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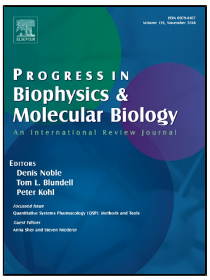

Low level laser therapy (LLLT) modulates ovarian function in mature female mice

Gonzalo Oubiña, Natalia Pascuali, Leopoldina Scotti, Mariana Di Pietro, Florenza A. La Spina, Mariano G. Buffone, Javier Higuera, Dalhia Abramovich, Fernanda Parborell

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TITLE PAGE

TITLE

Low level laser therapy (LLLT) modulates ovarian function in mature female mice

SHORT TITLE

Phototherapy modulates mice ovarian function

Gonzalo Oubiña[1], M. Sc; Natalia Pascuali[1], Ph. D; Leopoldina Scotti[1], Ph.D; Mariana Di Pietro[1], M.Sc; Florenza A. La Spina, M.Sc[1]; Mariano G. Buffone[1], Ph.D; Javier Higuera[2], Ph. D; Dalhia Abramovich[1], Ph.D and Fernanda Parborell[1], Ph.D.-

1 Instituto de Biología y Medicina Experimental (IByME) – CONICET, Buenos Aires, Argentina.

2 Higuera Dental Practice, Buenos Aires, Argentina.

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Correspondence: Fernanda Parborell, Instituto de Biología y Medicina Experimental, Vuelta de Obligado 2490, C1428ADN, Buenos Aires, Argentina.

FAX 54 011 4786 2564; e-mail: fparborell@gmail.com

ABSTRACT

It is known that LLLT has beneficial effects on several pathological conditions including wound healing, pain and inflammation. LLLT modulates biological processes, including cell proliferation, apoptosis and angiogenesis. In the present study, we examined the effect of local application of LLLT on follicular dynamics, ovarian reserve, AMH expression, progesterone levels, apoptosis, angiogenesis, and reproductive outcome in adult mice. LLLT (200 J/cm^2) increased the percentage of primary and preantral follicles, whilst decreasing the percentage of corpora lutea compared to control ovaries. LLLT-treated ovaries did not exhibit any changes regarding the number of primordial follicles. We observed a higher percentage of AMH-positive follicles (in early stages of development) in LLLT-treated ovaries compared to control ovaries. LLLT reduced the P_4 concentration and the apoptosis in early antral follicles compared to control ones. LLLT caused a reduction in the endothelial cell area and an increase in the periendothelial cell area in the ovary. Additionally, LLLT was able to improve oocyte quality. Our findings suggest that local application of LLLT modulates follicular dynamics by regulating apoptosis and the vascular stability in mouse ovary. In conclusion, these data indicate that LLLT might become a novel and useful tool in the treatment of several pathologies, including female reproductive disorders.

KEYWORDS: ovary; low level laser therapy, folliculogenesis; apoptosis; angiogenesis.

1. INTRODUCTION

Phototherapy is based on the interaction of light at low-energy density with cells and tissues, without the generation of thermal effects. Previous research has established that radiation at certain wavelengths can be beneficial to cells (Karu 1989, Karu *et al.* 2005). Low level laser therapy (LLLT) consists in irradiation within the visible to near infrared range of the light spectrum (Carroll *et al.* 2014), which has been applied in different aspects of regenerative medicine and dentistry (Huang *et al.* 2011, Carroll *et al.* 2014), with beneficial effects on wound healing (Schindl *et al.* 1999), pain (Kemmotsu *et al.* 1991) and inflammation (Hirschl *et al.* 2004, Mizutani *et al.* 2004). Moreover, many studies have shown that LLLT is a drug-free, safe and effective alternative to ameliorate these processes, whereas existing pharmaceutical treatments are not (Bjordal *et al.* 2011, Trawitzki *et al.* 2017, Ruh *et al.* 2018).

The positive biostimulatory impact of LLLT on tissue metabolism is well described in the literature (Forney & Mauro 1999, Ratkay-Traub *et al.* 2001) and it is known as "photobiomodulation". These photobiomodulatory effects are generated by the absorption of light energy by endogenous photoreceptors within the mitochondria (Karu & Kolyakov 2005, Poyton & Ball 2011). This leads to the production of reactive oxygen species (ROS) and ATP, and to a decrease in oxidative stress (Huang *et al.* 2009, Huang *et al.* 2013). In vitro studies, animal experiments and clinical studies have shown that LLLT can decrease cell apoptosis and induce cell proliferation, migration and adhesion (Bolton *et al.* 1995, Byrnes *et al.* 2005, AlGhamdi *et al.* 2012). LLLT has been shown to stimulate cell proliferation in vitro in several types of cells: fibroblasts (Yu *et al.* 1994, Carroll *et al.* 2014), keratinocytes (Grossman *et al.* 1998), endothelial cells (Moore *et al.* 2005) and

lymphocytes (Agaiby *et al.* 2000, Stadler *et al.* 2000). Besides, LLLT has been demonstrated to induce vascularization in several experimental models where blood vessel formation is critical (Corazza *et al.* 2007, Tuby 2009). In order to exert these effects, the laser parameters (wavelength, dose, power density, time of irradiation) must be combined properly (Karu 1991, Karu 1998, Karu 1999).

Despite considerable data obtained to date concerning the photobiomodulatory effect of LLLT on numerous clinical and in vitro studies, the effect of LLLT on reproductive medicine has not been elucidated. Few studies have demonstrated the effect of LLLT on the female reproductive system and, in particular, on the ovary. The first work in which LLLT is applied to ovarian granulosa cells was published in 1983 in a porcine model. The authors reported that 630nm He-Ne laser stimulates the 3-β-hydroxysteroid dehydrogenase, increasing the estrogen levels in the cells in culture and modulating progesterone levels (Gregoraszczuk *et al.* 1983). The application of the same laser wavelength generates an increase in the number of colonies in a Chinese hamster ovary (CHO) cell culture (Al Watban & Andres 2000). Additionally, it has been demonstrated that LLLT increases VEGF levels and MAPK activity in a human granulosa cell line (KGN) with a 830nm laser (Kawano *et al.* 2012). When applied to a more complex system of in vitro maturation of bovine embryos, LLLT increased the mitochondrial membrane potential in cumulus cells (Soares *et al.* 2014). In this system, the oocytes showed an increase in MAPK levels without affecting the meiotic progression or embryo production index, evidencing that LLLT can enhance ovarian cell metabolism. Recently, El Faham et al (2018) proposed that the use of LLLT in the field of infertility may have a strong impact as a new adjuvant therapy in enhancing endometrial receptivity and regeneration (El Faham *et al.* 2018).

However, to date no reports have addressed the in vivo effects of LLLT on ovarian function and the mechanisms involved. The hypothesis to be addressed in this study is that LLLT will improve reproductive function by modulating follicular dynamics, apoptosis and vascular stability in the ovary.

In the ovary, cell proliferation and angiogenesis are two intimately related processes in terms of follicular development, atresia, ovulation and luteogenesis (Zeleznik *et al.* 1981, Stouffer *et al.* 2001). Considering that LLLT modulates several biological processes that include cell proliferation, apoptosis, inflammation, migration and angiogenesis, we attempted to determine the in vivo effect of LLLT on the ovary from mature female mice under physiological conditions. Therefore, in this study, we examined the effect of local application of LLLT on follicular dynamics, ovarian reserve, anti-Müllerian hormone (AMH) expression, progesterone levels, ovarian apoptosis, blood vessel formation and stability, and reproductive outcomes in mature female mice.

2. MATERIALS AND METHODS

2.1 Hormones and drugs

Equine chorionic gonadotropin (eCG) was provided by Syntex S.A. (Buenos Aires, Argentina) and human chorionic gonadotrophin (hCG) was provided by Elea (Endocorion; Buenos Aires, Argentina). Proteinase K, acrilamyde, bis-acrilamyde, sodium dodecyl sulfate (SDS), ethylene diamine tetraacetic acid (EDTA), bovine serum albumin (BSA), Na2HPO4, NaH2PO4, sodium azide, NP-40, Tween-20 and biotin-conjugated lectin from *Bandeiraea simplicifolia* (BS-1) (L3579) were from Sigma-Aldrich (St. Louis, MO, USA). 3,3′-diaminobenzidine (DAB) was from Roche Applied Science (Mannheim, Germany).

The Apoptag Peroxidase in Situ apoptosis detection kit (S7100) was from Merck Millipore (Darmstadt, Germany). The details, suppliers and dilution of antibodies used in this study are reported in Table 1. All other chemicals were of reagent grade and were obtained from standard commercial sources.

2.2 LLLT parameters

Laser treatments were performed using a red 606 nm, continuous wave, diode laser (DMC Equipment LTDA, Brasil). Ovaries were irradiated with a power density of 2.5 W/cm^2 in a spot of 4 mm^2 for 40 or 80 seconds to achieve energy densities of 100 J/cm^2 or 200 J/cm^2 respectively.

2.3 Animals

Care and housing of mice were carried out at the Instituto de Biología y Medicina Experimental (IByME), Buenos Aires, Argentina. All animals were allowed food and water ad libitum and kept on a 12-hour light/dark cycle. All experimental protocols were approved by the Animal Experimentation Committee of the IBYME and conducted according to the guide for the care and use of laboratory animals of the National Institute of Health (USA).

2.4 *In vivo* ovarian LLLT application

Six to eight-week first filial generation hybrid mice of a cross between C57BL/6 male x Balb/c female mice in proestrus were used. Prior to the surgery, the proestrus stage of all mice was confirmed by vaginal citology as described by Byers et al. (Byers *et al.* 2012).

Animals were anesthetized with ketamine HCl (100 mg/kg; Holliday-Scott, Buenos Aires, Argentina) and xylazine (10 mg/kg; Konig Laboratories, Buenos Aires, Argentina). The ovaries were exteriorized through an incision made in the dorsal lumbar region. Subsequently, one ovary was irradiated with LLLT as detailed above. The contralateral ovary was used as a control, as it did not receive LLLT and remained exteriorized a similar amount of time to the irradiated ovary. After irradiation, ovaries were replaced, and the incision sutured. This experimental design represents a good model to study follicle development since it has the distinct advantage of allowing direct comparison between ovaries with synchronized cycles and similar levels of gonadotropins (Dhanasekaran & Moudgal 1989, Hughes & Gorospe 1991, Abramovich *et al.* 2006, Parborell *et al.* 2008, Choi *et al.* 2010).The animals were euthanized 24 h after surgery by CO_2 inhalation. The ovaries were removed, weighed and cleaned of adhering tissue in culture medium for subsequent assays.

2.5. Ovarian morphology

After removal, the ovaries were immediately fixed in Bouin solution for 12 h, dehydrated by graduated ethanol washes and embedded in paraffin. All follicle counts were performed by two independent researchers, blinded to the experimental groups. To prevent counting the same follicle twice, 5µm step sections were mounted at 50µm intervals onto microscope slides. To count the number of follicles and corpora lutea per ovarian section, slides were stained with hematoxylin and eosin (H&E). Structures were classified as previously described (Sadrkhanloo *et al.* 1987, Andreu *et al.* 1998, Pascuali *et al.* 2018) into the following groups: primary follicles (PriFs), preantral follicles (PAFs), antral follicles (AFs), atretic follicles (AtrFs) and corpora lutea (CLs). The number of PriFs, PAFs, AFs, AtrFs

and CLs was determined in four sections from each ovary (one control ovary and one treated ovary, n= 5 animals). The total number of ovarian structures was defined as 100%. Data are expressed as the percentage of each structure per ovary.

2.6. Immunohistochemistry (IHC)

For immunohistochemical localization of proteins, the avidin-biotin–peroxidase complex was used on ovarian sections as previously described (Pascuali *et al.* 2018). The utilized primary antibodies and their concentration are detailed in Table 1, lectin BS-1 was diluted 1:75 in PBS. Negative controls were obtained in the absence of the primary antibody. Stained sections were analyzed and digitally photographed at 40×, 100× or 400× magnification by conventional light microscopy as needed (Nikon, Melville, NY, USA).

2.7. Evaluation of Anti Müllerian Hormone (AMH) expression

Immunostaining for AMH was performed in ovarian sections to identify follicles in early stages of development. Follicles were scored positive if specific AMH staining was present in at least 80% of the granulosa cells. AMH–positive follicles were counted by two independent researchers, blinded to the experimental groups (4 sections/ovary, n=5) and the percentage of AMH-positive follicles over the total number of follicles was calculated.

2.8. Evaluation of apoptosis

To identify granulosa cell apoptotic nuclei, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) was used on ovarian sections following manufacturer's instructions (Apoptag S7100, Millipore). Quantification was performed by two independent researchers, blinded to the experimental groups. All early antral follicles

(EAFs) found in a section were photographed at 400x (four sections per ovary, n=5 animals) and were analyzed using the Image J software (Image Processing and Analysis in Java, National Institutes of Health, Bethesda, MD, USA). Percentages of apoptotic granulosa cells were calculated using the Cell Counter tool. The number of granulosa cells with labeled nuclei was manually determined for each EAF and divided by the total number of nuclei. EAFs were selected since in this stage follicles become most susceptible to atresia and thus are considered the most finely regulated checkpoint in folliculogenesis (Chun *et al.* 1996).

2.9. Evaluation of ovarian reserve

Immunostaining for the germ cell specific marker DDX-4 was performed in ovarian sections (4 sections/ovary, n=5) to identify primordial follicles. These follicles consist of an oocyte surrounded by one thin layer of flattened granulosa cells. Primordial follicle counts were performed by two independent researchers, blinded to the experimental groups. Primordial follicles were counted in four sections per ovary. The section area was measured using Image Pro Plus 3.0 (Media Cybernetics, Silver Spring, MA, USA) and the number of primordial follicles per mm^2 was calculated for each ovary treated with LLLT and its control.

2.10. Evaluation of vascular areas

Microphotographs of whole ovarian sections (4 sections/ovary; n=5 animals) from lectin BS-1 and α-SMA staining were processed using Image Pro Plus. In sections stained with lectin BS-1 (endothelial cell marker), the relative vascular area was measured. The total area occupied by follicles and stroma was manually delimited, excluding corpora lutea

from the analysis. The vascular area (lectin BS-1-positive cells) was determined by thresholding the lectin BS-1-positive stained area. Relative vascular area was calculated dividing the absolute vascular area by the corresponding total area. The presence of pericytes and vascular smooth muscle cells (VSMC) was detected by immunolabeling with a specific cell marker, α-SMA (Redmer *et al.* 2001, Robinson *et al.* 2009). The relative periendothelial area was calculated as described for relative vascular area.

2.11. Steroid extraction from ovarian tissue

Steroid extraction from irradiated whole ovaries and their controls was performed as previously described (Irusta *et al.* 2003, Irusta *et al.* 2007a). Labeled steroids were added as internal standards during extraction, with a recovery percentage between 60 and 80%. The final residues were resuspended in RIA buffer (Na2HPO4 40 mM; NaH2PO4 39.5 mM, NaCl 155 mM, sodium azide 0.1%, gelatin 1%, pH = 7.0) and stored at −20 °C until further analysis.

2.12. Radioimmunoassay (RIA)

Progesterone (P_4) concentration was measured by RIA in control and LLLT-treated ovaries (n = 5/group) (Irusta *et al.* 2003, Irusta *et al.* 2007b) by using a specific antibody supplied by Dr. G.D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO, USA). Under these conditions, the intra-assay and inter-assay variations were 8.0% and 14.2% for P_4. The values are expressed as ng hormone per mg ovary.

2.13. Western blot analyses

231 Protein extracts were obtained from control and contralateral irradiated ovaries as
232 previously described (Pascuali *et al.* 2018) . Total protein concentrations in the samples
233 were measured by the Bradford assay.
234 Protein resolution in SDS-polyacrylamide gel and transfer to nitrocellulose membranes was
235 performed as previously described (Pascuali *et al.* 2018). The utilized primary antibodies
236 and their concentration are reported in Table 1.
237 In each experiment, equal amounts of protein were loaded for all samples, and both groups
238 were loaded on the same gel. All the gels were run under the same experimental conditions.
239 Protein expression was compared and analyzed with densitometric studies by ImageJ. The
240 density of each band was normalized to the density of the β-actin or GAPDH band that was
241 used as an internal control. Arbitrary optical density data are expressed as arbitrary units ±
242 SEM.
243

244 **2.14. Superovulation and *in vitro* fertilization**

245 48 h after *in vivo* treatment with LLLT as described in section **2.4**, mice were injected
246 intraperitoneally with hormones as previously described by Buffone (Buffone *et al.* 2009).
247 Briefly, superovulation was induced using eCG (5 IU) at 6:30 PM, followed 48 h later by
248 hCG (5 IU). Cumulus enclosed, metaphase-arrested eggs were collected 14 h post-hCG in
249 Whitten's/Hepes containing 0.01% polyvinyl alcohol (PVA) (Whitten & Biggers 1968).
250 PVA was used prior to insemination in culture media devoid of BSA to keep the oocytes
251 from sticking to the culture dish. *In vitro* fertilization was performed as described
252 previously (Buffone *et al.* 2008) with modifications. Sperm were obtained from C57BL/6 ×
253 BalBc F1 males from the vasa deferentia and caudae epididymides and placed in a 900-µl
254 drop of TYH medium (Toyoda *et al.* 1971) supplemented with 4 mg/ml BSA under mineral

oil. The tissue was cut into sections and the sperm were allowed to swim out into the media for 10-20 min. Eggs and sperm (105/ml) were mixed in a 30 µl drop of TYH medium containing 4 mg/ml BSA and then cultured at 37°C in an atmosphere of 5% CO_2, 5% O_2, 90% N_2 for 3 h. Eggs were removed from the drop, washed three times in Whitten's/HEPES and then cultured in KSOM medium (Specialty Media) (Summers *et al.* 2000). Phase-contrast microscopy was used to evaluate fertilization. The success of fertilization was determined by morphological assessment of pronucleus formation and cleavage to the 2-cell stage after 27 hours. 2-cell embryos were cultured for 4 days and the developmental stage of each embryo was determined.

2.15. Data presentation

The data are expressed as mean ± SEM. Statistical analysis was performed using paired Student's t-test. Two-tailed values of $p<0.05$ were considered significant. Statistical analyses were performed with the Prism GraphPad 5.0 software (GraphPad Software, Inc., San Diego, CA, USA).

3. RESULTS

3.1. *In vivo* effect of LLLT on ovarian morphology

To evaluate the effect of LLLT on folliculogenesis dynamics in adult mice in proestrus, the percentages of follicles in various stages of development were determined in H&E stained ovarian sections. **Figure 1** shows that irradiation with 100 J/cm^2 of LLLT caused a significant increase in the percentage of PAFs (control: 13.0±1.8 vs LLLT 100 J/cm^2: 19.3±3.7, $p<0.05$) compared to the control group. Treatment with 200 J/cm^2 significantly

increased the percentage of PriFs (control: 7.6±0.6 vs LLLT 200 J/cm^2: 12.6±1.0, $p<0.05$) and PAFs (control: 13.0±1.8 vs LLLT 200 J/cm^2: 21.7±1.0, $p<0.05$), whilst it decreased the percentage of CLs (control: 24.9±4.5 vs LLL 200 J/cm^2: 10.3±1.7, $p<0.05$) compared to control. Additionally, we evaluated whether the changes observed in follicle growth by the 200 J/cm^2 treatment were maintained over time. Eight days post-irradiation, no differences in the percentages of any follicular structures were found between irradiated and control ovaries (*data not shown)*. Based on literature data and on these results, the dose of 200 J/cm^2 was used for the following assays.

3.2. *In vivo* effect of LLLT on ovarian reserve and expression of AMH

Considering that the ovarian reserve is composed by a limited pool of primordial follicles in a quiescent state, we decided to evaluate the effect of LLLT on this follicular population (Fig. 2). LLLT-treated ovaries did not show any change in the number of primordial follicles compared to untreated ovaries (control: 16.2±2.5 vs LLLT 200 J/cm^2: 14.0±2.0).

Since AMH is produced only in the early stages of follicular development, and is consequently used as an ovarian reserve marker, we performed an immunohistochemical detection of this hormone (Fig. 3E). We observed a higher percentage of follicles in early stages of development, as evidenced by higher rates of AMH-positive follicles in LLLT-treated ovaries compared to control ovaries (control: 57.6±1.9 vs LLLT 200 J/cm^2: 66.5±1.4, $p<0.05$)

3.3. *In vivo* effect of LLLT on ovarian weight and steroid hormone concentration

The effects of LLLT on ovarian weight and tissue P_4 concentration are summarized in *Table 2*. The weight of ovaries did not change in response to irradiation (n =12). Ovarian P4 concentration in LLLT-treated ovaries decreased compared to untreated ovaries ($p<0.05$).

3.4. *In vivo* effect of LLLT on ovarian apoptosis

Since LLLT has been shown to reduce apoptosis in several tissues, we decided to evaluate the apoptosis in EAFs by TUNEL assay (Fig. 4A, B). Treatment with LLLT significantly decreased the percentage of apoptotic granulosa cells in EAFs compared to non-irradiated ovaries (control: 5.01% ± 1.16 vs LLLT: 2.84% ± 0.73, $p<0.05$). Additionally, we assessed the expression of pro-apoptotic BAX protein and anti-apoptotic BCL-2 and BCLX-L proteins. No changes were observed in the BAX/BCL-2 and BAX/BCLX-L ratios from LLLT-treated ovaries in comparison with control ovaries (Fig. 4C, D).

3.5. *In vivo* effect of LLLT on ovarian angiogenesis

To determine if the changes observed in follicular dynamics following application of LLLT could be attributed to alterations in blood vessel formation and stability, staining with lectin BS-1 and α-SMA antibody were performed (Fig. 5 and 6, respectively). Relative vascular area in follicles and stroma significantly decreased in LLLT-treated ovaries compared to control ovaries (control: 2.97±0.65 vs LLLT: 2.05±0.39, $p<0.05$). Nevertheless, quantification of immunolabeling by α-SMA showed an increase in relative periendothelial

area in LLLT-treated ovaries in comparison with control ovaries (control: 9.35±1.36 vs LLLT: 13.56±0.84, $p<0.05$).

3.6. *In vivo* effect of LLLT on oocyte quality

To evaluate whether the local treatment with LLLT affected oocyte quality, we performed IVF with oocytes recovered from superovulated females (Fig. 7). The total number of recovered oocytes from LLLT-treated ovaries was significantly lower than control ovaries (control: 16.4±2.0 vs LLLT: 12.6±1.9, $p<0.05$). Oocytes were further incubated to evaluate fertilization ability. A higher percentage of oocytes obtained from irradiated ovaries developed into two-cell embryos compared to oocytes from control ovaries (control: 55.3% ± 7.5 vs LLLT: 86.5% ± 3.5, $p<0.05$). Additionally, to determine whether LLLT affects preimplantation embryo development, progression of the two-cell embryos was monitored. No significant differences in the percentage of two-cells embryos that reached blastocyst stage were found between oocytes obtained from irradiated and control ovaries (*data not shown*).

4. DISCUSSION

This study is the first to demonstrate that the ovarian application of LLLT modulates follicular development without altering the ovarian reserve, decreases apoptosis in granulosa cells and enhances oocyte quality in mature female mice. In addition, we showed that the application of LLLT decreases the endothelial cell area and increases the

342 periendothelial cell area in the ovaries from adult mice. These results suggest that LLLT
343 regulates folliculogenesis, vascular development and cell survival in the mice ovary.
344 In our study we used the contralateral ovarian model developed in mice to evaluate the
345 biomodulatory effect of the LLLT on ovarian function. The advantage of this model is that
346 the treatment can be performed in a single ovary and the contralateral ovary serves as a
347 control. Several authors and we have used this model to evaluate the ovarian physiology
348 and pathology (Woodruff *et al.* 1990, Nishimura *et al.* 1995, Abramovich *et al.* 2010,
349 Pascuali *et al.* 2015).

350 Ovarian histology showed that LLLT dose of 200 J/cm^2 caused an increase in the
351 percentage of PAFs and PriFs, as well as a reduction in the percentage of CL. Three
352 possible explanations emerge from these observations: i) a decrease percentage of CL is
353 caused by a higher percentage of CL in regression. However, a detailed examination of the
354 ovary did not display any evidence of CL regression after LLLT, ii) a decrease percentage
355 of CL occurred as a result of alterations in the ovulation. However, this also may not be the
356 case since the number of antral follicles is not significantly different compared to the
357 control group; iii) this dose of LLLT delays the early follicle development. In this case,
358 higher percentages of primary and preantral follicles are observed, suggesting that this may
359 be the possible cause of the alterations observed in follicular dynamics and the decrease in
360 the percentage of CL. Primordial follicles represent the most important follicular population
361 because the ovarian reserve is non-renewable. In our study, we observed that the LLLT did
362 not affect this follicular stage compared to the untreated ovaries. Based on primordial
363 follicle density, the ovarian reserve was unchanged in LLLT-treated ovaries. These results
364 suggest that the higher proportion of primary and preantral follicles in LLLT-treated

ovaries would be a consequence of delayed follicular development and not of the "burnout" phenomenon (follicular activation). More studies are needed to elucidate this point. Considering that LLLT does not provoke harmful effects on the population of primordial follicles, it could represent a possible treatment to regulate follicular dynamics in reproductive disorders with ovarian dysfunction.

AMH is synthesized by granulosa cells from primary to early antral follicles and its main function is to inhibit activation of primordial follicles and to maintain the dormant ovarian reserve. We observed a greater population of follicles in early stages of development with a positive signal for AMH in LLLT-treated ovaries compared to their contralateral ovaries. This result is consistent with the data from ovarian morphology. Besides, in our study, concentrations of P4 in the ovarian tissue were decreased by LLLT. This concurs with the results obtained from ovarian histology, which showed a lesser percentage of CL in LLLT-treated ovaries. Since luteal structures produce ovarian P4, the decrease in CL caused by LLLT treatment could be responsible for the low concentrations of this hormone.

Despite the presence of atresia at each follicular stage, the population of follicles with greater susceptibility to degenerate is the early antral stage (Hirshfield 1988). During this stage a greater contribution of hormones and survival factors is required, and the presence of a well-formed vascular network to provide them is essential (Chun *et al.* 1996).

The application of LLLT caused a decrease in the percentage of apoptotic cells in EAFs compared to untreated ovaries. This result suggests that LLLT possesses an anti-apoptotic effect on granulosa cells from EAFs, thereby modulating the process of atresia. These findings are consistent with previous research indicating that LLLT reduces apoptosis in *in vitro* studies, animal models and clinical studies (Bjordal *et al.* 2006, Hu *et al.* 2007, Zhang

388 *et al.* 2008). Several members of the Bcl-2 gene family have been described as the main
389 participants in the cascade of events that either activate or inhibit apoptosis (Boise *et al.*
390 1993). Bcl-2-related proteins can be separated into anti-apoptotic and pro-apoptotic
391 members, and the balance between these counteracting proteins determines cell fate (Oltval
392 *et al.* 1993). In our experimental model, no change was observed in the BAX/BCL-2 and
393 BAX/ BCLX-L ratios between LLLT-treated and control ovaries. Based on the data
394 described earlier, LLLT could be beneficial for suppressing apoptosis of follicular cells,
395 thus rescuing follicles from atresia. The possible modulation by LLLT of the expression or
396 activity of other pro- or anti-apoptotic proteins in the ovary is not ruled out. More studies
397 are required to elucidate the mechanisms involved in the anti-apoptotic effect of LLLT on
398 the ovary.

399 To determine whether the changes induced by LLLT in folliculogenesis and apoptosis in
400 EAFs can be attributed to differences in the quantity and maturity of blood vessels in the
401 follicular thecal compartment and ovarian stroma, we decided to quantify the endothelial
402 and periendothelial areas. The results showed that LLLT decreased the relative endothelial
403 area, but increased the relative periendothelial area in the ovaries. It is known that the
404 extension and maturity of the stromal vasculature that surrounds the earliest follicles during
405 their development is crucial for the initial recruitment (Stouffer *et al.* 2001, Fraser 2006).
406 As the follicles grow, vascular networks develop in their thecal compartments. The
407 presence of fewer blood vessels can be also due to a delay in the earliest stages of
408 folliculogenesis and, consequently, a lower proportion of CLs. This is consistent with the
409 results observed by histological analysis of the ovaries. Nevertheless, the blood vessels
410 present in the irradiated ovary have a greater coating of mural cells, which confers greater

411 vascular stability. In the current study, the results suggest that LLLT induces the
412 recruitment of mural cells to the vessels, providing a preferential supply of nutrients to the
413 follicles. Another explanation could be that the recruitment of these perivascular cells by
414 LLLT reduces new blood vessel sprouting from pre-existing vessels. These findings are
415 supported by previous observations in several animal models and clinical studies that
416 described beneficial LLLT effects on vasculature in numerous diseases. Gavish *et al*.
417 (2006) have demonstrated that LLLT stimulates cell proliferation and collagen synthesis,
418 and modulates the equilibrium between regulatory matrix remodelling enzymes in porcine
419 primary aortic smooth muscle cells (Gavish *et al.* 2006). It has also been demonstrated that
420 LLLT induces vascularization, promoting healing of acute and chronic wounds (Yu *et al.*
421 1997, Hopkins *et al.* 2004, Zhang *et al.* 2010).

422 According to the results obtained by ovarian morphology analysis, we decided to evaluate
423 the effect of LLLT on the quality of the oocytes. LLLT decreased the number of ovulated
424 eggs recovered from the ampulla of irradiated ovaries and increased the percentage of
425 embryos in two-cell stage. This decrease in recovered oocytes from irradiated ovaries is
426 consistent with our results of ovarian morphology, where we observed that LLLT reduces
427 the percentage of CL. However, the higher quality of the oocytes reinforces the hypothesis
428 that LLLT modulates the development of the ovarian vasculature by means of the
429 recruitment of mural cells to the endothelium, promoting the maturity and stability of the
430 blood vessel and follicular cell function. In agreement with our observations, Soares *et al.*
431 (2014) have shown the photobiological effect of low-level laser irradiation on a bovine
432 embryo production system. These authors have demonstrated that LLLT is able to modulate

the metabolism of granulosa cells and oocytes, enhancing *in vitro* maturation and embryo development (Soares *et al.* 2014).

More evidence is needed to elucidate the relevance of our observations regarding LLLT effects on vasculature and apoptosis during follicular development and to determine the molecular mechanisms of LLLT which allow a normal ovarian function. Therefore, our data indicate that LLLT is able to modulate the follicular dynamic, by regulating apoptosis and the development of a mature vasculature in the mouse ovary. Additionally, LLLT is able to enhance the quality of oocytes in mice.

Taking into account the permanent development of laser technology and the low cost of this therapy, LLLT might become a novel and useful tool in the treatment of several disorders including female reproductive disorders (premature ovarian failure, polycystic ovary syndrome, ovarian hyperstimulation syndrome and endometriosis). Nonetheless, further future studies are required to verify the effectiveness of this therapeutic strategy and to unravel the mechanisms underlying its beneficial effects.

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Legends

Figure 1. ***Effect of local LLLT on folliculogenesis in adult female mice***. A-F: photomicrographs of H&E stained sections from control and LLLT-irradiated ovaries. A, C, E: scale bars represent 200µm. B, D, F: insets. Scale bars represent 100µm. G, H: quantification of folicular structures from control and LLLT-irradiated ovaries 24h after treatment (G. 100 J/cm^2, n=5; H. 200 J/cm^2, n=5). PriF, primary follicles. PAF, preantral follicles. AnF, antral follicles. AtrF, atretic follicles. CL, corpora lutea. I: quantification of total structure density in control and LLLT-treated ovaries (200 J/cm^2, n=5) 24h after treatment. Data are expressed as mean ±SEM. * $p<0.05$; paired t-Student test.

Figure 2. ***Effect of local LLLT on the ovarian reserve in adult female mice.*** A-D. Representative photomicrographs of histological sections from control or LLLT-treated ovaries (200 J/cm^2) stained with anti-DDX4 for primordial follicle identification. A, B: scale bars represent 100µm. C, D: scale bars represent 50µm. D: inset of Fig. 2.B. Arrowheads indicate primordial follicles, arrows indicate primary follicles, pyramids indicate preantral follicles. E. Number of primordial follicles per mm^2 from control and LLLT-treated ovaries 24h after treatment (200 J/cm^2, n=5). Data are expressed as mean ±SEM. Numbers in parenthesis indicate the total of primordial follicles counted per group. Paired t-Student test showed no statistically significant differences between groups.

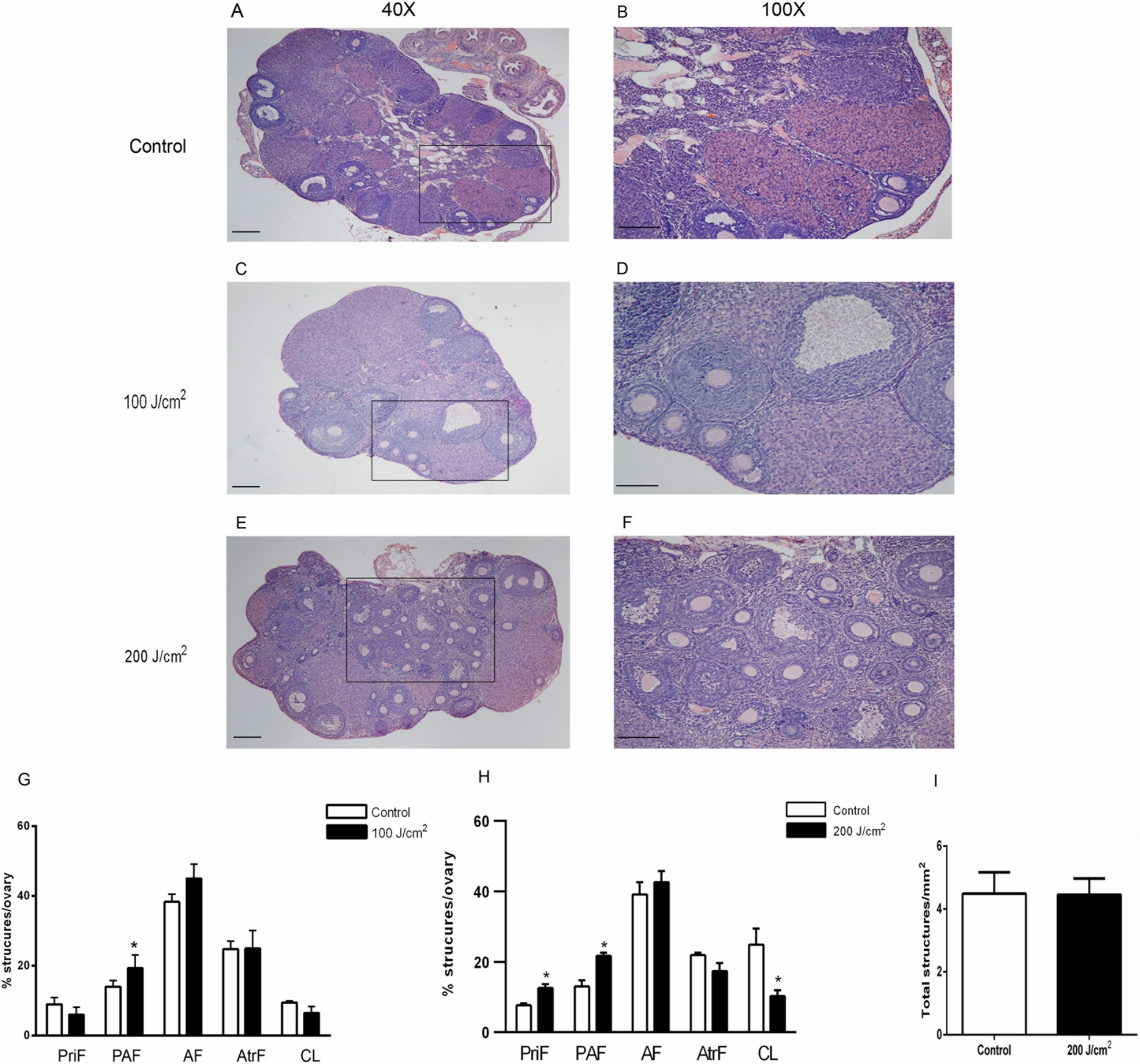
Figure 3. ***Effect of LLLT on expression of Anti-Müllerian Hormone (AMH) in adult female mice.*** A-D: representative photomicrographs of AMH immunostaining in control and LLLT-treated (200 J/cm^2) ovaries. A, C: scale bars represent 200 µm. B, D: scale bars represent 100 µm. E: percentage of AMH-positive follicles from control and LLLT-treated ovaries 24h after treatment (200 J/cm^2, n=5). Data are expressed as mean ±SEM. * $p<0.05$; paired t-Student test.

Figure 4. ***Effect of LLLT treatment on ovarian apoptosis.*** A: representative microphotographs of early antral follicles from control and LLLT-treated (200J/cm^2, n=5) ovaries stained with TUNEL. Scale bars represent 50 µm. B: TUNEL-based quantification of % of apoptotic granulosa cells in early antral follicles. C, D: western blot analyses of BAX, BCL-2, BCLX-L and β-actin expression in control and LLLT-treated (200J/cm^2, n=5) ovaries. A representative blot from each protein is shown. D: densitometric analyses of BAX/BCL-2 and BAX/BCLX-L ratios. Data are expressed as mean ±SEM. * $p<0.05$; paired t-Student test, n=5.

Figure 5. ***Effect of LLLT on ovarian endothelial cell area.*** A-D. Representative photomicrographs of histological sections from control or LLLT-treated (200 J/cm^2) ovaries stained with lectin BS-1 to identify vascular endothelium. A, C: scale bars represent 100 µm. B, D: scale bars represent 50 µm. Arrows indicate positive staining in the thecal vascular compartment. E: quantification of relative vascular area in ovarian follicles and stroma. Data are expressed as mean ±SEM. * $p<0.05$; paired t-Student test, n=5.

692 **Figure 6.** ***Effect of LLLT on ovarian periendothelial cell area.*** A-D. Representative
693 photomicrographs of histological sections from control or LLLT-treated (200 J/cm^2)
694 ovaries inmunostained for smooth muscle actin (α-SMA) to identify periendothelial
695 cells. A, C: scale bars represent 100 μm. B, D: scale bars represent 50 μm. E:
696 quantification of relative periendothelial area in ovarian follicles and stroma. Data are
697 expressed as mean ±SEM. * $p<0.05$; paired t-Student test, n=5.

698 **Figure 7.** ***Effect of LLLT of oocyte quality.*** A: number of recovered oocytes from
699 control and LLLT-treated (200J/cm^2, n=5) ovaries from superovulated females. B:
700 percentage of oocytes that reached 2-cell embryo stage after undergoing IVF. Data are
701 expressed as mean ±SEM. * $p<0.05$; paired t-Student test.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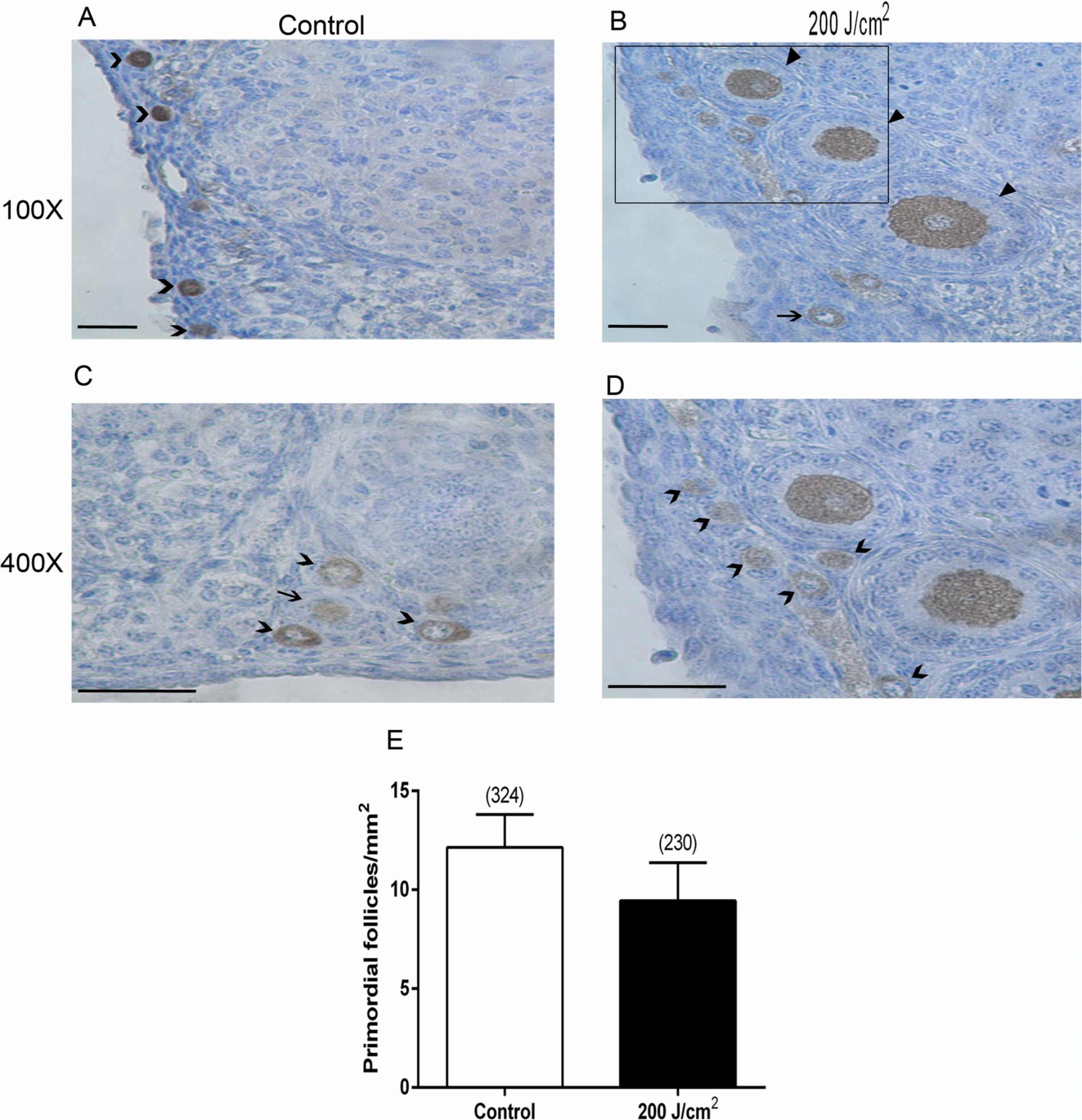
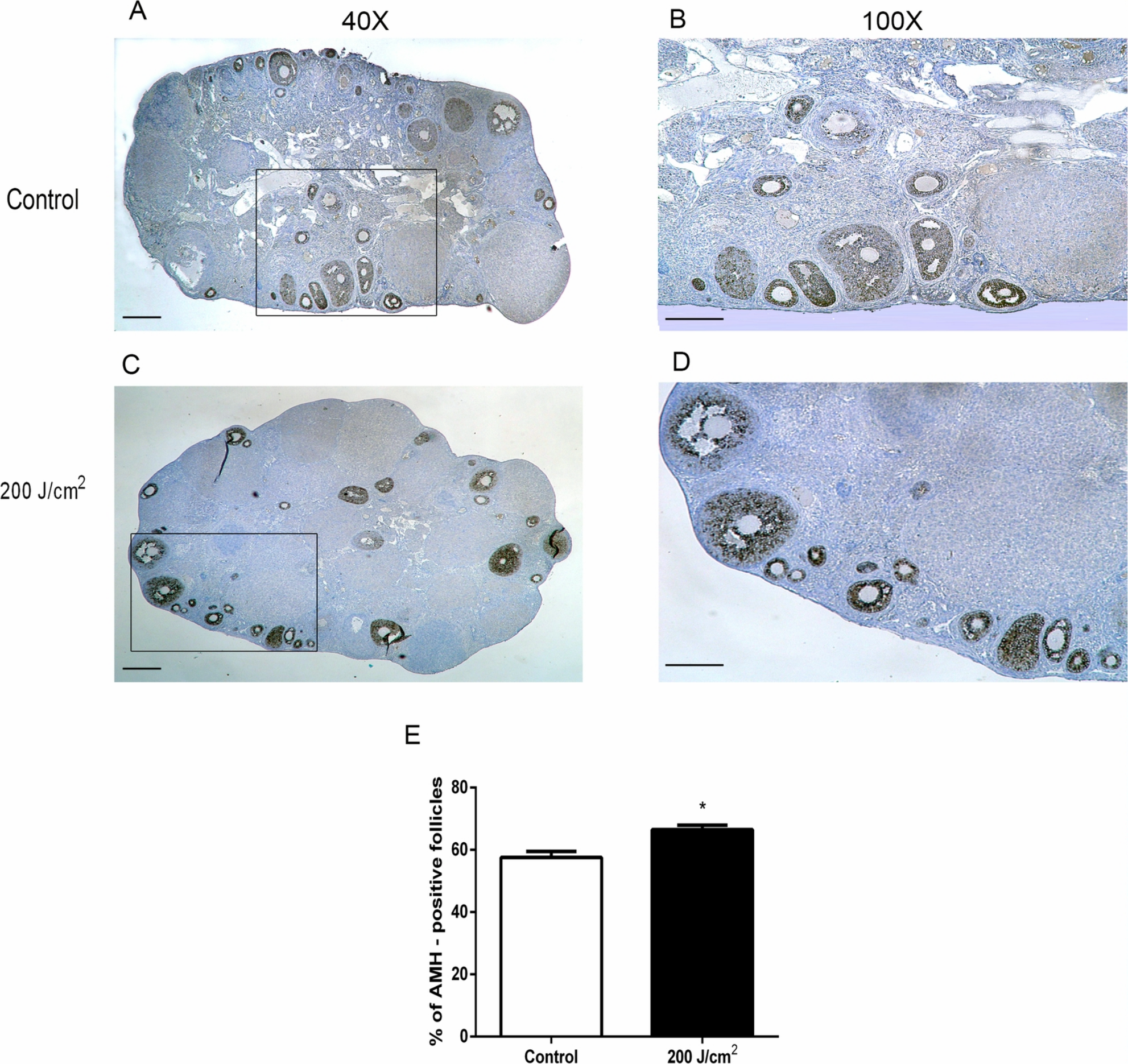
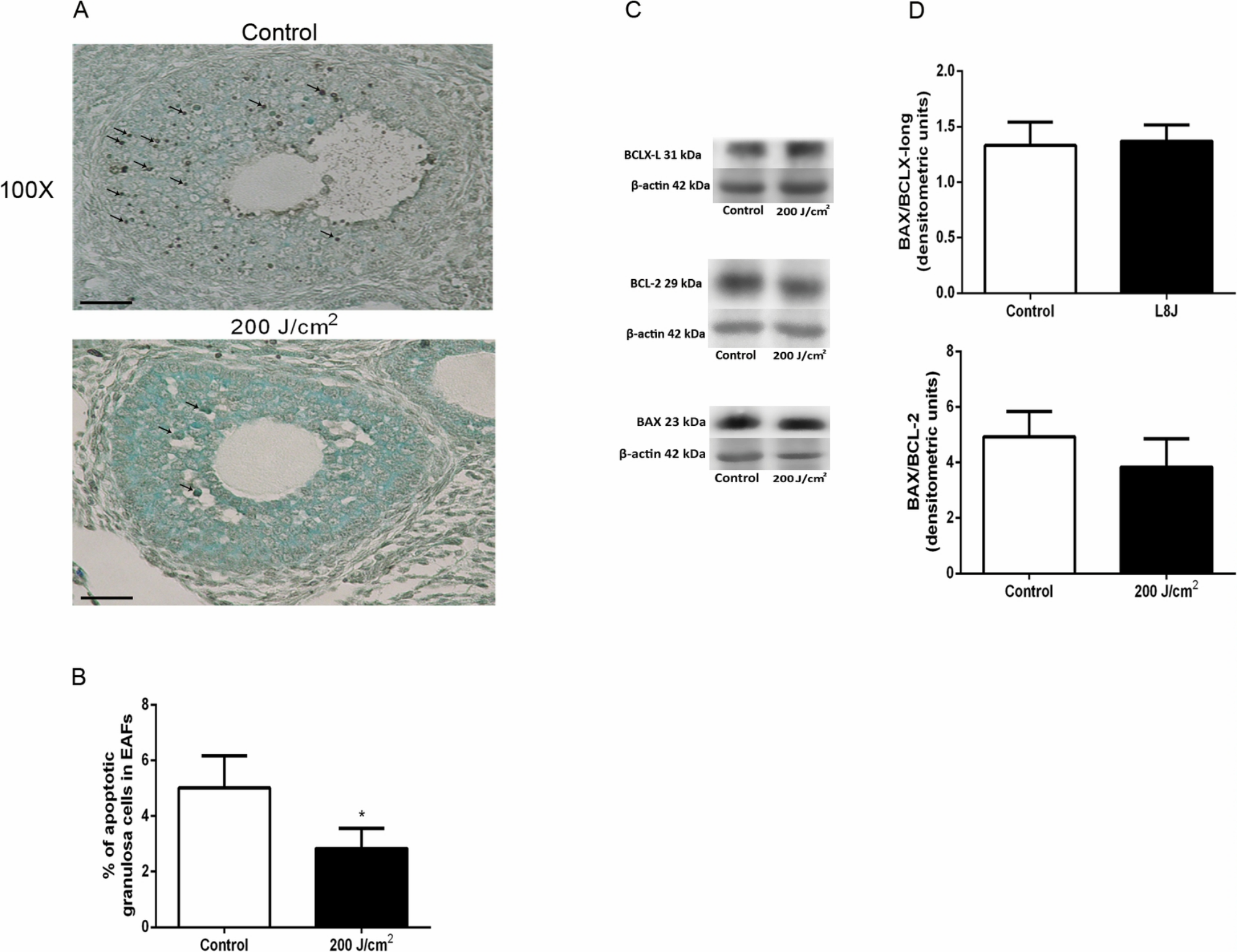
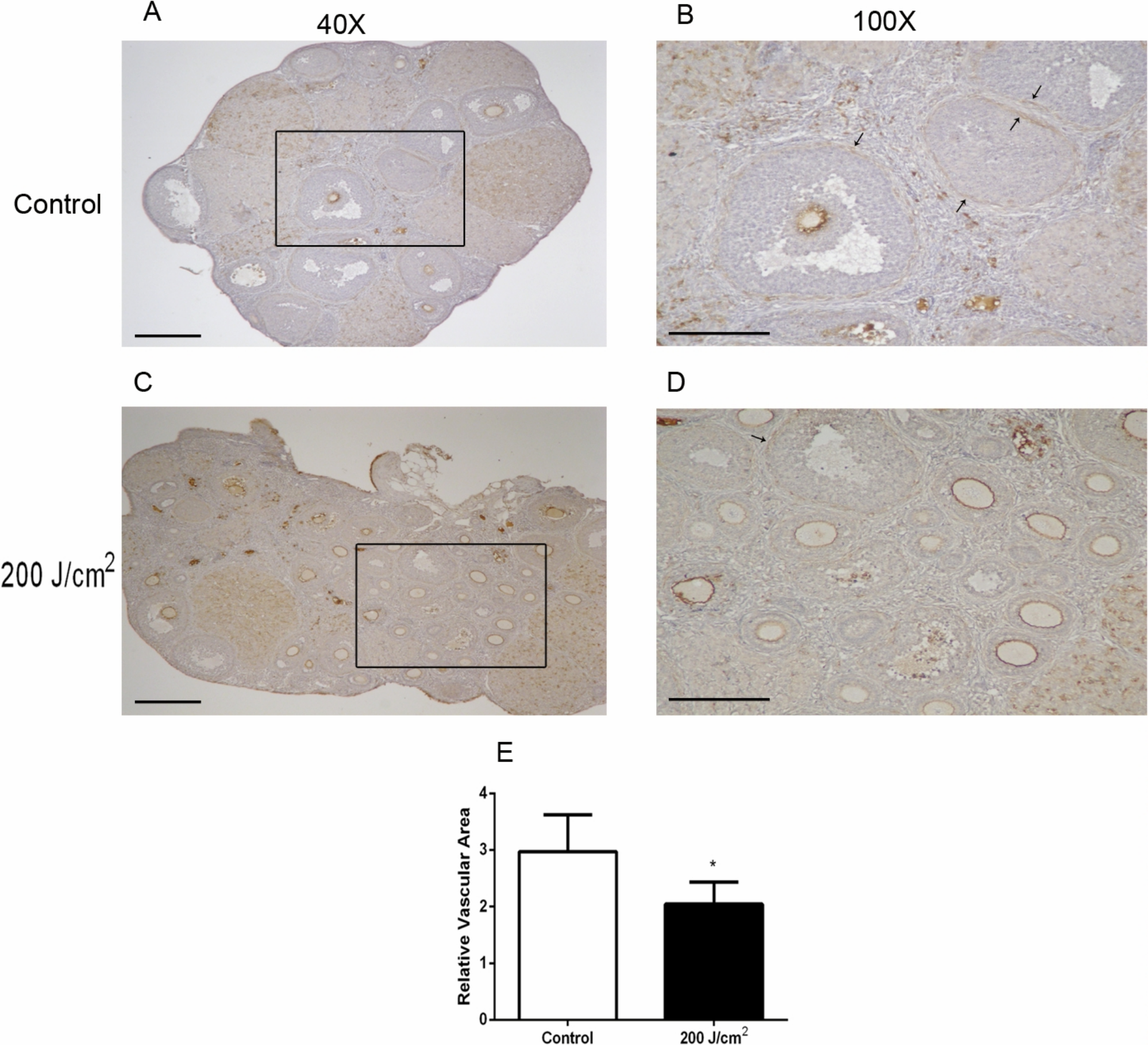
B
100X
Control
C
D
100 J/cm2
E
F
200 J/cm2
G
Control
100 J/cm2
% strucures/ovary
60
40
20
0
PriF
PAF
AF
AtrF
CL
H
Control
200 J/cm2
% strucures/ovary
60
40
20
0
PriF
PAF
AF
AtrF
CL
I
Total structures/mm2
6
4
2
0
Control
200 J/cm2


A
Control
B
200 J/cm2
100X
C
D
400X
E
Primordial follicles/mm2
15
10
5
0
(324)
(230)
Control
200 J/cm2


A
40X
B
100X
Control
C
D
200 J/cm2
E
% of AMH - positive follicles
80
60
40
20
0
*
Control
200 J/cm2


A
Control
100X
200 J/cm2
B
% of apoptotic granulosa cells in EAFs
8
6
4
2
0
*
Control
200 J/cm2
C
BCLX-L 31 kDa
β-actin 42 kDa
Control
200 J/cm2
BCL-2 29 kDa
β-actin 42 kDa
Control
200 J/cm2
BAX 23 kDa
β-actin 42 kDa
Control
200 J/cm2
D
BAX/BCLX-long (densitometric units)
2.0
1.5
1.0
0.5
0.0
Control
L8J
BAX/BCL-2 (densitometric units)
8
6
4
2
0
Control
200 J/cm2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D
200 J/cm2
E
Relative Vascular Area
4
3
2
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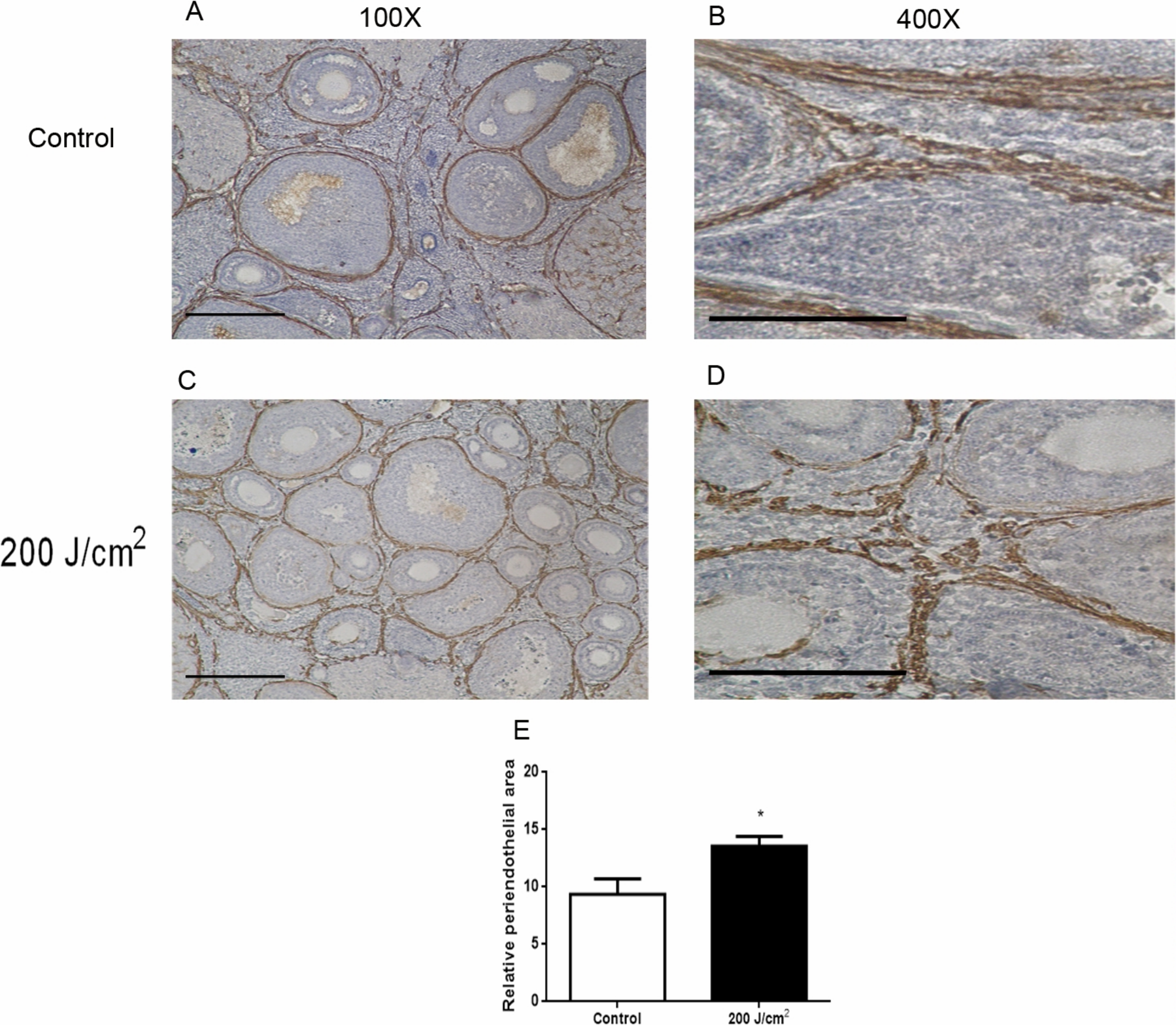
Control
200 J/cm2


A
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B
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Control
C
D
200 J/cm2
E
Relative periendothelial area
20
15
10
5
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*
Control
200 J/cm2

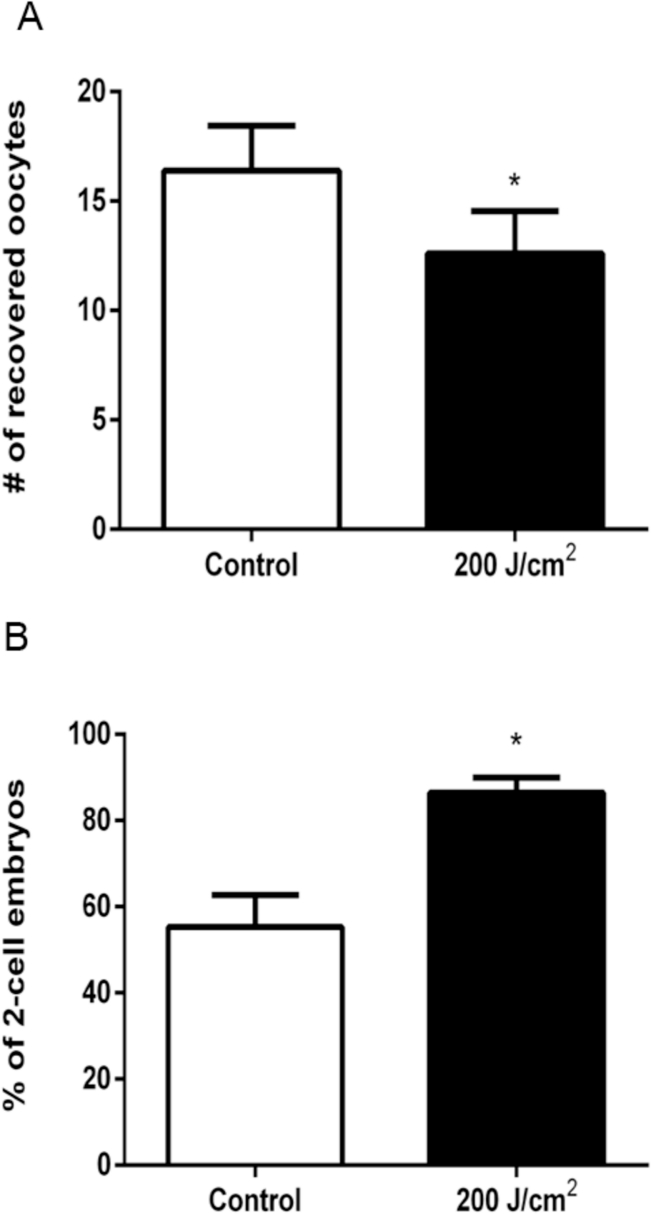
A
20
15
10
5
0
# of recovered oocytes
*
Control
200 J/cm2
B
100
80
60
40
20
0
% of 2-cell embryos
*
Control
200 J/cm2

Table 1
Antibodies used in immunohistochemistry (IHC) and Western blot.

Antibody target	Host/type	Catalog no.	Supplier	Technique	Dilution
Primary antibodies					
DDX4	Mouse polyclonal	ab27591	Abcam[b]	IHC	1:400
Anti-Mullerian Hormone	Goat polyclonal	sc-6886	Santa Cruz Biotechnology, Inc.[a]	IHC	1:400
α-Smooth muscle actin	Mouse polyclonal	ab18147	Abcam[b]	IHC	1:100
Bax	Rabbit polyclonal	sc-492	Santa Cruz Biotechnology, Inc.[a]	Western blot	1:300
Bcl-2	Rabbit polyclonal	sc-493	Santa Cruz Biotechnology, Inc. [a]	Western blot	1:200
Bcl-XL/S	Rabbit polyclonal	sc-634	Santa Cruz Biotechnology, Inc. [a]	Western blot	1:100
β-actin	Rabbit polyclonal	sc-1616	Santa Cruz Biotechnology, Inc. [a]	Western blot	1:3000
GAPDH	Rabbit monoclonal	2118	Cell Signaling[c]	Western blot	1:10000
Secondary antibodies					
Rabbit IgG (biotinylated)	Goat polyclonal	BA-1000	Vector Laboratories[d]	IHC	1:400
Mouse IgG (biotinylated)	Goat polyclonal	BA-9200	Vector Laboratories[d]	IHC	1:400
Goat IgG (biotinylated)	Rabbit polyclonal	BA-5000	Vector Laboratories[d]	IHC	1:400
Rabbit IgG (conjugated to HRP)	Goat polyclonal	A4914	Sigma-Aldrich[e]	Western blot	1:1000

a Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA.
b Abcam (Cambridge, Massachusetts, USA).
c Cell Signaling Technology (Beverly, MA, USA).
d Vector Laboratories, (Burlingame, CA, USA).
e Sigma-Aldrich (St. Louis, MO, USA).
IHC, immunohistochemistry; Ig, immunoglobulin; HRP, horse radish peroxidase.

Table 2
Effects of LLLT on ovarian weight and progesterone concentration in adult female mice
Data are expressed as mean ±SEM; paired t-Student test, n=5.

	Control n=5	LLLT 200 J/cm^2 n=5	P value
Ovarian weight (mg)	6,96±0,82	9±1,83	>0,05
Ovarian progesterone (ng/mg)	1,6±0,38	0,64±0,20	<0,05