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Endocannabinoids modulate apoptosis in endometriosis and adenomyosis☆

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ABSTRACT

Adenomyosis that is a form of endometriosis is the growth of ectopic endometrial tissue within the muscular wall of the uterus (myometrium), which may cause dysmenorrhea and infertility. Endocannabinoid mediated apoptotic mechanisms of endometriosis and adenomyosis are not known. We hypothesized that the down regulation of endocannabinoid receptors and/or alteration in their regulatory enzymes may have a direct role in the pathogenesis of endometriosis and adenomyosis through apoptosis. Endocannabinoid receptors CB1 and CB2, their synthesizing and catabolizing enzymes (FAAH, NAPE-PLD, DAGL, MAGL) and the apoptotic indexes were immunohistochemically assessed in endometriotic and adenomyotic tissues. Findings were compared to normal endometrium and myometrium. Endometrial adenocarcinoma (Ishikawa) and ovarian endometriosis cyst wall stromal (CRL-7566) cell lines were furthermore cultured with or without cannabinoid receptor agonists. The IC50 value for CB1 and CB2 receptor agonists was quantified. Cannabinoid agonists on cell death were investigated by Annexin-V/Propidium iodide labeling with flow cytometry. CB1 and CB2 receptor levels decreased in endometriotic and adenomyotic tissues compared to the control group ($p = 0,001$ and $p = 0,001$). FAAH, NAPE-PLD, MAGL and DAGL enzyme levels decreased in endometriotic and adenomyotic tissues compared to control ($p = 0,001$, $p = 0,001$, $p = 0,001$ and $p = 0,002$ respectively). Apoptotic cell indexes both in endometriotic and adenomyotic tissues also decreased significantly, compared to the control group ($p = 0,001$ and $p = 0,001$). CB1 and CB2 receptor agonist mediated dose dependent fast anti-proliferative and pro-apoptotic effects were detected in Ishikawa and ovarian endometriosis cyst wall stromal cell lines (CRL-7566). Endocannabinoids are suggested to increase apoptosis mechanisms in endometriosis and adenomyosis. CB1 and CB2 antagonists can be considered as potential medical therapeutic agents for endometriosis and adenomyosis.

1. Introduction

Endometriosis and adenomyosis are defined as the unusual location of the endometrial tissue at ectopic sites and in the myometrium. They can cause pelvic pain, dyspareunia, amenorrhea, dysmenorrhea and infertility (Irving and Clement 2011; Kruse et al., 2012; Lin et al., 2014; Lo Monte et al., 2013; Sznurkowski and Emerich, 2008; Gao et al., 2006; Vannuccini et al., 2016; Yang et al., 2013; Yamanaka et al., 2014). Endometriosis affects a large population and decreases quality of life. The pathogenesis of the disease remains unclear, although it is believed to relate with the quite aggressive behavior of endometriotic cells at migrating ectopic locations and the resistance of these cells to apoptosis (Agic et al., 2009; Nasu et al., 2011; Sbracia et al., 2016). Pathogenesis of adenomyosis is similar to endometriosis; adenomyotic cells have resistance to apoptosis as well (Yamanaka et al., 2014). The relationship between the endocannabinoids and adenomyosis is not yet studied.

Endocannabinoids, which are mostly located in the central nervous system and also in other organ systems, are Cannabis ligands that specifically act through their CB1 and CB2 receptors (Alger and Kim,

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Table 1
Age-matched control and experimental groups are presented with mean ± standard deviation/median (min–max) of their immune reactivities and apoptotic indexes.

Control	n	AGE mean ± SD	CB1 mean ± SD	CB2 mean ± SD	FAAH mean ± SD	NAPE-PLD mean ± SD	MAGL mean ± SD	DAGL median (min-max)	TUNEL median (min-max)
Proliferative	12	37,5 ± 1,27	0,80 ± 0,11	0,79 ± 0,10	0,79 ± 0,07	0,73 ± 0,15	0,69 ± 0,09	0,64 (0,49–0,79)	0,63 (0,0–0,85)
Secretory	7	41,4 ± 2,5	0,80 ± 0,11	0,76 ± 0,10	0,83 ± 0,11	0,71 ± 0,11	0,76 ± 0,10	0,72 (050–0,82)	0,65 (0,4–0,91)
Endometriosis									
Non-cystic	11	40,4 ± 2,07	0,35 ± 0,16	0,35 ± 0,21	0,37 ± 0,26	0,27 ± 0,23	0,22 ± 0,29	0,60 (0,0–0,66)	0,0 (0,0–0,70)
Cystic	9	35,6 ± 2,5	0,39 ± 0,20	0,40 ± 0,25	0,34 ± 0,34	0,32 ± 0,29	0,38 ± 0,24	0,52 (0,48–0,74)	0,11 (0,0–0,66)
Adenomyosis	17	42,5 ± 0,9	0,37 ± 0,19	0,34 ± 0,32	0,41 ± 0,21	0,43 ± 0,25	0,29 ± 0,26	0,48 (0,0–0,81)	0,05 (0,0–0,69)

2011; Coskun and Bolkent, 2014; Mercati et al., 2012; Muccioli, 2010; Scotchie et al., 2015; Yazulla, 2008). The most well-known endocannabinoids are anandamide (AEA) and di-arachidonoylglycerol (2-AG) (Alger and Kim, 2011; Muccioli, 2010; Scotchie et al., 2015; Yazulla, 2008). AEA is known to act more over the CB1 receptor in the female genital system, while 2-AG displays its effects usually through the CB2 receptor (Maccarrone, 2009; Taylor et al., 2010). In the female genital system, endocannabinoids and their receptors are generally located in the endometrium, the myometrium, the ovarian cortex and the medulla and the uterine tubes; they have critical roles in menstrual cycle, ovarian maturation, embryo transplantation and implantation (Brighton et al., 2011; El-Talatini et al., 2009, 2010; Karasu et al., 2011; Maccarrone, 2009; Scotchie et al., 2015; Sun et al., 2009; Taylor et al., 2010).

Recent researches suggested that endocannabinoids are involved in the pathophysiology of endometriosis in a variety of ways. Endocannabinoid agonists have anti-proliferative and analgesic effects on endometriotic cells or patients. Endometriosis-associated pain is shown to decrease by WIN 55212-2, CB1 and CB2 receptor agonist in experimental studies or by palmitoylethanolamide in patients with endometriosis (Cobellis et al., 2011; Dmitrieva et al., 2010; Giugliano et al., 2013; Indraccolo and Barbieri, 2010; Lo Monte et al., 2013). Cell proliferation in deep infiltrating endometriosis decreased with WIN-55212-2, both in vitro and in vivo (Leconte et al., 2010). In vitro stimulatory effect of endocannabinoid agonists on cell migration moreover was presented (Gentilini et al., 2010; McHugh et al., 2012). According to this, enhanced endometrial stromal cell migration via CB1 and GPR18 receptor with use of methanandamide, which is another endocannabinoid agonist, or *N*-arachidonyl glycine, which is an endogenous metabolite of anandamide, were shown through the activation of PI3K/Akt, ERK1/2 or MAPK pathways (Gentilini et al., 2010; McHugh et al., 2012). Although some activities of endocannabinoids are defined, we still do not know the effects of endocannabinoids on apoptosis in endometriosis. Anandamide leaded to apoptosis through CB1 receptor and p38 pathway on decidual cells (Almada et al., 2015; Fonseca et al., 2009).

Given the apoptotic and anti-proliferative effects of endocannabinoids, we hypothesized that the down regulation of endocannabinoid receptors and/or alteration in their regulatory enzymes may have a direct role in the pathogenesis of endometriosis and adenomyosis through apoptosis. We aimed to define the potential apoptosis related classical receptor mediated effects of endocannabinoids on endometriosis and adenomyosis. We investigated the differences of immune labelings of CB1 and CB2 receptors, AEA and 2-AG catabolizing and synthesizing enzymes, as well as apoptotic index between the endometriotic and adenomyotic patients and age-matched controls. Depending on the supposedly pro-apoptotic effects of endocannabinoids (Almada et al., 2015; Siegmund et al., 2016), endometrial adenocarcinoma cell line (Ishikawa) and ovarian endometriosis cyst wall stromal cell line (CRL 7566) were cultured with or without cannabinoid classical receptor agonists. The xCELLIgence cell impedance based system was used to calculate the IC50 value for CB1 and CB2 receptor agonists. Cannabinoid agonists' effect on cell death was investigated with flow cytometry by Annexin-V/propidium iodide labeling. Outcomes of these experiments may explain endocannabinoid effects of cell survival and death mechanisms in endometriosis.

2. Materials and methods

2.1. Design

A double blind randomized experimental study was designed. We received endometrial archive samples belonging to patients having been diagnosed as endometriotic and adenomyotic from January 2010 to July 2012. Age-matched paraffin endometrial tissue blocks of 20 endometriosis, 17 adenomyosis patients and 19 normal controls between 24 and 52 years were obtained from Hacettepe University Pathology Department (Table 1). Control tissues were obtained from patients undergoing dilatation and curettage surgery for benign gynecological conditions other than endometrial disease. Control endometrial tissues were sub-grouped according to the phase of menstrual cycle as proliferative (n = 12) and secretory phases (n = 7). The endometriotic tissues were also sub-grouped as cystic (n = 9) and non-cystic (n = 11). The use of endometriotic cells and the paraffin blocks of endometrial tissue was approved by the Hacettepe University Non-invasive Clinical Researches Ethical Committee (TBK 12/05-08), Ankara, Turkey.

2.1.1. CB1 and CB2 receptors and FAAH, NAPE-PLD, MAGL, DAGL enzymes immune labeling

5–6 μm Thick paraffin sections were deparaffinized. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide (cat# 216763, Sigma-Aldrich, St. Louis, USA) after antigen retrieval. Nonspecific binding was blocked with 5% normal mouse serum (cat#M5905, Sigma-Aldrich, St. Louis, USA) for 30 min. Slides were incubated with following primary antibodies overnight at 4 °C: CB1(cat#C2866, rabbit polyclonal;1/100 dilution; Sigma-Aldrich, St. Louis, USA), CB2 (cat#HPA028718, rabbit polyclonal; 1/100 dilution; Sigma-Aldrich, St. Louis, USA), FAAH (cat#HPA007425, rabbit polyclonal, 1/50 dilution; Sigma-Aldrich, St. Louis, USA), MAGL (cat#100035, rabbit polyclonal, 1/100 dilution, Cayman, Michigan, USA), DAGL (cat#ab106979, rabbit polyclonal, 1/100 dilution, Abcam, Cambridge, USA). Incubation with NAPE-PLD (cat#HPA019832, rabbit polyclonal, 1/100 dilution, Sigma-Aldrich, St. Louis, USA) was performed overnight at RT. The secondary antibody incubation (cat#EXTRA3, mouse monoclonal, Sigma-Aldrich, St. Louis, USA) was performed for 30 min at RT at 1/20 dilution. After washing slides and incubating with DAB (cat#D3939, Sigma-Aldrich, St. Louis, USA) we used haematoxylin for counterstaining. Digital images were analyzed and captured using the Leica DM6000B microscope equipped with a DFC480 digital camera.

2.1.2. Image analysis

Two pathologists according to pathological criteria for the diseases selected endometriotic and adenomyotic foci under the microscope (Fu et al., 2013; Yu et al., 2015).

Ten endometriotic foci or equal amount of glands have been selected at non-overlapping fields of each endometrial, endometriotic and adenomyotic sections by the motorized stage module of a Leica DM6000B microscope (Lin et al., 2014). Photomicrographs of each focus were generated by the microscope (Leica DM6000B) attached computerized digital camera (DFC 480, Leica Westlar Germany) and captured as TIFF at 200× magnification. The bright-field images were analyzed quantitatively by image processing program (LAS 3.8 Leica Inc., Westlar Germany version 3.8). Areas of interest (ROI) consisting of endometrial, adenomyotic or normal glandular (for control group) foci have been chosen at the x and y stages at the binary mode; and the total ROI was calculated for 10 foci. The measurements were done at minimum 45,876 um^2- maximum 125,214 um^2 for each endometriotic, adenomyotic or glandular focus (Hey-Cunningham et al., 2013). Brown stained particles (immune labeled cells) were counted in the binary defined area, at counting mode of LAS. Haematoxylin was extracted from DAB by RGB level of the software. The blue threshold value was 106,49 px for the nuclei, and the brown threshold value was 65,22 px for peroxidase labeling. The number of total immune reactive cell percentage was expressed as the ratio of immune positive particles (both the glandular epithelial and stromal cells) to total ROI.

2.1.3. TUNEL analysis for apoptosis

Slides were rinsed after de-waxing and dehydrating. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide for 10 min. Sections were treated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 8 min at room temperature. We used the in-situ cell death detection kit (Roche, Indianapolis, IN, USA) for detecting the DNA fragments. Digital images were analyzed and captured using the Leica DM6000B microscope equipped with the DC500 digital camera after DAB incubation and haematoxylin staining. The apoptotic index was expressed as a percentage of apoptotic glandular and stromal cells over total glandular and stromal cells at 200× magnification. Average of 4 analyzed non-overlapping fields was reported (Shen et al., 2010) in every specimen.

2.1.4. Cell culture

Ishikawa cell line (99040201, Sigma, Germany) was used to simulate normal endometrial glandular cells with their well-known phenotypic similarity and their response to steroids, resembling the physiological conditions (Tamm-Rosenstein et al., 2013). Ishikawa cell line is provided at passage 15 and authenticated by the manufacturer (European Collection of Authenticated Cell Cultures, London, UK). Endometriosis cyst wall stromal cell line (CRL-7566, ATCC, USA) was used at passage 15, to analyze the cannabinoid agonistic effect on endometriosis model. CRL-7566 is provided at passage 15 and, authenticated by DNA-based method. Although stated as mycoplasma free by the providers, the cell lines were tested by EZ-PCR Mycoplasma test kit (cat#20-700-10, Biological Industries, Kibbutz Beit Haemek, Israel) before use. Ishikawa cells were incubated in DMEM F-12 with 10% FBS and 2% L-glutamine and 1% pen-strep solution at 37 °C and 5% CO_2. CRL-7566 cells were incubated in DMEM with % 20 FBS and % 2 L-glutamine and % 1 pen-strep solution at 37 °C and 10% CO_2. Cells were used for the experiments at passage 18.

2.1.5. Impedance-based real-time cell proliferation analysis

Real-time cell proliferation was assessed with the xCELLIgence system (Roche Applied Science, Mannheim, Germany; ACEA Biosciences, San Diego, CA). Disposable 96-well e-plates were coated with 10 μgr/ml fibronectin, cells were seeded and incubated at 37 °C, % 5 CO_2 until cell index was 1 at 22 h (Lowin et al., 2012).

Ishikawa cell line was used to detect half maximal inhibitory concentration (IC_{50}) of selective CB1 and CB2 agonists ACPA (1318, Tocris Bioscience, Bristol, UK), respectively. ACPA (100 nM, 1 μM, 10 μM, 100 μM) and CB 65 (1 μM, 10 μM, 100 μM) were applied at different concentrations and the IC_{50} was calculated accordingly (to determine the of cannabinoids RTCA software was used) (Fig. 5A and C). Because our experimental procedure took about 146 h; IC50 concentrations were calculated both at 126th and 46th hours (Fig. 5B and D). After detecting IC50 concentrations, Ishikawa and CRL-7566 cells were exposed to the determined IC_{50} concentration of ACPA (9.3×10^{-6} M) and CB65 (1.9×10^{-4} M) for 46 h and monitored at every 15 min for 146 h (Fig. 6).

2.1.6. Flow cytometry analysis

We incubated cells with the determined IC_{50} values of ACPA ($9{,}3 \times 10^{-6}$ M) and CB65 ($1{,}9 \times 10^{-4}$ M) for 46 h after expansion. Cells with and without cannabinoid agonists were analyzed by Annexin-V/propidium iodide labeling in FACS Aria flow cytometer (Becton, Dickinson Biosciences, USA) at 46th hour. Cells were classified as live (Annexin V−, PI−), necrotic (Annexin V−, PI+), early apoptotic (Annexin V+, PI−), and late apoptotic (Annexin V+, PI+) cells. The acquired data was analyzed by using BD FACSDiva software v6.1.2 (Becton Dickinson Biosciences, USA) (Fig. 7). The ratio of apoptosis was reported as early apoptotic percentage plus late apoptotic percentage in the text.

2.2. Statistics

Distribution of normality of immune labeling was evaluated by the Shapiro-Wilk test. Age and CB1, CB2, FAAH, NAPE-PLD, MAGL immune labelings of stromal and glandular cell variables were evaluated by one-way ANOVA followed by post hoc Tukey testing. DAGL immune labeling in stromal and glandular cells was not normally distributed. Therefore, they were evaluated by the Kruskal-Wallis and the Mann-Whitney *U*-tests with Bonferroni correction. Correlation analysis was performed using the Pearson's (for parametric data) or the Spearman correlation (for nonparametric data) tests. Parametric data were presented as the mean ± standard error of mean, while others were presented as minimum, median, and maximum values. Confidence interval was 95% and statistical significance was defined as $p < 0.05$. The SPSS (15.0 version) program and the NCSS-PASS 2007 software were used for analysis.

3. Results

3.1. CB1 and CB2 receptor immune labeling

CB1 and CB2 receptor immune labeling was cytoplasmic and intense in both endometrial glandular and stromal cells in the control group (Fig. 1A–F). Immune labeling percentages for CB1 and CB2 receptors in the experimental groups were significantly ($p = 0{,}001$) lower than that of the control group (Fig. 1G). The CB1 and CB2 receptor immune labeling was similar in the endometriosis and the adenomyosis groups. Endometrial glandular and stromal cells in proliferative and secretory phases of the control group exhibited similar CB1 receptor immune labeling. The CB2 receptor labeling of the glandular cells was significantly ($p = 0{,}020$) higher in the proliferative phase than the secretory phase however it remained unchanged in the stromal cells between proliferative and secretory menstrual phases in this group. CB2 immune labeling for glandular epithelial and stromal cells decreased with age ($r = -0{,}612$, $p = 0{,}012$; $r = -0{,}53$, $p = 0{,}033$) in the adenomyosis group.

3.2. FAAH and NAPE-PLD immune labeling

FAAH and NAPE-PLD enzymes presented a compatible pattern of immune labeling with CB1 receptor (Fig. 2A–F). Immune labeling analysis indicated that FAAH and NAPE-PLD enzyme immune labeling were significantly lower ($p = 0{,}001$) in glandular and stromal cells in the endometriosis and the adenomyosis groups when compared to the control group (Fig. 2G). Although FAAH enzyme levels in the glandular

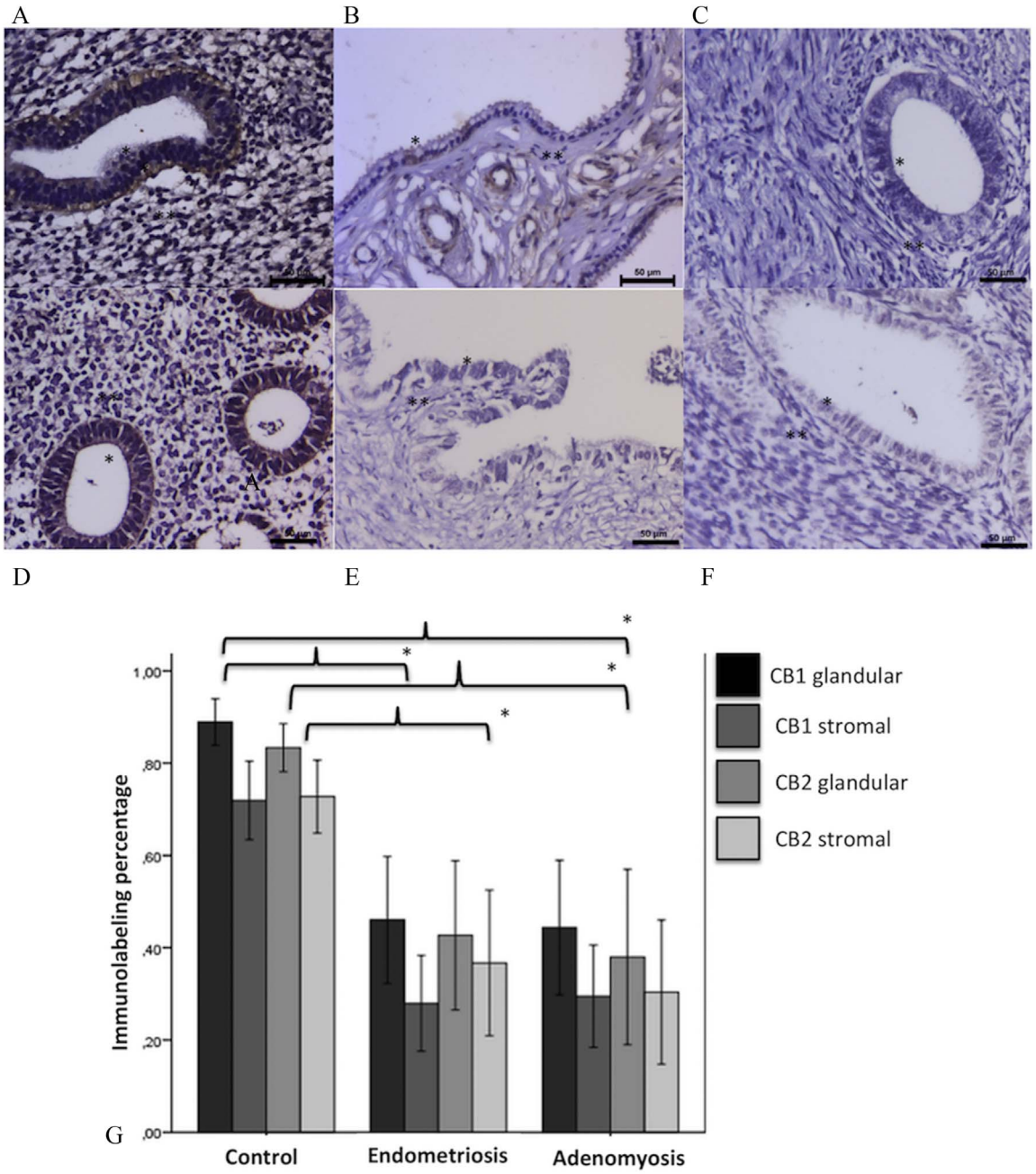

Fig. 1. A–F are endometrial micrographs exhibiting cytoplasmic CB1 (A–C) and CB2 (D–F) receptor immune labeling on glandular epithelial (*) and stromal cells (**), Haematoxylin 400×. (G) Immune labelings for CB1 and CB2 receptor distribution in control and experimental groups are shown. (*) p = 0,001. Note the significantly decreased immune labeling in adenomyosis (C and F) and endometriosis groups (B and E) comparing to control (A and D) in the micrographs and the graphic (n = 19 for control; n = 20 for endometriosis and n = 17 for adenomyosis).

cells was significantly lower in the proliferative phase when compared to the secretory phase (p = 0,004), FAAH enzyme immune labeling did not show any difference in stromal cells between menstrual phases of the control group. The control group furthermore showed similar NAPE-PLD enzyme expression in endometrial glandular and stromal cells in proliferative and secretory phases.

3.3. MAGL and DAGL enzyme immune labeling

MAGL and DAGL showed a compatible immune labeling pattern with the CB2 receptor (Fig. 3A–F). Immune labeling analysis indicated that immune labeling of MAGL (p = 0,001 both for glandular and stromal cells) and DAGL (p = 0,002 for glandular cells) in the experimental groups compared to the control group (Fig. 3G–I).

3.4. TUNEL assay for apoptosis

Lower TUNEL positivity was detected in the endometriosis and the adenomyosis groups compared to the control group in glandular and stromal cells (p = 0,001) (Fig. 4A–E). Apoptotic index revealed no difference in the glandular and stromal cells among the phases of the cycle in the control group and between the endometriosis and the adenomyosis groups as well as cystic and solid subgroups of endometriotic patients.

3.5. Impedance-based real-time cell proliferation analysis

Optimal anti-proliferative effect of ACPA and CB65 at IC50 concentrations was at the 46th hour (Fig. 5B and D). The IC_{50} concentra-

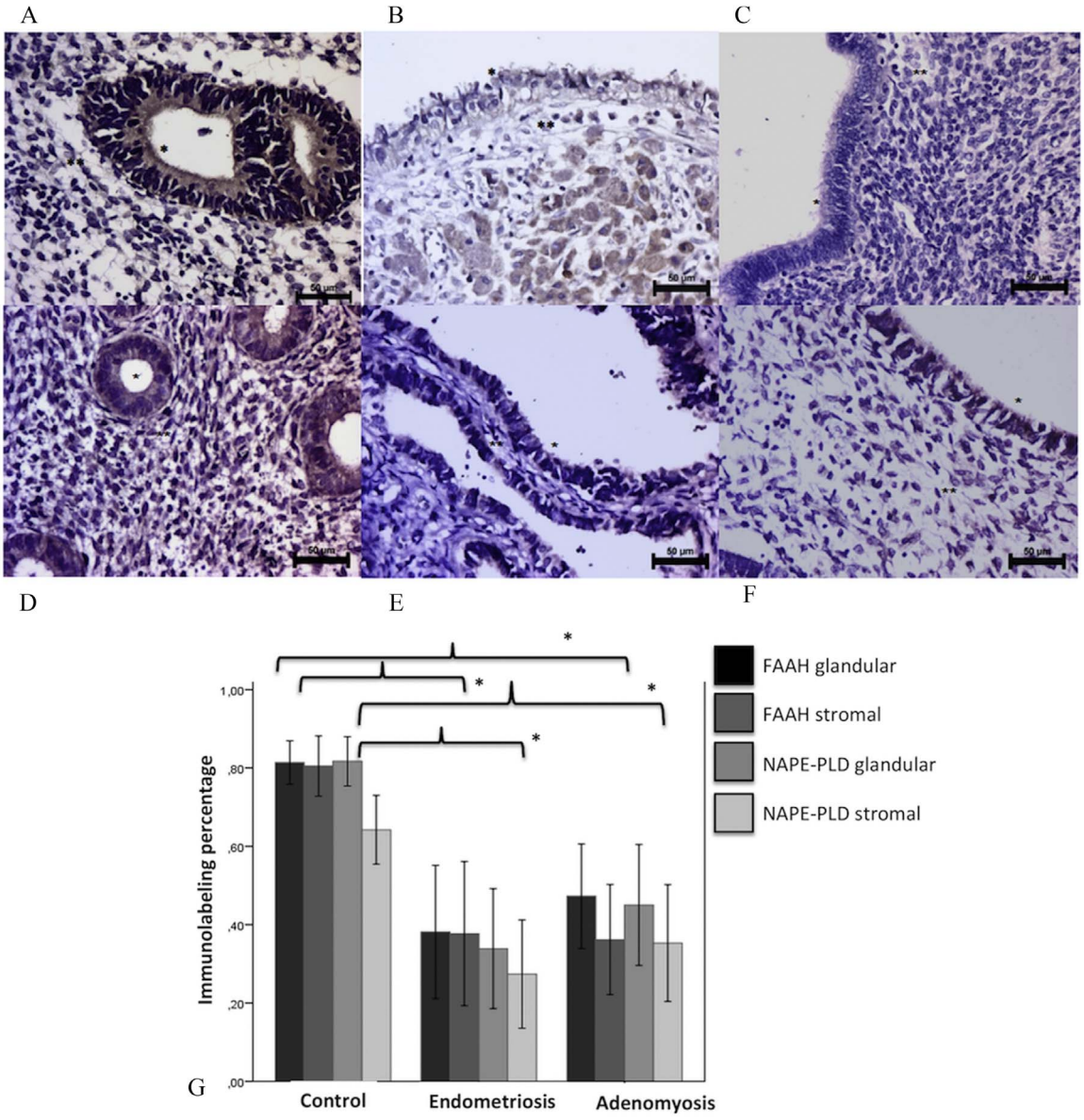

Fig. 2. A–F are endometrial micrographs showing cytoplasmic AEA catabolizing FAAH (A–C) and synthesizing NAPE-PLD (D–F) enzyme immune labeling on glandular epithelial (*) and stromal cells (**), Haematoxylin 400×. (G) Graphic shows the immune labeling for FAAH and NAPE-PLD in control and experimental groups. (*) p = 0,001. Note that both enzymes significantly decreased in endometriosis (B and E) and adenomyosis (C and F) groups comparing to control (A and D). (n = 19 for control; n = 20 for endometriosis and n = 17 for adenomyosis).

tions of CB1 and CB2 agonists were detected as 9.3×10^{-6} M for ACPA and 1.9×10^{-4} M for CB 65 on Ishikawa cells (Fig. 5A and C).

Ishikawa and CRL-7566 endometriotic cells exhibited decreasing cell indices immediately after application of the IC50 concentration of ACPA and CB 65. Cell proliferation index for CRL-7566 cells decreased 76% with ACPA and 86% with CB65 (Fig. 6A and B). Cell proliferation index for Ishikawa cells decreased 95% with ACPA and 81% with CB65 (Fig. 6C and D).

3.6. Flow cytometry analysis

Annexin-V/propidium iodide labeled total (early and late) apoptotic Ishikawa and CRL-7566 cell numbers increased with ACPA and CB65 exposure compared to the untreated control (Fig. 7). The 71,7% of Ishikawa cells and 81,7% of CRL-7566 cells were apoptotic (early and late) with ACPA (Fig. 7A and D). The 80,5% of Ishikawa cells and 78,3% of CRL-7566 cells were apoptotic (early and late) (Fig. 7B and E) with CB65. In the untreated control group, only 0,8% of Ishikawa cells, but 76,9% of CRL-7566 cells were apoptotic (early plus late) (Fig. 7C and F).

4. Discussion

Endometriosis and adenomyosis that co-exist with endometriosis (Garavaglia et al., 2015), affecting nearly 10–15% of the female population (Jeung et al., 2016) increase the risk of gynecological malignancies (Krawczyk et al., 2016). Adenomyosis was furthermore recognized in 16–34% of endometrial carcinoma hysterectomy specimens (Gizzo et al., 2016). Endometriotic cells tend to have cancer cell like aggressive features for migrating to ectopic locations and they are resistant to apoptosis (Agic et al., 2009; Nasu et al., 2011; Sbracia et al., 2016). Adenomyotic cells have resistance to apoptosis as well (Yamanaka et al., 2014).

Cannabinoid receptors (CB1, CB2) and the NAPE-PLD, FAAH, DAGL, MAGL enzymes exhibited different regulation in the glandular epithelial cells and stromal cells of the normal endometrial, the

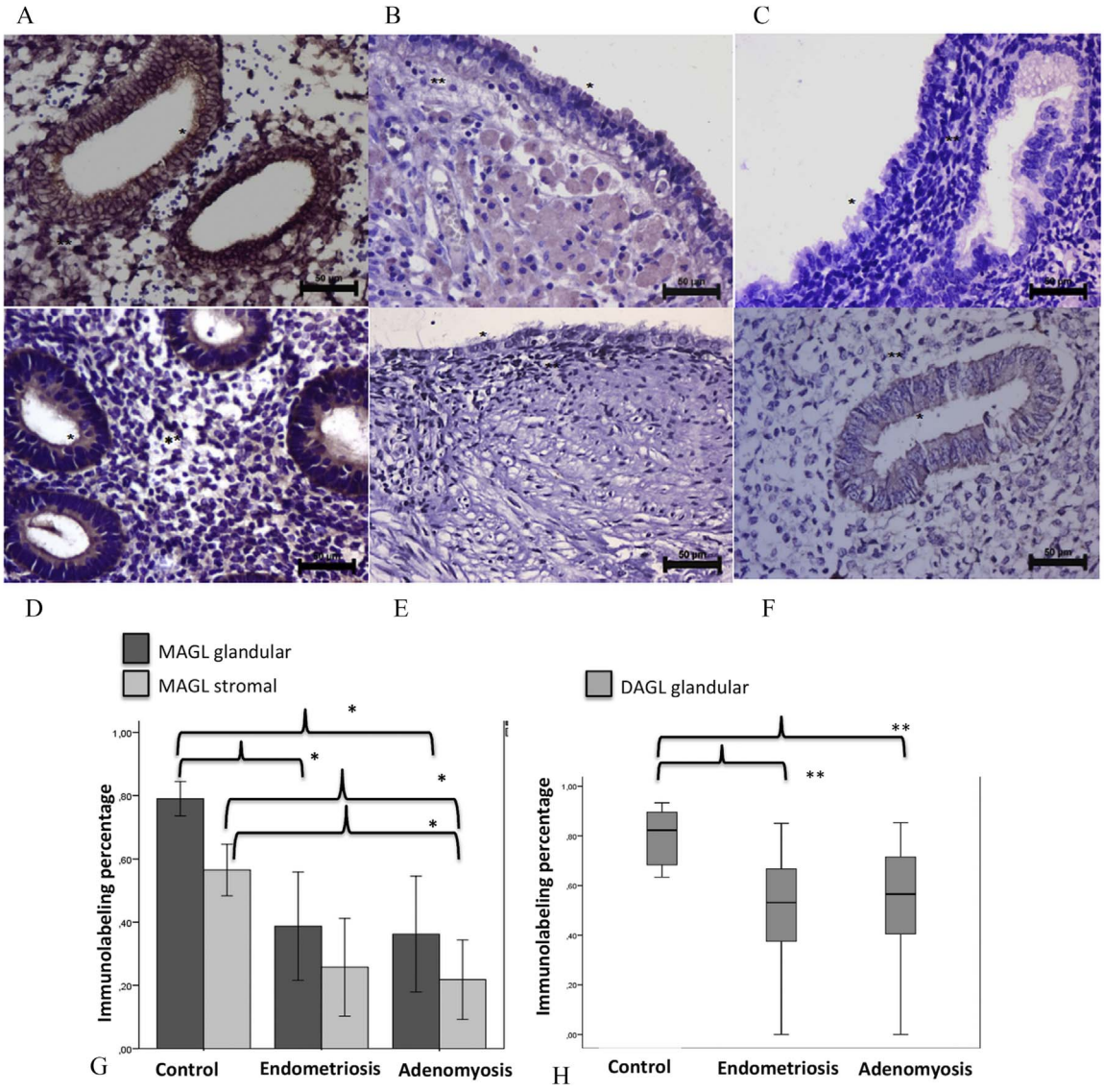

Fig. 3. A–F are endometrial micrographs showing cytoplasmic 2-AG synthesizing MAGL (A–C) and 2-AG catabolizing DAGL (D–F) enzyme immune labeling on glandular epithelial (*) and stromal cells (**), Haematoxylin 400×. (G) Graphic shows the immune labeling percentages for MAGL enzyme distribution in control and experimental groups. (*) p = 0,001. (H) (boxplot graph) shows nonparametric distributed immune labeling scores for DAGL on glandular epithelial cells of all groups respectively. (**) p = 0,002. (n = 19 for control; n = 20 for endometriosis and n = 17 for adenomyosis).

endometriotic and the adenomyotic tissues in this study. Immune labeling values for CB1 and CB2 receptors in glandular and stromal cells of endometriosis and adenomyosis groups were lower than that of the control group. Our findings for CB1 immune labeling were similar with the results of Resuehr et al. (2012) who showed that CB1 immune reactivity decreased in endometriosis compared to the control group, however they were different than that of Leconte et al. (2010) who suggested no difference for the CB1 receptor expression level between endometriosis and controls (Leconte et al., 2010; Resuehr et al., 2012).

CB1 and CB2 receptor distribution in the endometriosis and adenomyosis groups were not significantly different in our study. Findings of this study demonstrated lower immune labeling for cannabinoid receptors in adenomyosis and endometriosis compared to the control group.

Labeling for NAPE-PLD and FAAH, synthesizing and catabolizing enzymes of AEA, decreased in glandular epithelial cells and stromal cells in both endometriosis and adenomyosis compared to the control. Taylor et al. (2010) showed the existence of the receptors of AEA (CB1) together with its synthesizing and catabolizing enzymes in the endometrium at different stages of the menstrual cycle and in the ovary by immunohistochemistry (Taylor et al., 2010). It is known that AEA is more common in endometrium than 2-AG at physiological conditions (Maccarrone, 2009; Taylor et al., 2010). Although Sanchez et al. (2016) detected increased systemic levels of AEA, 2-AG and OEA in patient derived serum, lower expressions of CB1 mRNA was detected in the same cases' endometriotic cells compared to controls at secretory phase of menstruation (Sanchez et al., 2016). Our study is the first that searched for the synthesizing and catabolizing enzymes of AEA in endometriosis and adenomyosis patients. Immune reactivity of synthesizing and catabolizing enzymes were detected to be similar in endometriosis and adenomyosis. Since the expression of both NAPE-PLD and FAAH decreased in endometriosis and adenomyosis groups in line with receptor immune reactivity, we suggest that synthesis and degrading of AEA get slower together in both epithelial and stromal cells during the pathogenesis of the disease.

Tissues from the proliferative and secretory phases of the normal endometrium exhibited the same immune labeling pattern for CB1 in this study. This finding revealed that CB1 receptor immune reactivity is not menstrual cycle dependent. This finding correlated well with the data of Taylor et al. (2010) and colleagues (Taylor et al., 2010).

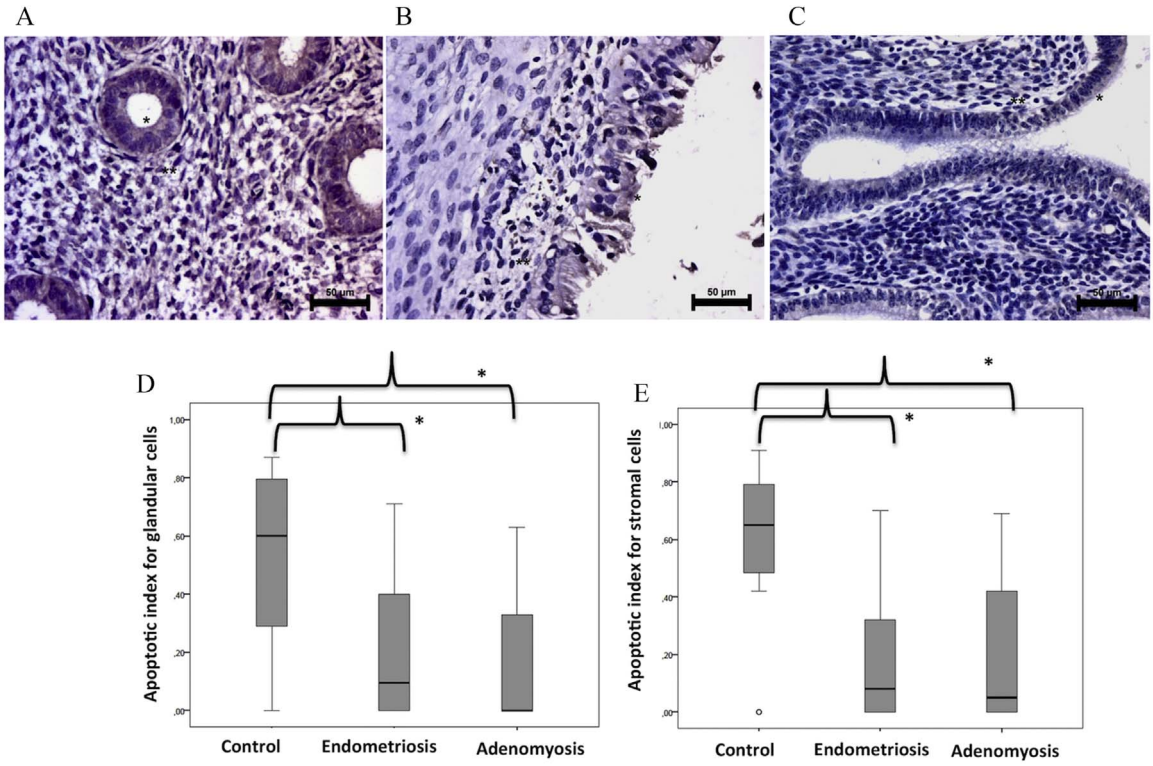

Fig. 4. From A to C are endometrial micrographs from control and experimental groups exhibiting apoptotic stromal (**) and epithelial (*) cells undergoing apoptosis with their nuclei labeled in dark brown. Haematoxylin 400×. D and E show boxplot graphs of apoptotic indices for glandular epithelial and stromal cells respectively (*) $p < 0,05$. Note that control group exhibits significantly higher apoptotic rate comparing to both adenomyosis and endometriosis groups. ($n = 19$ for control; $n = 20$ for endometriosis and $n = 17$ for adenomyosis).

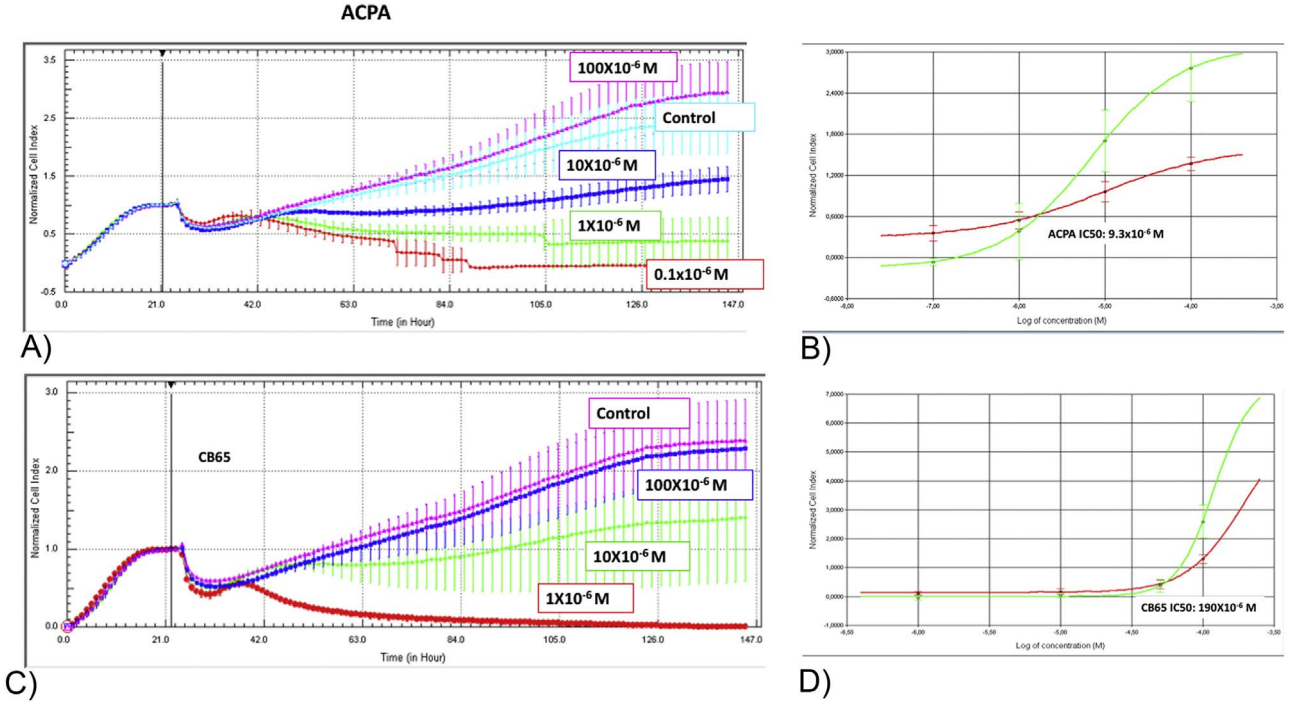

Fig. 5. (A and C) Real time cell proliferation curves of Ishikawa cells following application of different doses of ACPA (100 nM, 1 µM, 10 µM, 100 µM) and CB65 (1 µM, 10 µM, 100 µM) are shown. (B) and (D) are logarithmic graphics representing the calculated value for IC_{50} concentration. The anti-proliferative effect of ACPA (9.3×10^{-6} M) and CB65 (1.9×10^{-4} M) at IC50 concentrations was observed from 46th hour. All plots were generated using the RTCA Software 1.1.

Resuehr et al. (2012) however reported that secretory phase of the normal endometrium exhibits increased CB1 immune reactivity (Resuehr et al., 2012). The strength of our study is the larger number of patients and also larger panel of labeling comparing to previous reports.

FAAH immune labeling was higher in the secretory compared to the proliferative phase in glandular epithelial cells in this study. This revealed that glandular epithelial cells at secretory phase were independent from CB1 receptor activity. We found that immune labeling for NAPE-PLD was similar at different phases of the control group.

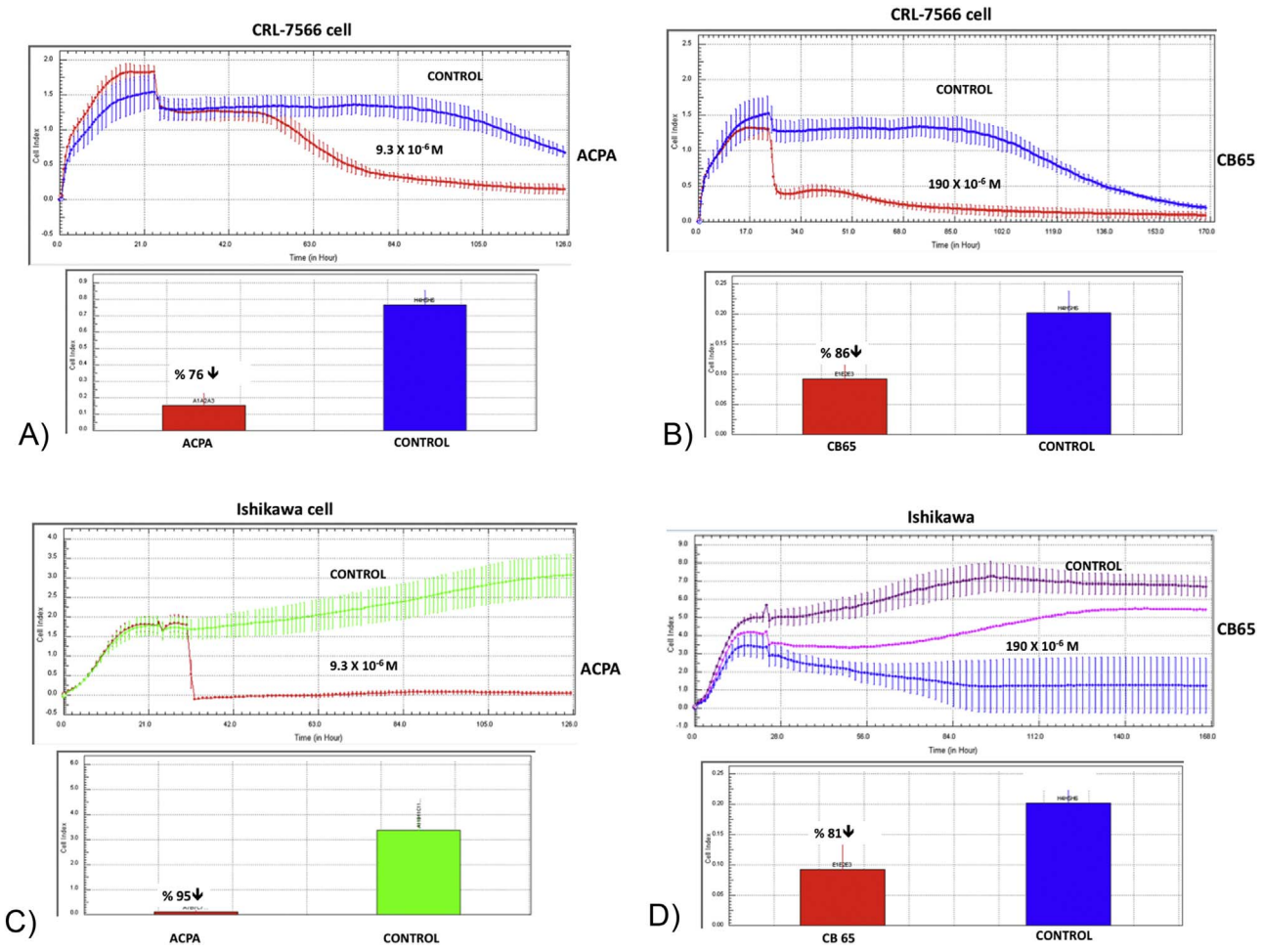

Fig. 6. (A–D) Real time cell proliferation curves and bar graphics are shown following application of ACPA (9.3×10^{-6} M) and CB65 (1.9×10^{-4} M) at IC50 concentrations on control (Ishikawa) and endometriotic (CRL-7566) cells. Note that the cell proliferation indices of CRL-7566 (A and B) and Ishikawa (C and D) cell lines decreased through hours comparing to untreated controls. All plots were generated using the RTCA Software 1.1.

According to Taylor et al. (2010), FAAH enzyme level is higher at late secretory phase and lower at early proliferative phase of the menstrual cycle, while NAPE-PLD immune reaction is higher at late secretory and early proliferative phases than late proliferative and early secretory phases in glandular epithelial cells of normal endometrium (Taylor et al., 2010). Our findings for phase distribution of FAAH are consistent with the data of Taylor et al. (Taylor et al., 2010). We suggest that the FAAH enzyme rather than the CB1 receptor or the NAPE-PLD enzyme regulates endocannabinoid activity. The limitation of our study was using archive blocks but not fresh tissue samples. Working on archived paraffin blocks is a well-established method for evaluating homogenous patient groups.

Levels of synthesizing and catabolizing enzymes (DAGL and MAGL respectively) of 2-AG were correlated with CB2 receptor immune labeling in all groups. DAGL enzyme activities in glandular and stromal cells increased with age in solid subgroup of endometriosis, suggesting that 2-AG synthesis decreases with aging at disease. Immune labeling for both enzymes of 2-AG decreased in glandular and stromal cells in the experimental groups compared to that of the control group. Our findings regarding synthesizing and catabolizing enzymes of 2-AG and its receptor are original since their immune labeling pattern has not been studied in the normal endometrium in comparison with endometriosis and adenomyosis until now. It is likely that 2-AG might be a molecule playing an important role in the pathogenesis of endometriosis and adenomyosis.

CB2 receptor reactivity was higher in the proliferative phase than the secretory phase in the control group. This finding was consistent with the results of Taylor et al. (2010), while the enzyme reactivity score was similar in both phases (Taylor et al., 2010). These findings reveal that the enzymes do not regulate the effect of 2-AG through CB2 receptors in the modulation of the menstrual cycle and these receptors do not mediate the effects of other endocannabinoids. We suggest 2-AG activity might be taking an active role in endometrium progressing to late phases of reproductive ages as we detected increased MAGL enzyme activity in the proliferative phase on glandular cells with increasing age. We suggest that the pathophysiological mechanism of endometriosis could be different than adenomyosis. Aging may play role on dysregulation of endocannabinoids via CB2 receptors, taking into consideration the decreased CB2 receptor reactivity with increasing age in adenomyosis.

Our data from TUNEL analysis was in line with the literature and correlated with our immune labeling results. The proliferation capacity of glandular epithelial and stromal cells of endometrium is higher in endometriosis than normal endometrium (Agic et al., 2009; Nasu et al., 2011; Sanchez et al., 2012).

The IC_{50} value was confirmed as 9.3×10^{-6} M for ACPA and 1.9×10^{-4} M for CB 65 on Ishikawa cells. ACPA exhibited stronger anti-proliferative effect on Ishikawa cells and, CB65 caused stronger anti-proliferation on CRL-7566 respectively. Our study is the first to examine the real time direct and dose dependent anti-proliferative effect of cannabinoid agonists in both control and endometriotic cells. We report CB65 exhibits stronger pro-apoptotic effect on Ishikawa cells and, ACPA causes stronger pro-apoptosis on CRL-7566 respectively. Truthfully, there is more than one cell death mechanism (Galluzzi et al.,

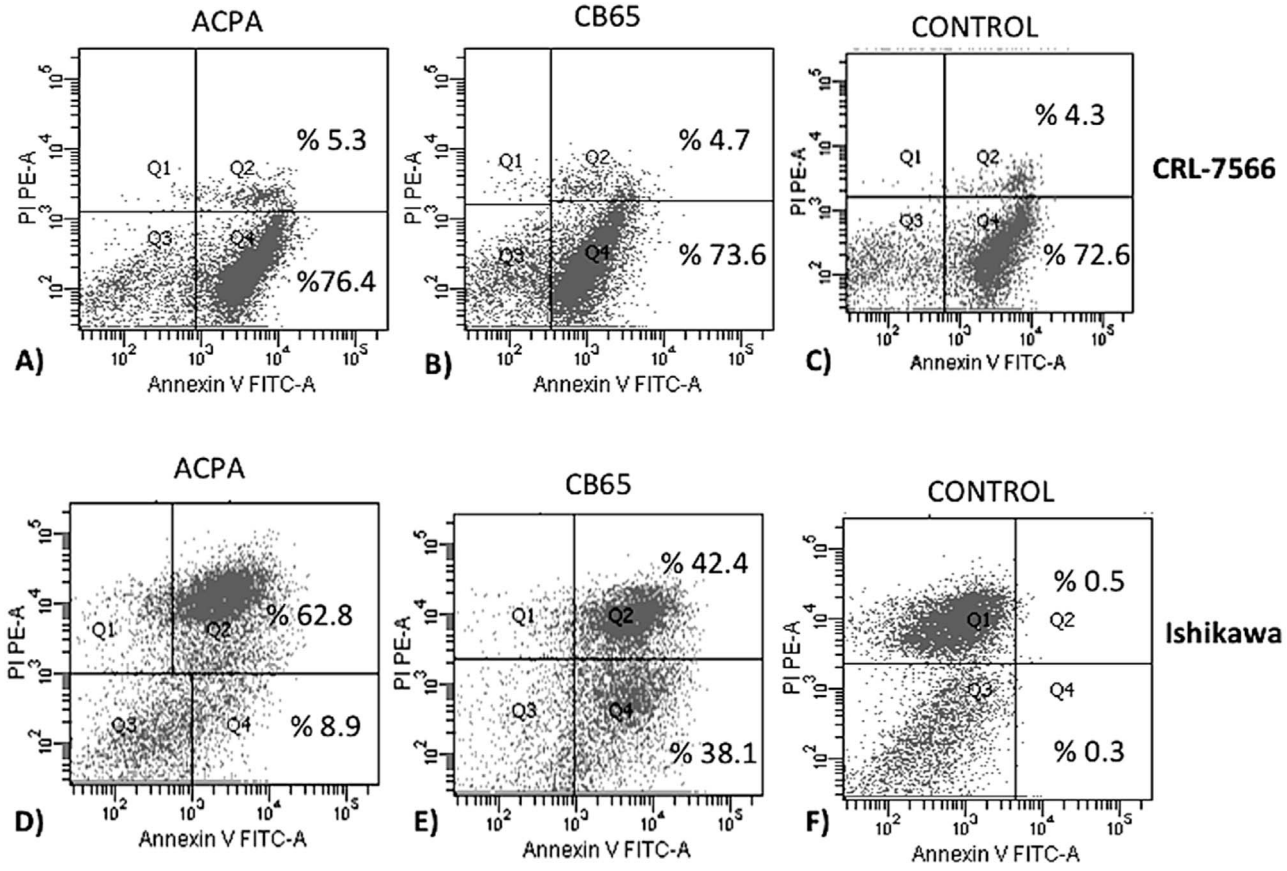

Fig. 7. From A to F are representative flow cytometry analysis of CRL-7566 and Ishikawa, cells that were labeled with Annexin-V/PI. The percentage of apoptotic cells is equal to $Q_2 + Q_4$ (early apoptotic and late apoptotic cells) areas. Both o cell lines were exhibited apoptosis with cannabinoid agonists. CB65's effect was more prominent on Ishikawa and, ACPA's was more prominent on CRL-7566 cells. All the quadrants arranged according to the isotyping control values. All analyses were done using BD FACSDiva software v6.1.2 (Becton Dickinson Biosciences, USA).

2012). The deviation of our results may be because of the different apoptotic pathways, which play role on endometrial cell death. Cannabinoid agonists and their receptors have been shown in endometrial cancers (Ayakannu et al., 2013, 2015; Guida et al., 2010). AEA and 2-AG synthesis and degradation pathways are known in cancer angiogenesis and overall gene expression levels were reported for endometrial carcinoma (Ayakannu et al., 2015). Although both synthetic CB1 and CB2 agonists increased the apoptotic cell percentage compared to control group and decreased the cell proliferation indexes in our study, the molecular pathways of cannabinoid-dependent cell mechanisms need to be searched. We suggest that cannabinoid agonists can potentially inhibit endometriotic cell proliferation. Palmitoylethanolamide was recently used to reduce chronic pelvic pain in endometriotic patients (Angioni, 2015). Based upon this finding, we suggest that cannabinoid agonists can be assessed for their molecular mechanisms on endometriotic cell proliferation regression and apoptosis and, can be a potential therapeutic agent.

In conclusion, endocannabinoids and their receptor distribution on endometriotic and adenomyotic tissue samples were compared with healthy endometrial tissue samples. We presented that expression of endocannabinoid receptors and synthesizing and catabolizing enzymes and apoptotic cell ratio decrease in endometriosis and adenomyosis, compared to normal endometrium. Cannabinoid agonist presented antiproliferative and apoptotic effect on cell culture.

Conflict of interest

The authors listed above have no financial interest with any company or organization in the subject matter or materials discussed in this manuscript.

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