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Original Contribution

17β-Estradiol and steady-state concentrations of H_2O_2: antiapoptotic effect in endometrial cells from patients with endometriosis

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ABSTRACT

Increased levels of hydrogen peroxide (H_2O_2) can initiate protective responses to limit or repair oxidative damage. However, H_2O_2 signals also fine-tune responses to growth factors and cytokines controlling cell division, differentiation, and proliferation. Because 17β-estradiol (E_2) also plays important roles in these processes, and is considered a major risk factor in the development and progression of endometriosis, this study evaluated whether E_2 has an antiapoptotic effect on oxidative stress in endometrial cells in combination with steady-state H_2O_2 levels ([H_2O_2]ss). Endometrial stromal cells were prepared from the eutopic endometrium of 18 women with and without endometriosis to produce primary cells. These cells were stimulated with E_2 for 20 h, exposed to [H_2O_2]ss, and examined for cell viability, proliferation, and apoptosis. The endometrial cells from women with endometriosis maintained the steady state for 120 min at high H_2O_2 concentrations. When they were pretreated with E_2 and exposed to [H_2O_2]ss, a decrease in apoptosis level was observed compared to the control cells ($p < 0.01$). The endometrial cells from patients with endometriosis subjected to both E_2 and [H_2O_2]ss showed increased ERK phosphorylation. These findings suggested that H_2O_2 is a signaling molecule that downregulates apoptosis in endometrial cells, supporting the fact that endometriosis, albeit a benign disease, shares some features with cancer such as decreased catalase levels. These results link the E_2 effects on [H_2O_2]ss to resistance to apoptosis and progression of endometriosis.

Introduction

Endometriosis is an estrogen-dependent disease causing pelvic pain and infertility in 10% of reproductive-age women [1]. Despite the poor understanding of the underlying pathogenic mechanisms involved in this disease, it is well established that endometriosis occurs and regresses in an estrogen-dependent fashion. Locally produced cytokines and reactive oxygen species (ROS), which act as secondary messengers, are implicated in the development and progression of endometriosis. The possibility that hydrogen peroxide (H_2O_2; one of the most common ROS in cells) has a modulatory effect in endometrial cells [2] was investigated in this study. H_2O_2 has been linked to the redox regulation of cell proliferation [3,4], apoptosis [5–7], and inflammatory processes [8]. It is continuously produced in vivo [6,9] and remains in a semi-steady state, i.e., its concentration changes at a slower pace than its turnover. Hence, exposing cells to steady-state H_2O_2 concentrations ([H_2O_2]ss), as opposed to bolus additions, constitutes a superior method of oxidant delivery that mimics the physiological setting. In the cellular environment, H_2O_2 plays a pivotal role in the inflammatory process by acting as a signaling molecule. Some investigators suggest that H_2O_2 plays a dual role in the regulation of inflammatory processes, acting as both

Abbreviations: E_2, 17β-estradiol; [H_2O_2]ss, Steady-state H_2O_2; ERK, Extracellular signal-regulated kinase; ROS, Reactive oxygen species; FOX, Ferric–xylenol orange; XO, Xylenol orange; BHT, Butylated hydroxytoluene; DAPI, 4′,6-diamidino-2-phenylindole; Gox, Glucose-1-oxidase; Cat, catalase; GSH, Glutathione; LDH, Lactate dehydrogenase; Ac-DEVD-AMC, 7-acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]propane sulfonate; IL, Interleukin; TNF, Tumor necrosis factor; MCP-1, Monocyte chemoattractant protein-1; ICAM-1, Intercellular adhesion molecule; FACS, Fluorescence-activated cell sorter; 8-OHdG, 8-hydroxy-2′-deoxyguanosine

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a proinflammatory and an anti-inflammatory agent [10,11]. In addition, H_2O_2 controls opposing cellular metabolic processes such as cell proliferation, when at low concentrations, and cell death signaling at high concentrations [9]. Normal cell proliferation correlates with production of endogenous ROS through the activation of growth-related signaling pathways, including the mitogen-activated protein kinase ERK1/2 (an extracellularly regulated kinase) [12]. Thus, increased cell proliferation and decreased apoptosis levels emerge as the major mechanisms responsible for the development of endometriosis, even though they are associated with the important role estrogens play in the growth and maintenance of the ectopic endometrium [2,13]. 17β-Estradiol (E_2) is essential to cell proliferation, development, growth, and differentiation of both female and male secondary sex characteristics. It is well known that estrogen is a major risk factor in the disease-associated biological changes and clinical manifestations of endometriosis [14]. Nevertheless, the biochemical mechanisms underlying the influence of estrogen in endometriosis remain unclear. Mounting evidence indicates that estrogens such as E_2 have a protective effect on oxidative stress [15,16]. Therefore, the effect of E_2 on the survival of endometrial cells from patients with endometriosis after oxidative challenge induced by $[H_2O_2]$ss was examined in this study through the assessment of cell viability, apoptosis, proliferative capability, and cytokine regulation. This study investigated the interactions between endometrial cells, E_2, and $[H_2O_2]$ss with the goal of identifying biochemical strategies for improving the clinical management of patients with endometriosis. We thus hypothesized that endometriosis is a condition resulting more from the development of a pseudo-tumoral disease than from an additive process caused by menstrual regurgitation, which is the traditional theory about the cause of endometriosis.

Materials and methods

Chemicals and biochemicals

All chemical reagents were of analytical grade. Deionized and ultrafiltered water from the Milli-Q ultrafiltration system was used. The biochemical assays were conducted using commercially available kits.

Sample collection

The study was carried out in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Review Board (CEP0858/10) from the São Paulo Federal University (UNIFESP). A written informed consent was signed by each patient, who volunteered to participate, before the study start. Endometrial specimens were obtained from these volunteers in the Endometriosis Unit/Department of Gynecology at UNIFESP. Eutopic endometrium specimens, to be used as controls, were collected from 7 patients without macroscopic endometriosis while undergoing laparoscopy for other reasons (tubal infertility, nonendometriotic ovarian cyst, or myoma). Thus, seven primary endometrial control cell lines were produced from these biopsies. Eutopic endometrium biopsies were performed in 11 patients with endometriosis reportedly suffering from chronic pelvic pain. Demographic and disease-related data from the patients are summarized in Table 1.

Tissue isolation and cell culture

Primary endometrial cell culture was prepared from biopsies as described by D'Amora et al. [17] with minor modifications. Tissue samples were rinsed, minced into small pieces, and digested with collagenase IA (0.05 mg/ml) for 30 min at 37 °C. Cells were placed onto primary flasks (Becton–Dickinson Labware, Le Pont de Claix, France) and cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 without phenol red and with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 mg/ml streptomycin in a humidified incubator at 37 °C and 5% CO_2. After 24 h of incubation, nonadherent cells were washed out and adherent stromal cells were characterized by immunostaining with FITC-labeled anti-cytokeratin and Cy3-labeled anti-vimentin antibodies.

Table 1
Patient characteristics.

	Patients with endometriosis ($n=11$)	Controls ($n=7$)
Age (mean ± SEM)	34 ± 1.1	36 ± 1.4
American Fertility Society (AFS) stage		
Cycle number		
Proliferative phase	6	4
Secretory phase	5	3
I–II	5	—
IV	6	—

The concentration of FBS in the medium was changed from 10 to 0.5% for 24 h before treatment with E_2 and $[H_2O_2]$ss when the endometrial cells reached 70 to 80% confluence.

Biochemical measurement of the total peroxide concentration in endometrial cells by the ferric–xylenol orange (FOX) assay

The FOX assay is based on the ability of hydroperoxides to react with an excess of Fe^{2+} at a low pH when the XO dye is present. The amount of Fe^{3+} generated by this reaction is measured as an Fe–XO complex at 560 nm [18] and is demonstrated in the following equations:

$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + OH^- + RO^{\bullet}, \quad (1)$$

$$Fe^{3+} + XO \rightarrow Fe\text{-}XO. \quad (2)$$

The FOX reagent was prepared by dissolving ammonium ferrous sulfate (9.8 mg) in 250 mM H_2SO_4 (10 ml) to a final concentration of 250 mM ferrous iron in acid. This solution was added to 90 ml of HPLC-grade methanol containing 79.2 mg of butylated hydroxytoluene (BHT). Finally, 7.6 mg of XO was added with stirring to make the working reagent (250 mM ammonium ferrous sulfate, 100 mM XO, 25 mM H_2SO_4, and 4 nM BHT, in 90% (v/v) methanol in a final volume of 100 ml). The blank reagent did not contain ferrous sulfate. Nearly confluent endometrial cells in six-well tissue culture plates were harvested by trypsinization, washed with cold phosphate-buffered saline (PBS), and centrifuged. The cell pellet was mixed with the FOX reagent and incubated at room temperature for 30 min. The samples were subsequently centrifuged at 13,000 *g* for 10 min, cell debris was removed, and the absorbance of the supernatant was determined at 560 nm. The total peroxide content of the endometrial cells was determined as a function of the difference in absorbance between the testing and the blank samples, using a H_2O_2 solution as the standard. This procedure was performed in triplicates using harvested cells at 0, 1, 3, and 8 h, and after that, the cell pellet was mixed with the FOX reagent as described above.

Treatment of cells with 17β-estradiol

Endometrial cells (10^4 per well) seeded to display monolayers at 70% confluence were cultured in DMEM/F-12 without phenol,

supplemented with 10% FBS, 100 IU/ml penicillin, 100 mg/ml streptomycin, and treated with 100 nmol/L E_2 for 20 h.

Extracellular pH

The extracellular pH was measured in the medium removed from the cultured cells described previously. Independent triplicate measurements were taken and reported as average ± SD between the replicates. The *p* values were calculated using the independent *t* test and Welch's correction.

Treatment of cells with 17β-estradiol and measurement of extracellular pH

Endometrial cells from women with and without endometriosis seeded (10^4 per well) to display monolayers at 70% confluence were cultured in phenol-free DMEM/F-12 supplemented with 10% FBS, 100 IU/ml penicillin, and 100 mg/ml streptomycin and treated with 100 nmol/L E_2 for 20 h. These cultures were incubated in a humidified incubator at 37 °C with 5% CO_2.

The extracellular pH was measured in the medium removed from the wells; independent triplicate measurements were taken and reported as average ± SD between the replicates. The *p* values were calculated using the independent *t* test and Welch's correction.

Calibration of steady-state concentrations of H_2O_2: modulation of in vitro H_2O_2 production

Endometrial cells from patients with and without endometriosis were exposed to dissolved O_2 (ambient, i.e., 21% O_2 to 1%) and $[H_2O_2]$ss (1–100 μM) in combination for 2 h (30, 60, and 120 min). The $[H_2O_2]$ss level was maintained using a two-enzyme system that employs glucose-1-oxidase (Gox), to consume O_2 and produce H_2O_2, and catalase (Cat), to consume H_2O_2 while partially replenishing O_2. This assay is based on the exposure of cells to $[H_2O_2]$ss as described by Antunes and Cadenas [6]. Briefly, Gox and Cat are added at different concentrations to the culture medium. The Gox activity and O_2 diffusion distances between the ambient air (21% O_2) and the bottom of the well determine the concentrations of O_2 at the bottom of the well available for the attached cells. The H_2O_2 concentration at the cell level depends on the ratio between the Gox and the Cat activities, which were experimentally determined at 37 °C ($k_{Gox} = 4.8 \times 10^{-2}$ M/s and $k_{Cat} = 1200$/s). This model was validated by O_2 and H_2O_2 measurements. The consumption of H_2O_2 in the endometrial cells showed first-order decay kinetics with a constant rate (k_{cell}) of 1.5×10^{-3}/s/million cells [6,19]. The DMEM/F-12 medium alone did not consume significant amounts of H_2O_2. The effects of hormone treatment were examined after the calibration of the steady-state system. A $[H_2O_2]$ss of 2 h was achieved by the addition of an initial concentration of H_2O_2 together with glucose oxidase at an activity that compensated for the rapid consumption of the initial H_2O_2 concentration by the cells. However, it was observed experimentally that the capacity of cells to consume H_2O_2 was slightly decreased at high levels of $[H_2O_2]$ss and consequently, the amount of glucose oxidase given had to be adjusted to achieve the desired steady-state concentration. The concentration of FBS in the medium was changed from 10 to 0.5% in the subsequent experiments for the observation of the E_2 effect on the H_2O_2-induced oxidative stress. The H_2O_2 level was measured with an oxygen electrode after the addition of catalase [19]. The glucose oxidase activity was measured following O_2 consumption by using the Clark-type oxygen electrode (YSI, Inc., Yellow Springs, OH, USA). Consequently, the activities of catalase and glutathione peroxidase, which are important complex antioxidant enzymes that remove H_2O_2, were measured to define the low-to-moderate steady-state H_2O_2 dose (see supplementary material).

Cellular production of ROS

Endometrial cells were plated (10^4 per well) in triplicate into 96-well plates, incubated for 18 h, treated with 100 nmol/L E_2 for 20 h, and exposed to a period of $[H_2O_2]$ss. These cells were subsequently incubated at 37 °C in 5% CO_2 for 12 h, washed three times in PBS, and incubated with 100 μl of 5 μmol/L monochlorobimane in PBS per well for the GSH (glutathione) determination. The cellular levels of O_2, H_2O_2, and GSH were assessed by a Gemini EM fluorimeter (Molecular Devices, USA). Fluorescence intensity was recorded hourly for 5 h. The fluorescence excitation/emission maxima for monochlorobimane were 380/485 nm, respectively. The levels of GSH were calculated in each sample as follows: ROS rate (arbitrary units $\cdot \min^{-1} \times 10^{-4}$ cells) = (fluorescence intensity (arbitrary units) at T 300 min − fluorescence intensity (arbitrary units) at T 0×300 $\min^{-1}$). The number of adherent cells was determined by the crystal violet assay [2]. The production and metabolism of GSH were assessed in the cells extracted from the 18 endometrial biopsies (7 control and 11 endometriosis patients).

Determination of catalase activity

Endometrial cells were seeded (10^4 per well) in six-well plates at 80% confluence, treated with 100 nmol/L E_2 for 20 h, and exposed to $[H_2O_2]$ss. These cells were subsequently incubated at 37 °C in 5% CO_2 for 12 h and washed twice with PBS before the evaluation of the catalase activity by ultraviolet spectroscopy at 240 nm according to Aebi [20]. The catalase measurements were compared to the amount of proteins determined by the BCA protein assay reagent kit in each sample (Pierce, Rockford, IL, USA). The catalase activity was assessed in the cells extracted from the 18 endometrial biopsies.

Lactate dehydrogenase assay

The cell viability was measured in the presence of 100 nmol/L E_2 after incubation with $[H_2O_2]$ss. These cells were washed once, resuspended in fresh medium after 12 h incubation with H_2O_2, plated (1.5×10^5 cells per well) into 12-well tissue culture plates, and incubated overnight at 37 °C before evaluation with the lactate dehydrogenase (LDH) release method. The LDH activity was measured spectrophotometrically according to a slightly modified version of the method described by Rego and Oliveira [21]. A small volume of medium was collected for the determination of extracellular LDH; the attached cells were lysed for the determination of intracellular LDH. The enzyme activity was assessed at room temperature on a microplate reader (Packard) by monitoring the rate of conversion of reduced nicotinamide adenine dinucleotide (0.28 mM NADH) to oxidized nicotinamide adenine dinucleotide (NAD^+) for 3 min at 340 nm. Pyruvate (0.32 mM in phosphate buffer, pH 7.4) was used as substrate, and the LDH released into the extracellular medium was expressed as a percentage of the total LDH activity (extracellular + intracellular LDH).

Measurement of cell death

Endometrial cells at approximately 80% confluency (10^4 per well) were harvested and assayed at the given times. Annexin V–FITC staining was conducted on each sample of 1×10^4 cells using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) follow-

ing the manufacturer's protocol. The cell staining was measured using a FACS CANTO II four-color flow cytometer (BD Biosciences) and analyzed using the FACS DIVA (BD Biosciences). The samples were independently assayed in triplicates. Apoptosis was induced by treatment with 100 nmol/L E_2 for 20 h and subsequent incubation with $[H_2O_2]$ss (30, 60, and 120 min). If not stated otherwise, the incubation period was 60 min and controlled by the addition of an excess of catalase, at the desired times, to virtually nullify the H_2O_2 concentration. These cells were washed once and resuspended in fresh medium, and apoptosis was measured at 12 h after the start of H_2O_2 incubation. This was achieved by following the flip-flop movement of phosphatidylserine between the inner and the outer plasma membrane leaflets as described. First, upon increased exposure to $[H_2O_2]$ss, and only after a long exposure period (30, 60, and 120 min), the cells started to appear in the early (annexin V positive) and late apoptosis (annexin V and propidium iodide (PI) positive) states. It is well known that when there is an increased dosage of H_2O_2, there is a shift from apoptotic to necrotic cell death [5,22]. Second, the onset of apoptosis was studied with respect to time (12 h) after a 60-min incubation with $[H_2O_2]$ss (30 μM) to maintain the concentration of H_2O_2 at 50 μM. Cells were incubated for 2 h at a glucose oxidase activity adequate to maintain a steady state of 30 μM and an excess of catalase to assess whether the product from the reaction catalyzed by the glucose oxidase or oxygen consumption in this reaction would affect the results. The level of cell death in this sample was not significantly different from that in the controls (not shown). The qualitative features of the titration curves shown in this study were well reproduced among experiments; however, the amount of cell death together with the threshold and saturation values of H_2O_2 that induced apoptosis showed some variation. The titration curves shown are representative of at least three experiments.

Fluorogenic caspase substrate

The caspase activity was measured using the fluorogenic substrate Ac-DEVD-AMC (7-acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin). Nearly confluent endometrial cells growing in six-well tissue culture plates were treated with 100 nmol/L E_2 for 20 h and subsequently incubated with $[H_2O_2]$ss as described. Cells treated with 20 μg/ml staurosporine were used as the positive control for apoptosis. After treatment, the cells were harvested, washed with cold PBS, resuspended in 50 μl of cell lysis buffer (50 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% Chaps, 1 mM dithiothreitol (DTT), 100 μM EDTA), incubated on ice for 20 min, and centrifuged at 10,000 *g* for 10 min at 4 °C. The protein concentrations in the supernatants were measured using a BCA protein assay reagent kit (Pierce). Equal amounts of protein in 50 μl of assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% Chaps, 10 mM DTT, 100 μM EDTA, 10% glycerol) were added per well to a black 96-well plate; cell lysate supernatants containing equal amounts of protein in a final volume of 50 μl were added per well to the same plate, followed by 5 μl of 1 mM Ac-DEVD-AMC. The reaction mixture was incubated for 1 h at 37 °C. The fluorescence was measured with a Gemini EM fluorimeter (Molecular Devices) using 360 nm excitation and 460 nm emission filters.

Quantification of 8-OHdG in endometrial cells

Endometrial cells at approximately 70% confluency (10^4 per well) were treated with 100 nmol/L E_2 for 20 h and incubated with $[H_2O_2]$ss. After this incubation, the genomic DNA was extracted as described previously [23]. The DNA pellets were solubilized in 0.1 mM desferrioxamine before enzymatic digestion. The HPLC-LC/MS samples were injected into an HPLC-MS/MS (Shimadzu, Kyoto, Japan) by using a C18-DB column to measure 8-OHdG (Shimadzu) and the corresponding ^{18}O- and $^{15}N_5$-labeled derivatives. In addition to the MS detector, a UV detector set at 260 nm was used to quantify the amount of DNA by using the 2′-deoxyguanosine peak area and external calibration [15]. In addition, the amount of contaminating RNA was determined using the area under the guanosine peak.

Western blot analysis

The protein extraction and Western blot analysis were performed according to standard procedures. Briefly, the total protein was isolated from the control and experimental cells using an extraction buffer containing 50 mM Tris–HCl (pH 7.4), 100 mmol/L NaCl, 50 mM NaF, 1 mM $NaVO_4$, 0.5% NP-40, and a Complete protease inhibitor cocktail (Roche, Mannheim, Germany). The cell extracts were clarified by centrifugation at 10,000 *g* and the protein concentration was estimated with a BCA protein assay reagent kit (Pierce). Equal amounts of protein (30 μg) were separated through 10% SDS–polyacrylamide gel electrophoresis. After transfer and blocking, the nitrocellulose membrane was incubated overnight at 4 °C with a 1:1000 dilution of either rabbit anti-human ERK1/2 IgG anti-phospho-ERK (pERK) at 1:1000 (Cell Signaling, Beverly, MA, USA) or anti-α-tubulin (Sigma–Aldrich, St. Louis, MO, USA) at 1:1000 dilution. The detection was carried out using the SuperSignal West Pico chemiluminescent substrate (Pierce).

Cytokines

Cytokines were assayed using the Inflammation CBA Detection Kit (BD Biosciences). The protein levels of interleukin (IL)-8, IL-1β, IL-6, IL-10, tumor necrosis factor (TNF), and IL-12p70 were quantitatively measured in endometrial cells seeded (10^4 per well) into six-well tissue culture plates. These cells were serum-deprived for 24 h after reaching subconfluency, i.e., 50 to 70% confluence; subsequently treated with E_2 for 20 h; and exposed to 30 μM $[H_2O_2]$ss. The medium removed from the wells was analyzed by FACS DIVA in a FACS CANTO II four-color flow cytometer and with BD CellQuest using standard curves (BD Bioscience).

Statistical analysis

The results from the in vitro studies are presented as the means of independent triplicate experiments. Statistical analysis was performed using GraphPad Prism 5. Means were compared by the Student *t* test and Welch's correction. In all figures, the error bars represent the SEM. A level of $p < 0.05$ was accepted as significant.

Results and discussion

Endometrial cell culture

Eutopic endometrial biopsies, from 11 patients with endometriosis and 7 women without endometriosis, were cultured into viable primary cell cultures as described in D'Amora et al. [17]; these cells reached 90% confluency within 3 ± 1 days (mean $\pm$ SEM) from the initial isolation (Fig. 1A and B). The characterization of the primary cells was performed on the third passage, which allowed these cells to present a phenotype very similar to that of the cells in the original tissues from women with and without endometriosis. The results showed approximately 90% vimentin-positive cells in all samples as determined by immunofluorescence microscopy (Fig. 1C and D).

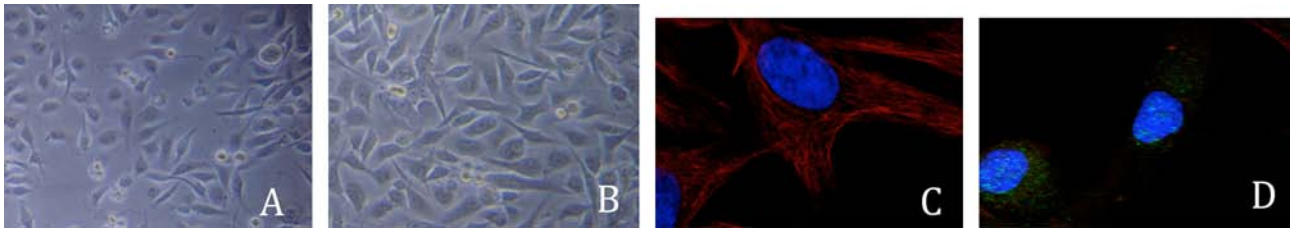

Fig. 1. The sample of mesenchymal cells (stromal endothelial) used in all experiments. Cells are shown under contrast-phase microscopy and by indirect immunofluorescence for vimentin (red), cytokeratin (green), and DAPI (blue, for nuclei). (A) Contrast phase–confluent culture after 2 days. (B) Contrast phase—confluent culture after 3 days. (C) Immunofluorescence was performed with Cy3–anti-vimentin antibodies. (D) Immunofluorescence-positive control for cytokeratin.

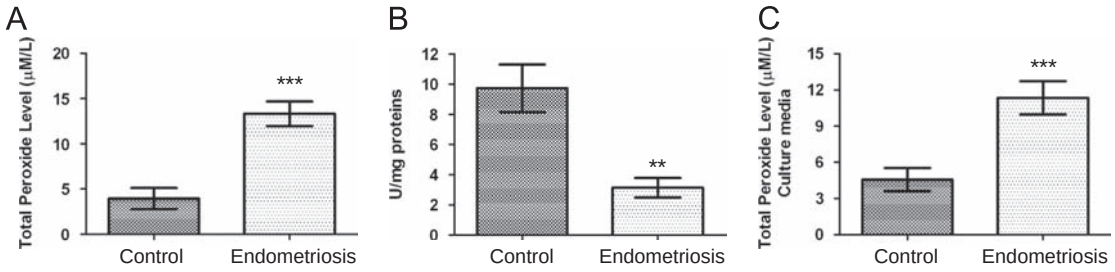

Fig. 2. Production and detoxification pathways of peroxides. (A) Peroxide levels in endometrial cells (10^4 cells), control and with endometriosis, measured by standard FOX analysis using XO reagent, $^{***}p < 0.001$. (B) Detoxification of hydrogen peroxide. Catalase activity results are expressed as catalase activity U/mg of protein, $^{**}p < 0.01$. (C) Peroxide levels in cell culture medium of endometrial cells, control and with endometriosis, measured by standard FOX analysis, $^{***}p < 0.01$. Each bar represents the mean ± SD.

Oxidative stress index

The level of oxidants and antioxidant capacity were simultaneously measured to assess oxidative stress and determine the antioxidant capacity and H_2O_2 levels in the control endometrial cells and cells from patients with endometriosis. The H_2O_2 concentration was determined by using the FOX assay with the XO indicator. The control endometrial cells, not exposed to any agent, showed no increase in H_2O_2 levels (Fig. 2A). Conversely, the endometrial cells obtained from women with endometriosis (at various stages) showed a significant increase in H_2O_2 at 16 μM, which is close to the levels produced by eukaryotic cells during an inflammatory response. The number of markers for H_2O_2 production was 2.4-fold higher in the endometrial cells from women with endometriosis (at various stages) than in the controls ($p < 0.001$) (Fig. 2A). The oxidative stress index was comparable between endometrial cells from patients with and without endometriosis, which is in agreement with results reported in previous studies [2,24]. The detoxification of H_2O_2 can be achieved through two different enzymatic systems, namely, GSH peroxidase and Cat. In this study, the Cat activity was 3-fold lower in the endometrial cells obtained from women with endometriosis than in the control cells ($p < 0.01$; Fig. 2B). The H_2O_2 levels produced in the endometrial stromal cells were similar to the levels observed in tumor cells. Furthermore, increased H_2O_2 concentration is associated with decreased catalase activity in tumor cells [25,26], which was also observed in the endometriotic cells in our study. The total peroxide levels in the medium used to culture the control endometrial cells and those with endometriosis were also investigated, measured, and analyzed as described. The medium from the control endometrial cells showed no differences in peroxide production indicative of H_2O_2, whereas the endometrial cells from patients with endometriosis showed increased H_2O_2 formation at ~13 μM (Fig. 2C). We show that the dysregulation of endogenous ROS in endometrial cells from patients with endometriosis shares numerous features with that observed in tumor cells [27].

The treatment of endometrial cells with 17β-estradiol alters the pH in the culture medium

Changes in the extracellular pH are generally not obvious during standard endometrial cell culture (with and without endometriosis). However, during the course of these studies, it was noticed that treatment with E_2 altered the pH in the medium to such a degree that it was visible during a cursory examination of the endometrial cell cultures. This effect was apparent after 16 h of treatment and more prominent after 20 h. The pH of the medium in which the control endometrial cells without treatment were grown ranged from 7.1 to 7.4 after 16 h. The treatment of the endometrial cells from women with endometriosis with E_2 prevented acidification of the medium, resulting in a pH of 8.01 after 20 h. The addition of control endometrial cells further increased the pH to 8.16 (Fig. 3). These results suggested that treatment with E_2 increases the pH, thereby preventing acidification of the medium that usually occurs during long incubation periods. Intracellular acidification has also been associated with apoptosis; necrotic cell death is more common at pH 6.5 [28,29]. Although the role of the extracellular pH in hormone treatment-induced apoptosis is unclear, previous reports indicate that changes in pH contribute to the induction of apoptosis by various agents [30,31].

Concentration and gradients of H_2O_2: induction of apoptosis in endometrial cells by steady-state concentrations of H_2O_2

Many cells can adapt to oxidative stress by showing increased resistance to subsequent high doses after exposure to low, nonlethal doses of a particular ROS [8,32]. This study provides the first report of $[H_2O_2]$ss levels in endometrial cells. An important and primary consideration is that the cell culture process itself imposes oxidative stress, either by facilitating the generation of reactive species or by hindering the adaptive upregulation of cellular antioxidants [32,33]. A quantitative approach

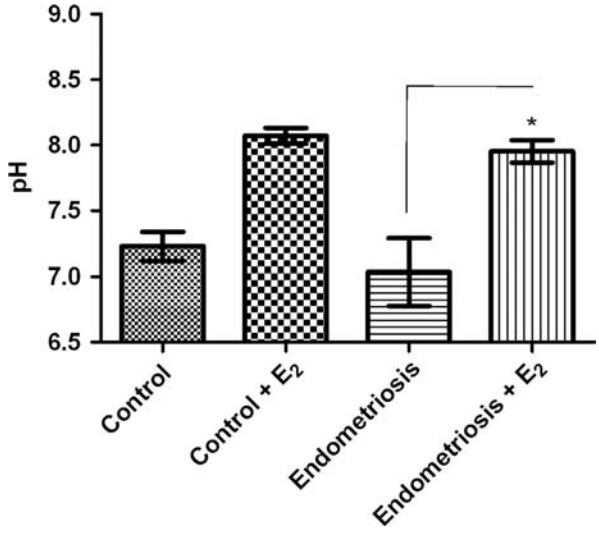

Fig. 3. Endometrial cell controls and with endometriosis at various stages have altered extracellular pH when treated with E_2. Approximately 2×10^5 cells were cultivated and, after 3 days of growth, the medium (in the absence of phenol red) was removed from each well, and pH was measured using a standard pH meter. The assay was conducted in triplicate, and the average pH values are reported ($\pm$ standard deviation, with $^*p < 0.05$).

to the induction of apoptosis by H_2O_2 was achieved by exposing the control endometrial cells and cells obtained from patients with endometriosis to $[H_2O_2]$ss. This process entailed the simultaneous addition of H_2O_2, glucose oxidase, and Cat. Fig. 4A shows the changes in H_2O_2 concentration over time and illustrates H_2O_2 in the presence of endometrial cells obtained from women with and without endometriosis. A steady state was maintained for 120 min in the endometrial cells (control and those with endometriosis) at low extracellular H_2O_2 concentrations (10 μM). Comparatively, at higher H_2O_2 concentrations (60 μM), only the endometrial cells obtained from patients with endometriosis (at various stages) were able to maintain the steady state for 120 min; a continuous increase in the extracellular concentration of H_2O_2 followed, which indicated a decreased capacity of the cells to consume H_2O_2. The well-defined saturation level of H_2O_2 at 30 μM was associated with the induction of apoptosis (Fig. 4B). A $[H_2O_2]$ss of 5 μM did not induce significant apoptosis (annexin V positive) after 30, 60, or 120 min of incubation. After the completion of these incubations, these cultures were washed with PBS and their media replaced with DMEM/F-12 supplemented with 0.5% FBS for 16 h. As expected, the concentration of H_2O_2 produced after the incubation was about 15 μM. This result indicated that increased cell proliferation (Figs. 7A and B) correlates with endogenous production of ROS. In addition, these results suggested the following: (i) a critical level of H_2O_2 is required to induce apoptosis; (ii) endometrial cells from patients with endometriosis better tolerated H_2O_2 exposure, thus indicating that H_2O_2 is a signaling molecule with a pivotal role in the induction of proliferation or cell death by apoptosis; and (iii) long incubation periods with low $[H_2O_2]$ss are not sufficient to trigger apoptosis. This scenario can be considered similar to what is observed in tumor cells. The saturation level of $[H_2O_2]$ss in endometrial cells from patients with endometriosis was defined at 30 μM based on the curves obtained from the 60- and 120-min incubations. The apoptosis levels increased from near control values to maximum levels (40%) when the $[H_2O_2]$ss was in the range of 20–30 μM (Fig. 4C, 60-min incubation curve). The duration of incubation with H_2O_2 is important for the determination of the H_2O_2 concentration required to induce apoptosis.

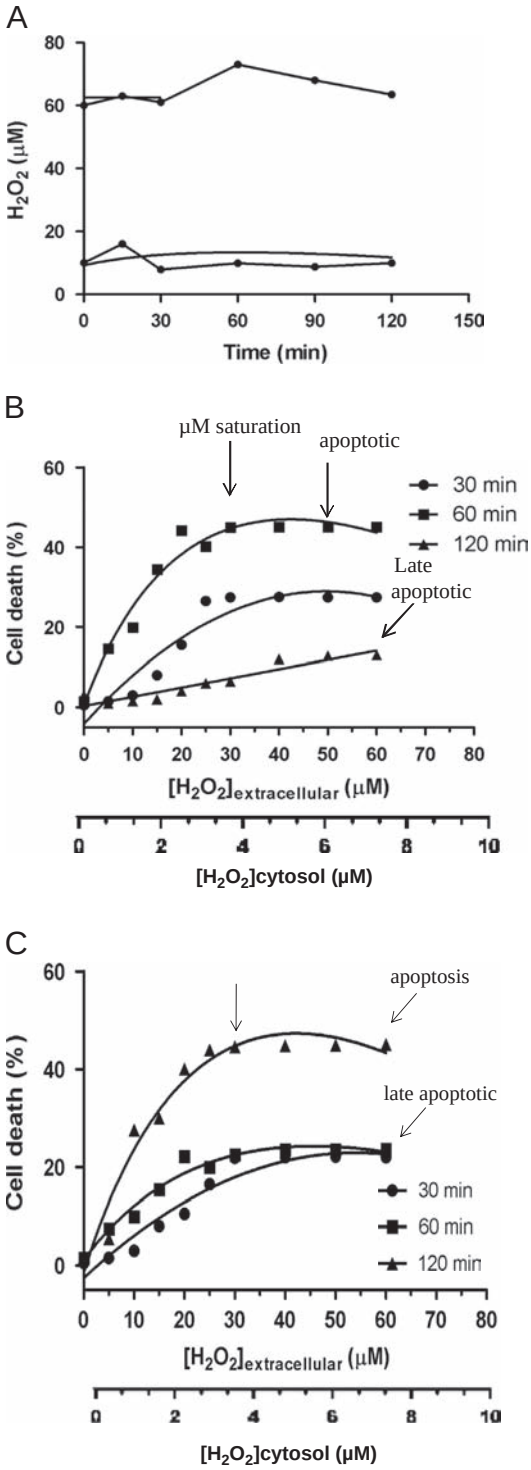

Fig. 4. Concentration of H_2O_2—induction of apoptosis in endometrial cells by steady-state concentrations of H_2O_2. (A) As described by Antunes and Cadenas [6], time courses of H_2O_2 concentrations are shown, one for a relatively low and the other for a relatively high $[H_2O_2]$ss. The dotted line represents the endometrial cells obtained from women with endometriosis. The solid line represents endometrial cell controls. Glucose oxidase was added to the cell suspension. H_2O_2 was measured in aliquots of the cell suspension at the indicated times as described under Materials and methods. (B and C) Cell death was measured 12 h after starting H_2O_2 incubation in cells incubated for 30, 60, and 120 min. (B) Endometrial cell control (μM saturation is indicated). The 120 min curve could not be used to define the saturation level because the H_2O_2 concentration did not remain at a steady-state level. (C) Endometrial cells from patients with endometriosis (μM saturation is indicated). Intracellular concentrations of H_2O_2 were estimated from the FOX assay (see text).

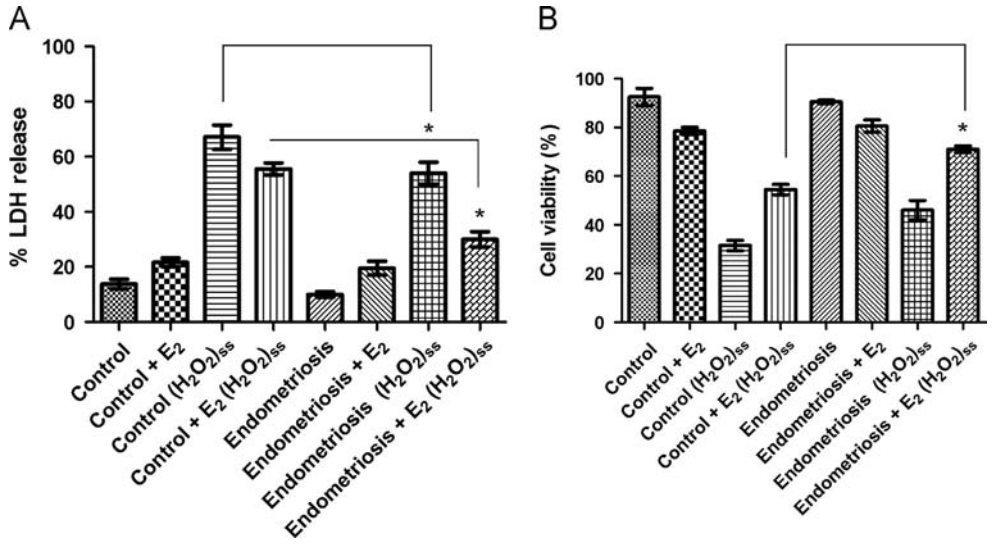

Fig. 5. LDH release test. (A) Cell lysis results in lactate dehydrogenase release into the culture medium, and LDH catalyzes the conversion of NADH to NAD^+. Therefore increased NADH oxidation, which was measured by the change in absorbance at 340 nm, was indicative of increased cell lysis and increased LDH release in endometrial cells, with and without endometriosis, treated with 30 μM H_2O_2. LDH assays were conducted in triplicate, and the averages are reported (± standard deviation, $^*p < 0.05$). (B) Percentage of cell viability ($^*p < 0.05$).

The antiapoptotic effect of 17β-estradiol on endometrial cells from patients with endometriosis

Cells are exposed to ROS, such as H_2O_2, during the course of normal aerobic metabolism or after exposure to diverse biological and environmental free radical-generating conditions. A previous study with endometrial stromal cells showed the effects of H_2O_2 by bolus addition [34]. This technique, in which a single dose of H_2O_2 is applied at the beginning of the experiment, represents the most commonly used method to expose cultured cells to H_2O_2 and does not result in a constant exogenous H_2O_2 level during the course of an experiment. This H_2O_2 cellular consumption, catalyzed by antioxidant enzymes such as Cat, requires the use of high initial doses of H_2O_2 (typically 100 μM to 1 mM), which may affect the redox homeostasis of cells and cause oxidative stress and negative responses [8,34]. In contrast, exposing cells to $[H_2O_2]$ss (using a calibrated incubation period and H_2O_2 concentrations), as opposed to bolus additions, constitutes a superior method of oxidant delivery that mimics the physiological setting [6,8,35]. E_2 is identified as playing an important role in the protection against oxidative stress, regulation of longevity signals, and enhancement of resistance to apoptotic processes [14,16]. Therefore, the antiapoptotic effect of 100 nmol/L E_2 for 20 h on oxidative stress was tested by exposing cultured eutopic endometrial cells from patients with endometriosis to $[H_2O_2]$ss.

It has been reported in the literature that E_2 induces the production of the major antioxidant GSH [36]. In this study, whether treatment with E_2 and exposure to $[H_2O_2]$ss increase the levels of GSH was investigated. The results showed that E_2 increased the levels of reduced GSH, compared to the controls, to untreated endometrial cells with endometriosis, and to endometrial cells with endometriosis treated with E_2 for 20 h and exposed to 30 μM $[H_2O_2]$ss (data not shown). The level of oxidized glutathione in the control cells was ~1.8 nmol/10^4 cells and was not affected by the E_2 treatment (data not shown). These findings support previously reported results about the protective effects of estrogens, such as E_2 [37], against oxidative stress [15,16].

Taken together, these results suggested that the oxidative environment of endometrial cells from women with endometriosis displays high levels of endogenous oxidative stress associated with an increase in ROS production and a simultaneous decrease in ROS detoxification [27,38]. These results also demonstrated that E_2 has an antiapoptotic effect on oxidative stress conditions, contributing to the progression of the disease.

Effect of 17β-estradiol on cell survival after exposure to $[H_2O_2]$ss

Cellular viability was determined through the LDH release test in the control cells and cells from patients with endometriosis treated with 100 nmol/L E_2 and followed by 30 μM $[H_2O_2]$ss exposure.

The cell viability upon hormone treatment after exposure to 30 μM $[H_2O_2]$ss for 120 min was 53% in the controls. Prior treatment with 100 nmol/L E_2 for 20 h prevented H_2O_2 (30 μM)-induced cell damage (with endometriosis) by 1.6-fold of the control level (Figs. 5A and B). The effects of treatment with 100 nmol/L E_2 and $[H_2O_2]$ss on apoptosis were studied. The results demonstrated a trend toward decreased apoptosis in the endometrial cells from patients with endometriosis when they were exposed to 30 μM H_2O_2 (Fig. 6A).

It is well known that caspase-3 has a primordial role in triggering the cascade of events that lead to apoptosis. Cell lysates from the experimental cultures were tested for functional caspase activity using the caspase-3- and caspase-7-specific substrate Ac-DEVD-AMC; this peptide–coumarin conjugate is nonfluorescent until coumarin is cleaved from the peptide by an active caspase, and consequently, increased fluorescence is indicative of caspase activity. The endometrial cells from patients with endometriosis were effectively protected from cell death after a 20-h pretreatment with E_2 in the 100 μM $[H_2O_2]$ss environment compared to the untreated control cells (Fig. 6B).

Quantification of 8-OHdG in the genomic DNA from endometrial cells

Several studies have shown that permanent oxidative damage occurs in lipids from cellular membranes, proteins, and DNA. In the nuclear and mitochondrial DNA, 8-OHdG or 8-oxo-7,8-dihydro-2′-deoxyguanosine is one of the predominant forms of free radical-induced oxidative lesions and has, therefore, been widely used as a biomarker for oxidative stress, aging, and carcinogenesis [39]. Endometriosis is considered a benign disease; however, it shares some characteristics with cancer, such as the propensity for invasion,

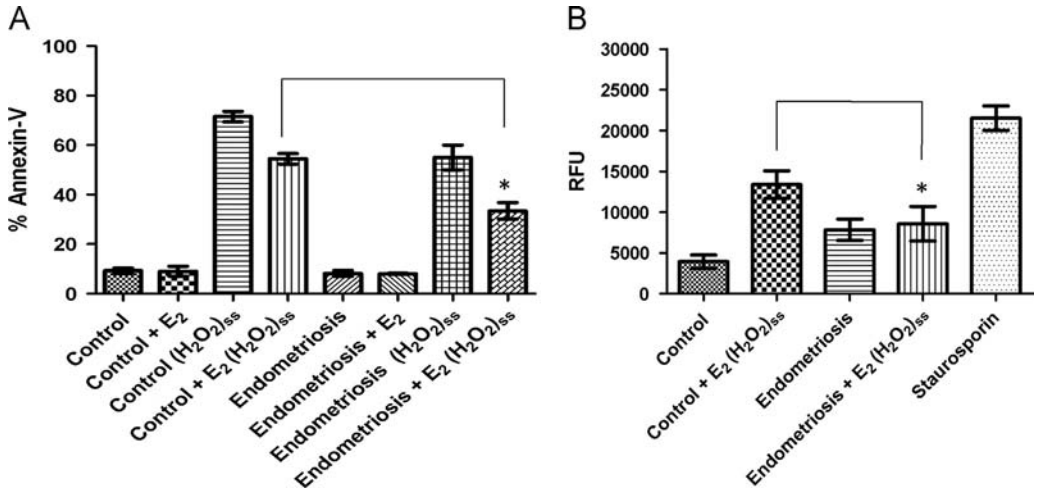

Fig. 6. Endometrial cells were exposed to steady-state 30 μM H_2O_2, harvested by trypsinization on the third day, stained with annexin V–FITC and PI, and analyzed using the FasCanto II flow cytometer. (A) Time-course analysis of annexin V–FITC staining. Flow cytometry results are reported as the percentage of annexin V–FITC-positive cells, $^*p < 0.01$. (B) Endometrial cell exposure to steady-state H_2O_2 (30 μM) induces caspase activation. Approximately 1×10^4 cells were harvested at 3 days and equal amounts of cell lysates were incubated 1 h with the fluorogenic caspase substrate Ac-DEVD-AMC. Fluorescence was reported in arbitrary relative fluorescence units (RFU) as an indicator of caspase activation. The staurosporin-treated cells were included as positive controls. Each bar indicates the average of six samples with the indicated standard deviation. The data shown are representative of three independent experiments, $^*p < 0.05$.

unrestrained growth, neoangiogenesis, and distant spreading. The treatment of cells with H_2O_2 induces the formation of the hydroxyl radical ($^\bullet$OH). These radicals can be produced by various mechanisms, specifically, the Fenton reaction of H_2O_2 (which diffuses into the nucleus), metals, and other endogenous/exogenous ROS. The interaction between $^\bullet$OH and DNA bases, such as guanine, leads to a modification that results in 8-OHdG. This modification was observed and E_2 did not protect against the formation of 8-OHdG in the endometrial cells from women with endometriosis (see the supplementary material).

Control of the proliferative properties of endometrial cells

Oxidative stress is known to induce ERK activation. The Raf/MEK/ERK signaling pathway is involved in the proliferative response induced by endogenous ROS [12,40,41]. The ERK pathway was investigated through Western blots with proteins from the endometrial cells, untreated or treated with 100 nmol/L E_2 for 20 h and exposed to 30 μM $[H_2O_2]$ss. Increased ERK phosphorylation with a pERK/α-tubulin ratio of 0.454 ± 0.226 was observed in the untreated endometrial cells from women with endometriosis compared to 0.268 ± 0.146 in the control cells ($p > 0.05$). As expected, the treatment with E_2 and exposure to $[H_2O_2]$ss resulted in a continuous increase in ERK phosphorylation or pERK/α-tubulin of 0.514 ± 0.137 in the endometrial cells from women with endometriosis ($p < 0.05$) (Figs. 7A and B). These results suggested that the presence of E_2 during $[H_2O_2]$ss has a synergistic effect on cell proliferation, thus corroborating previous studies that link ERK activation with ROS production in endometriosis [42].

Dual regulation of cytokines by $[H_2O_2]$ss in endometrial cells

H_2O_2 plays a dual role in inflammatory processes and is involved in the upregulation of a set of proinflammatory (MCP-1, IL-8, ICAM-1) and/or anti-inflammatory (IL-6) proteins. In summary, H_2O_2 upregulates the expression of proteins important in the spread of inflammation (IL-8); however, paradoxically, it leads to the production of proteins that will lower the inflammatory response level or contribute to its resolution [11,43]. Therefore, the effect of H_2O_2 associated with the treatment of 100 nmol/L E_2 for 20 h on endometriosis as a chronic inflammation was evaluated. An increase in the levels of IL-8, a

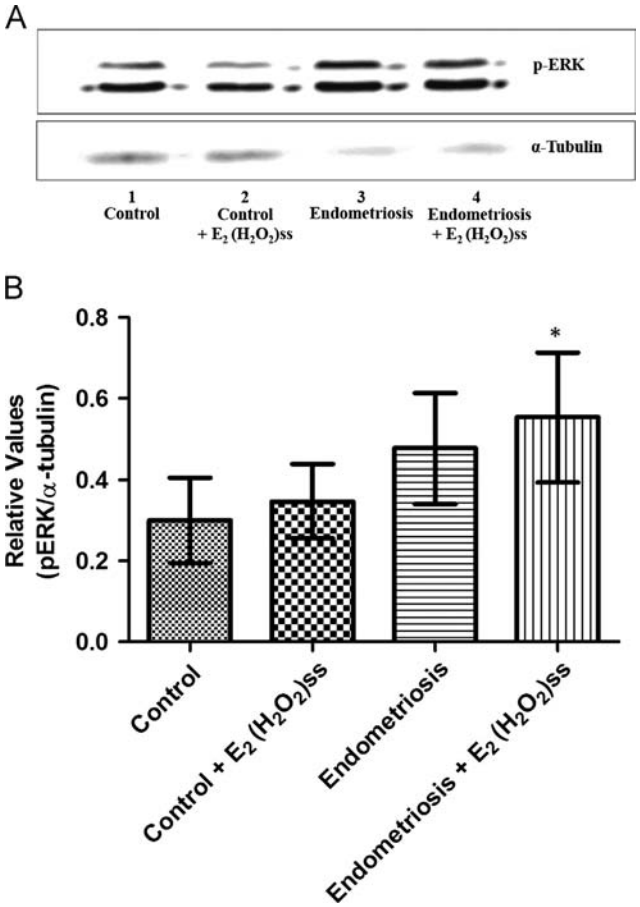

Fig. 7. (A) Presence of ERK and pERK was determined by Western blot on lysates of endometrial control cells (lanes 1 and 2) and endometrial cells with endometriosis (lanes 3 and 4). Endometrial cells were lysed and total protein lysates were immunoblotted with anti-pERK or with α-tubulin antibody for normalization. (B) Normalized values for the expression of pERK are reported in the bar graphs and expressed as means ± SD. The results are the means of three independent cell lines, $^*p < 0.05$.

proinflammatory mediator, was observed in the medium from cultured untreated endometrial cells from patients with endometriosis (Fig. 8). Conversely, the endometrial cells treated with E_2

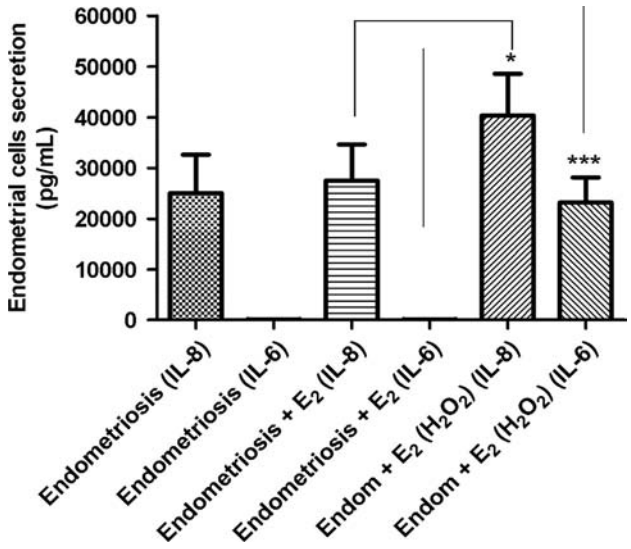

Fig. 8. Regulation of cytokines in endometrial cells with endometriosis. Cells were treated with E_2 for 20 h and then exposed to $[H_2O_2]$ss. The culture medium was recovered for endometrial cell protein secretion measurement by flow cytometer (endometrial cell protein secretion in pg/ml). Values are the means ± SEM of duplicate determinations in cultures from endometrial cells with endometriosis (at various stages), $^{*}p < 0.05$, $^{***}p < 0.001$.

and exposed to $[H_2O_2]$ss presented increased levels of IL-6, a mediator associated with acute phase response to inflammation and increased IL-8 levels. The untreated control endometrial cells did not express detectable amounts of cytokines. Interestingly, these cells presented a slight increase in IL-8 secretion when treated with E_2 and exposed to $[H_2O_2]$ss (data not shown). It is important to emphasize that cytokines such as TNF-α, IL-6, and IL-8 can be detected at increased levels in the peritoneal fluid of women with endometriosis [44]. The results from this study suggested that $[H_2O_2]$ss in the presence of E_2 could have a fine-tuning role in the regulation of the expression of these cytokines. E_2 regulates both proinflammatory cytokine mediators (such as IL-8) and low-grade inflammatory mediators (such as IL-6); both are important in spreading and potentiating inflammatory responses. The ability to avoid exacerbated inflammatory responses, such as in endometriosis, is essential in managing this disease.

Conclusions

The results in this study link the effects of E_2 on $[H_2O_2]$ss to several processes involved in the progression of endometriosis such as apoptosis resistance, increased cell proliferation, and spatial regulation of H_2O_2 and E_2 signals. These findings indicate that endometrial cells from patients with endometriosis show the dysregulation of endogenous ROS and share numerous features observed in tumor cells. Our work demonstrates that E_2, in steady-state concentrations of H_2O_2, has an antiapoptotic effect in endometrial cells from patients with endometriosis. Consequently, these cells present an altered phenotype of ROS production that leads to an increase in the cells proliferative capabilities and favors the progression of the disease. These results could lead to new therapeutic strategies for the treatment of endometriosis targeting the modulation of the signaling pathways of H_2O_2 and E_2 with antioxidant molecules.

Authors' contributions

Sheila S. Andrade and Aline Azevedo contributed the assay design, interpretation of the data, statistical analysis, and manuscript drafting. Izabel C.G. Monastério performed the in vitro assays. Ismael I.D.C.G. da Silva directed the design and coordination of the study and contributed in drafting the manuscript and interpreting the results. All the authors read and approved the final version of the manuscript.

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References

[1] Olive, D. L.; Schwartz, L. B. Endometriosis. *N. Engl. J. Med.* **328:**1759–1769; 1993.
[2] Ngo, C.; Chereau, C.; Nicco, C.; Weill, B.; Chapron, C.; Batteux, F. Reactive oxygen species controls endometriosis progression. *Am. J. Pathol.* **175:** 225–234; 2009.
[3] Novogrodsky, A.; Ravid, A.; Rubin, A. L.; Stenzel, K. H. Hydroxyl radical scavengers inhibit lymphocyte mitogenesis. *Proc. Natl. Acad. Sci. USA* **79:** 1171–1174; 1982.
[4] Burdon Alliangana, R. H.; Gill, D. V. Hydrogen peroxide and the proliferation of BHK-21 cells. *Free Radic. Res.* **23:**471–486; 1995.
[5] Lennon, S. V.; Martin, S. J.; Cotter, T. G. Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. *Cell Prolif.* **24:**203–214; 1991.
[6] Antunes, F.; Cadenas, E. Cellular titration of apoptosis with steady state concentrations of H_2O_2: submicromolar levels of H_2O_2 induce apoptosis through Fenton chemistry independent of the cellular thiol state. *Free Radic. Biol. Med* **30:**1008–1018; 2001.
[7] Antunes, F.; Cadenas, E.; Brunk, U. T. Apoptosis induced by exposure to a low steady-state concentration of H_2O_2 is a consequence of lysosomal rupture. *Biochem. J* **356:**549–555; 2001.
[8] Oliveira-Marques, V.; Marinho, H. S.; Cyrne, L.; Antunes, F. Role of hydrogen peroxide in NF-kappaB activation: from inducer to modulator. *Antioxid. Redox Signaling* **11:**2223–2243; 2009.
[9] Veal, E.; Day, A. Hydrogen peroxide as a signaling molecule. *Antioxid. Redox Signaling* **15:**147–151; 2011.
[10] Otterbein, L. E.; Bach, F. H.; Alam, J.; Soares, M.; Tao Lu, H.; Wysk, M.; Davis, R. J.; Flavell, R. A.; Choi, A. M. Carbon monoxide has anti-inflammatoryeffects involving the mitogen-activated protein kinase pathway. *Nat. Med* **6:**422–428; 2000.
[11] de Oliveira-Marques, V.; Cyrne, L.; Marinho, H. S.; Antunes, F. A quantitative study of NF-kappaB activation by H_2O_2: relevance in inflammation and synergy with TNF-alpha. *J. Immunol.* **178:**3893–3902; 2007.
[12] McCubrey, J. A.; Steelman, L. S.; Abrams, S. L.; Lee, J. T.; Chang, F.; Bertrand, F. E.; Navolanic, P. M.; Terrian, D. M.; Franklin, R. A.; D'Assoro, A. B.; Salisbury, J. L.; Mazzarino, M. C.; Stivala, F.; Libra, M. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Adv. Enzyme Regul.* **46:**249–279; 2006.
[13] Akoum, A.; Jolicoeur, C.; Boucher, A. Estradiol amplifies interleukin-1-induced monocyte chemotactic protein-1 expression by ectopic endometrial cells of women with endometriosis. *J. Clin. Endocrinol. Metab* **85:**896–904; 2000.
[14] Dassen, H.; Punyadeera, C.; Kamps, R.; Delvoux, B.; Van Langendonckt, A.; Donnez, J.; Husen, B.; Thole, H.; Dunselman, G.; Groothuis, P. Estrogen metabolizing enzymes in endometrium and endometriosis. *Hum. Reprod.* **22:**3148–3158; 2007.
[15] Hirota, J.; Baba, M.; Matsumoto, M.; Furuichi, T.; Takatsu, K.; Mikoshiba, K. T-cell-receptor signalling in inositol 1,4,5-trisphosphate receptor (IP3R) type-1-deficient mice: is IP3R type 1 essential for T-cell-receptor signalling? *Biochem. J* **333(Pt 3):**615–619; 1998.
[16] Urata, Y.; Ihara, Y.; Murata, H.; Goto, S.; Koji, T.; Yodoi, J.; Inoue, S.; Kondo, T. 17Beta-estradiol protects against oxidative stress-induced cell death through the glutathione/glutaredoxin-dependent redox regulation of Akt in myocardiac H9c2 cells. *J. Biol. Chem.* **281:**13092–13102; 2006.
[17] D'Amora, P.; Maciel, T. T.; Tambellini, R.; Mori, M. A.; Pesquero, J. B.; Sato, H.; Girão, M. J. B. C.; Guerreiro da Silva, I. D. C.; Schor, E. Disrupted cell cycle control in cultured endometrial cells from patients with endometriosis harboring the progesterone receptor polymorphism PROGINS. *Am. J. Pathol.* **175:**215–224; 2009.
[18] Gay, C.; Collins, J.; Gebicki, J. M. Determination of hydroperoxides by the ferric-xylenol orange method. *Redox Rep.* **4:**327–328; 1999.
[19] Antunes, F.; Cadenas, E. Estimation of H_2O_2 gradients across biomembranes. *FEBS Lett.* **475:**121–126; 2000.

[20] Aebi, H. Catalase in vitro. *Methods Enzymol.* **105:**121–126; 1984.
[21] Rego, A. C.; Oliveira, C. R. Dual effect of lipid peroxidation on the membrane order of retinal cells in culture. *Arch. Biochem. Biophys.* **321:**127–136; 1995.
[22] Antunes, F.; Cadenas, E. Estimation of H_2O_2 gradients across biomembranes. *FEBS Lett.* **475:**121–126; 2000.
[23] Ravanat, J. L.; Gremaud, E.; Markovic, J.; Turesky, R. J. Detection of 8-oxoguanine in cellular DNA using 2,6-diamino-8-oxopurine as an internal standard for high-performance liquid chromatography with electrochemical detection. *Anal. Biochem* **260:**30–37; 1998.
[24] Szczepanska, M.; Mikolajczyk, M.; Raczynska, P.; Skrzypczak, J. [The evaluation of IL-12 levels in peritoneal fluid and serum of women with endometriosis]. *Ginekol. Pol.* **72:**408–417; 2001.
[25] Amstad, P.; Peskin, A.; Shah, G.; Mirault, M. E.; Moret, R.; Zbinden, I.; Cerutti, P. The balance between copper–zinc superoxide dismutase and catalase affects the sensitivity of mouse epidermal cells to oxidative stress. *Biochemistry (Moscow)* **30:**9305–9313; 1991.
[26] Rodríguez, A. M.; Carrico, P. M.; Mazurkiewicz, J. E.; Meléndez, J. A. Mitochondrial or cytosolic catalase reverses the MnSOD-dependent inhibition of proliferation by enhancing respiratory chain activity, net ATP production, and decreasing the steady state levels of H_2O_2. *Free Radic. Biol. Med* **29:**801–813; 2000.
[27] Arnold, R. S.; Shi, J.; Murad, E.; Whalen, A. M.; Sun, C. Q.; Polavarapu, R.; Parthasarathy, S.; Petros, J. A. Lambeth, J. D. Hydrogen peroxide mediates the cell growth and transformation caused by the mitogenic oxidase Nox1. *Proc. Natl. Acad. Sci. USA* **98:**5550–5555; 2001.
[28] Goossens, J. F.; Henichart, J. P.; Dassonneville, L.; Facompre, M.; Bailly, C. Relation between intracellular acidification and camptothecin-induced apoptosis in leukemia cells. *Eur. J. Pharm. Sci.* **10:**125–131; 2000.
[29] Gottlieb, R. A.; Nordberg, J.; Skowronski, E.; Babior, B. M. Apoptosis induced in Jurkat cells by several agents is preceded by intracellular acidification. *Proc. Natl. Acad. Sci. USA* **93:**654–658; 1996.
[30] Lee, Y. J.; Song, J. J.; Kim, J. H.; Kim, H. R.; Song, Y. K. Low extracellular pH augments TRAIL-induced apoptotic death through the mitochondria-mediated caspase signal transduction pathway. *Exp. Cell Res.* **293:**129–143; 2004.
[31] Sharma, M.; Sahu, K.; Dube, A.; Gupta, P. K. Extracellular pH influences the mode of cell death in human colon adenocarcinoma cells subjected to photodynamic treatment with chlorin p6. *J. Photochem. Photobiol. B* **81:** 107–113; 2005.
[32] Halliwell, B.; Whiteman, M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br. J. Pharmacol* **142:**231–255; 2004.
[33] Halliwell, B. Oxidative stress in cell culture: an under-appreciated problem? *FEBS Lett* **540:**3–6; 2003.
[34] Foyouzi, N.; Berkkanoglu, M.; Arici, A.; Kwintkiewicz, J.; Izquierdo, D.; Duleba, A. J. Effects of oxidants and antioxidants on proliferation of endometrial stromal cells. *Fertil. Steril.* **82(Suppl. 3):**1019–1022; 2004.
[35] Grant, C. M. Regulation of translation by hydrogen peroxide. *Antioxid. Redox Signaling* **15:**191–203; 2011.
[36] Yang, S. H.; Liu, R.; Perez, E. J.; Wen, Y.; Stevens Jr S. M.; Valencia, T.; Brun-Zinkernagel, A. M.; Prokai, L.; Will, Y.; Dykens, J.; Koulen, P.; Simpkins, J. W. Mitochondrial localization of estrogen receptor beta. *Proc. Natl. Acad. Sci. USA* **101:**4130–4135; 2004.
[37] Dabrosin, C.; Ollinger, K.; Ungerstedt, U.; Hammar, M. Variability of glutathione levels in normal breast tissue and subcutaneous fat during the menstrual cycle: an in vivo study with microdialysis technique. *J. Clin. Endocrinol. Metab* **82:**1382–1384; 1997.
[38] Szatrowski, T. P.; Nathan, C. F. Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res.* **51:**794–798; 1991.
[39] Valavanidis, A.; Vlachogianni, T.; Fiotakis, C. 8-Hydroxy-2′-deoxyguanosine (8-OHdG): a critical biomarker of oxidative stress and carcinogenesis. *J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev* **27:**120–139; 2009.
[40] Laurent, A.; Nicco, C.; Chereau, C.; Goulvestre, C.; Alexandre, J.; Alves, A.; Levy, E.; Goldwasser, F.; Panis, Y.; Soubrane, O.; Weill, B.; Batteux, F. Controlling tumor growth by modulating endogenous production of reactive oxygen species. *Cancer Res.* **65:**948–956; 2005.
[41] Kageyama, N.; Miura, M.; Ichinose, M.; Tomaki, M.; Ishikawa, J.; Ohuchi, Y.; Endoh, N.; Shirato, K. Role of endogenous nitric oxide in airway microvascular leakage induced by inflammatory mediators. *Eur. Respir. J.* **10:**13–19; 1997.
[42] Murk, W.; Atabekoglu, C. S.; Cakmak, H.; Heper, A.; Ensari, A.; Kayisli, U. A.; Arici, A. Extracellularly signal-regulated kinase activity in the human endometrium: possible roles in the pathogenesis of endometriosis. *J. Clin. Endocrinol. Metab.* **93:**3532–3540; 2008.
[43] Xing, Z.; Gauldie, J.; Cox, G.; Baumann, H.; Jordana, M.; Lei, X. F.; Achong, M. K. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J. Clin. Invest.* **101:**311–320; 1998.
[44] Harada, T.; Iwabe, T.; Terakawa, N. Role of cytokines in endometriosis. *Fertil. Steril.* **76:**1–10; 2001.