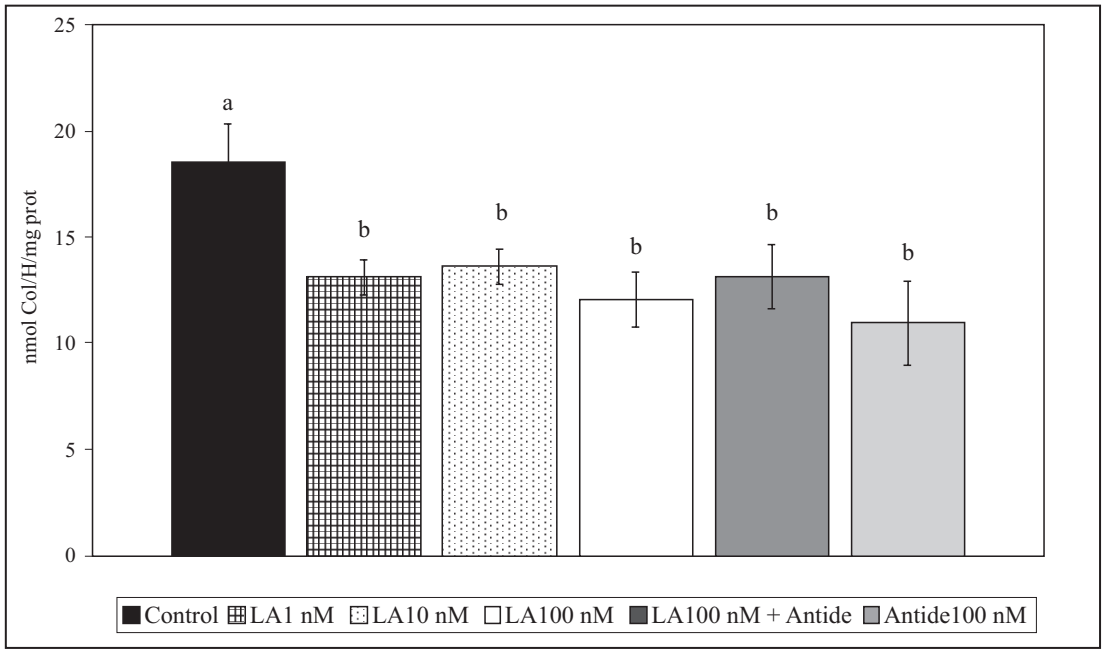
Graph 1: Apoptosis measured by staining with hematoxylin in BGC-1 treated for 24 hours with 100 nM LA and Antide (Test 2). (Two-way ANOVA, n = 9, p = 0.0017, Bonferroni).



Graph 2. Caspase 3 activity (µmol p-NA/well/µg protein/20 hs) in BGC-1 treated with LA and Antide for 24 and 48 h. For experimental design 1 (LA 0, 1, 10 and 100 nM) and 2 (0 to 100 nM and 100 nM Antide or only with 100 nM LA) three experiments were performed in triplicate. (n = 9 for treatment with 1 and 10 nM and with Antide, n = 15 to 100 nM control and LA (since both designs are repeated).



Graph 3: Expression of Bcl family proteins (Bax and Bcl-2) by Western blot (WB) in BGC-1 treated for 48 h. Test 3. Two experiments were plated in triplicate and a representative of each treatment by being street each corresponding to a repeat run. One of the membranes was discarded by presenting patches (n = 5, pBAX = 0.065, pbax/bcl-2 = 0.1, Dunnett's multiple test against control).



Graph 4: PLD activity (phospholipase D) in BGC-1 treated for 24 h with 0, 1, 10 and 100 nM LA (leuprolide acetate) and with or without pre-incubation with 100 nM Antide (Test 3). Two experiments in triplicate were analyzed in the same activity assay. (n = 6, p = 0.0079; Bonferroni).

conditions set forth above, there was a significant increase in apoptosis induced by LA[3, 4], a GnRH agonist used in the synchronization protocol as preparatory assisted reproduction (AI-IA, in vitro fertilization-IVF, etc.).

Inhibition of local inductive apoptosis effect by the LA could lead to the improvement of the quality of oocytes obtained by follicular puncture in animals induced to superovulate with GnRH agonists.

Through the data obtained by staining with hematoxylin, incubation of 100 nM LA for 24 h induced apoptosis in BGC-1[5]. Preincubation with 100 nM Antide (competitive antagonist of GnRH) partially inhibits apoptosis this induced concentration. Also, in this same cell line was determined an increase in caspase 3 activity in the treatment with 100 nM LA at 24 and 48 h incubation. Furthermore, it was shown that pre-incubation with 100 nM Antide partially inhibits caspase activity induced by 100 nM LA. Thus, the antagonistic effect of Antide, detected morphologically showed no consistency determinations against caspase 3 activity. The Antide showed inducing effect of caspase 3 activity after 48 h incubation. Keep in mind that the activity of caspase 3 involves not only the apoptosis but also relates to other processes such as cellular differentiation and mitosis [29, 37].

The WB determination of proteins Bax and Bcl-2 revealed regulation of apoptosis by the intrinsic pathway, showing activation of the expression of Bax at 48 h incubation with 100 nM LA, this effect was inhibited by the antagonist action.

Our results in BGC-1 treated for 48 h with 100 nM LA show increased Bax/Bcl-2 relationship determined by WB and coincide with the observed *in vivo* experiments with pre-pubertal mice treated with eCG, except for the fact that Parborell et al. found no variation in levels of Bax or Bcl-2 but of Bcl-XL in pre-ovulatory follicles [21]. These results together with those obtained for the product ICQ Bax, indicate that the intracellular mechanism by which induces apoptosis in these bovine ovarian GC would be mitochondrial pathway.

The results obtained by determination of the PLD in BGC-1 showed that the LA also acts by inhibiting PLD activity responsible for apoptosis by inhibiting the extrinsic pathway. The analysis of the extrinsic pathway of regulating apoptosis through the PLD activity determination revealed inhibition of this enzyme activity in all treatments. The fact that PLD activity also decreased with Antide, lead to the idea that Antide *per se* induced the activation of caspase 3, possibly through the extrinsic pathway of regulating apoptosis, as Bax levels and Bcl-2 were not changed by Antide.

Therefore, in BGC-1, apoptosis induced by LA as dose-dependent manner via the intrinsic pathway by stimulating the expression of Bax, and through the extrinsic pathway by inhibiting PLD activity. Antide antagonist character is questionable considering the results presented.

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