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EFFECTS OF THE NATURAL PROTEASE INHIBITOR AND HORMONE THERAPY, ALONE AND IN COMBINATION, ON UTERINE LEIOMYOMA CELLS

KEY WORDS: Anoikis; cell death; 17 -estradiol; leiomyoma; progesterone; protease inhibitor.

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ABSTRACT

Uterine leiomyoma is a benign tumor, common in women of reproductive age. It is considered a public health problem because surgery is the most effective treatment followed by treatment of major relapse. The EcTI protease inhibitor extracted from *Enterolobium contortisiliquum* seeds was tested as a modulator of leiomyoma conditions and its effects on the adhesion pathway targeting FAK/Src and p130cas proteins, cell cycle, and cell death were analyzed. We used primary leiomyomatous cells and their respective adjacent tissues cells in culture, treated with 17β-estradiol (E_2), progesterone (P_4), and EcTI, alone and in combination. The FAK/Src phosphorylated form was increased in adjacent tissue cells while a significant decrease was observed in leiomyomatous cells, which may explain the loss of focal adhesion. In addition, the increased p130cas protein expression in adjacent cells may suggest a protection from apoptosis induced by Anoikis; unlikely, the decrease of this component resulted from treatments in the leiomyomata's cells stimulated the process. In the cell death pathway, the treatment of leiomyomatous cells with progesterone and EcTI leads to death; this effect is corroborated by the increased expression of the pro-apoptotic Bax protein and decreased expression of the anti-apoptotic protein BcL-2. EcTI, through recruiting the p130cas protein, activates key aspects of the Anoikis process, established by the relationship between the Bax and BcL-2 cell death proteins.

Introduction

Uterine leiomyoma, also known as uterine fibroids, are the most common benign smooth muscle tumors of the female genital tract. It is the main indication for hysterectomy, affecting approximately 70–80% of women in reproductive age. Although they are commonly benign, they cause hemorrhagic disorders, discomfort, or pelvic pain [1-2]. The fact that fibroids appear during the period of reproductive life, increase during gestation, and regress after menopause suggests its dependence on ovarian hormones [3-4].

It is characterized by a single or multiple tumor nodules of varying sizes and classified according to its location. They are considered monoclonal tumors with independent origin between nodules in the same uterus, and histologically as benign neoplasms of smooth muscle cells that are immersed in a large extracellular matrix disorder [5-6].

The occurrence of uterine leiomyoma demonstrates a familial and racial predisposition being 2.2 times more frequent among first-degree relatives in families with two or more affected limbs, and two or three times higher in African-American women than in Caucasian women. Reproductive factors also appear to affect the development of leiomyoma with a protective role of parity in relation to its incidence [6,7-8].

Two models for fibroids outset, growth, and maintenance have been proposed: (1) increased levels of17β-estradiol (E_2), Progesterone (P_4), and their receptors lead to increased mitoses and somatic mutations; and (2) increased levels of estrogen receptors in the myometrium of a fibroid uterus may be due to inherent abnormality [9-10].

Ovarian steroid hormones

Ovarian steroid hormones are responsible for controlling the differentiation, proliferation, and degeneration of cells that compose the uterus throughout the female reproductive life. The response of uterine tissue to steroid hormones is complex and involves biochemical and morphological events resulting in uterine growth and differentiation. Although the mitogenic effect of these hormones, especially 17β-estradiol, appears to be in the endometrium, the myometrium also exhibits sensitivity to the hypertrophic and hyperplasic actions of steroids [11-12]. Estradiol plays an important role in the regulation of reproductive function. Two estradiol receptors (Erα and Erβ) are known to bind estradiol and mediate its hormonal effects [13-14].

Ishikawa et al. [15] suggested that estradiol could maintain progesterone receptor (PR) levels and thus, promote leiomyoma growth. In addition, estradiol would significantly decrease the expression of the p53 protein (tumor suppressor protein) in leiomyoma cells when compared to normal myometrial cells, suggesting that leiomyoma growth is partially due to the inhibition of p53 functions Progesterone is a key hormone in the female reproductive system, playing an important role in preparing the uterus for implantation, and establishment and maintenance of

pregnancy. The action of progesterone on the uterine tissues including the endometrium, myometrium, and colon are mediated by the combined effects of their receptors [12].

EcTI (*Enterolobium contortisiliquum* Trypsin Inhibitor)

Enterolobium contortisiliquum belongs to the Mimosoideae subfamily and is widely found in Brazil as trees that are more than 20 meters high; its fruits are rich in tannins and saponins [16]. *E. contorsiliquum* seeds contain a 20 kDa molecular mass trypsin inhibitor, the ***E***nterolobium ***c***ontortisiliquum **T**rypsin **I**nhibitor – EcTI, in the concentration of 5.6 mg of inhibitor per gram of seeds, which indicates a potential alternative to commercial inhibitor preparation to be used as a biochemical tool [17]. The tertiary structure of EcTI was determined confirming its structural similarity with the plant Kunitz family of protease inhibitors and presenting the same conserved amino acid residues, including disulfide bridges [18]. It inhibits the activity of trypsin, chymotrypsin, human plasma kallikrein (huPK), plasmin, and human neutrophil elastase, and prevents the activation of MMP-2 and MMP-9. EcTI is cytotoxic to gastric cancer cells without affecting normal remodeling tissue (fibroblast) and regenerative hMSCs [19]. Furthermore, studies by Paula et al. [20] have shown that EcTI inhibits the invasion of gastric cancer cells by changing the integrin-dependent cell signaling pathway.

Materials and Methods

Inhibitor purification

EcTI was purified using a protocol described by Batista et al. and modified by de Paula, [20-21]. Briefly, E. contortisiliquum seeds were crushed and homogenized in 20 mM Tris/HCl (1:10, w/v). The crude extract was centrifuged at 5000x g at 4°C for 15 min, and proteins were collected from the supernatant through acetone precipitation (80%, v/v) at 4°C. The precipitate was vacuum-dried and solubilized in 50 mM Tris/HCl buffer at pH 8.0 and 50 mM NaCl and applied to a DEAE-Sephadex column (2x25 cm), pre-equilibrated with 100 mM Tris/HCl buffer at pH 8.0. The inhibitor activity was measured by the inhibition of trypsin hydrolysis using 1 mM N-α-benzoyl-DL arginine *p*-nitroanilide (Bachem) as substrate. Fractions were sequentially applied to trypsin-Sepharose and a Superdex 75 column (coupled to the ÄKTA, GE Helthcare), equilibrated with 50 mM Tris/HCl buffer at pH 8.0. The purification profile was evaluated by reserve phase chromatography on a C18 protein/peptide column (15 cm x 4.6 mm, Vydac) with a gradient of acetonitrile (0-100%) of trifluoroacetic acid (TFA) 1% (v/v) at room temperature and a flow rate of 0.7 mL/min.

Cell culture

The standardization of the primary culture of leiomyoma and adjacent tissue cells was performed according to Bonazza et al. [22]. This study was approved by the Institutional Ethics Review Board (CEP0858/10) from São Paulo Federal University (UNIFESP) and performed in compliance with the Declaration of Helsinki. Patients (N= 8) who volunteered to participate in the study signed the Informed Consent Form before the study start. The primary cell culture was maintained in DMEM-F12 (Dulbecco's modified Eagle's medium) without phenol red and high glucose (Sigma–Aldrich), 1% antibiotic mix containing 100 mL penicillin and 100 mg/mL streptomycin, and 10% FBS (fetal bovine serum); cultures were maintained in a humidified incubator at 37°C and 5% CO_2 for three days. Cell culture expansion was performed using a PBS solution (10 mM phosphate buffered saline at pH 7.4) with 0.02% ethylenediaminetetraacetic acid (EDTA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay

The MTT reaction is a colorimetric assay that relies on the ability of mitochondrial and cytosolic dehydrogenases of viable cells to reduce the MTT reagent to purple formazan crystals [23]. The viability of primary cells in the conditions of serum, collagen coated plates, and treatment with E_2 (17β-estradiol), P_4 (progesterone), and EcTI was determined by measuring the cell redox activity using the MTT assay in different conditions. In the first condition, cells were plated at 5 × 10^3 density on 96-well microplates (Corning Inc.); medium was removed after cell attachment and replaced with DMEM-F12 with 2% FBS and plates were incubated for 24 h at 37°C and 5% CO_2. In the second condition, 5 × 10^3 cells were plated on 96-well microplates (Corning Inc.) coated with 10 µg collagen I as described by de Paula et al., 2012, and left to adhere for 4 h in a humidified atmosphere at 37°C and 5% CO_2. In the third condition, the primary culture of leiomyoma and adjacent tissue cells was submitted to different concentrations of EcTI (0, 5.0, 25.0, 50.0, and 100.0 µM) in the presence and absence of a type I collagen coat during 24 h at 37°C and 5% CO_2 for the standardization of the EcTI concentration; myometrial cells (5 × 10^3 per well) were seeded to display 80% confluence in collagen type I coated plates and cultured in DMEM-F-12 without phenol red and supplemented with 2% FBS, 100 IU/ml penicillin, 100 mg/ml streptomycin, and treated with 100 nM E_2, 100 nM P_4, and different EcTI concentrations (0, 5.0, 25.0, 50.0, and 100.0 µM) for 24 h. Under all conditions, cells were added to 5 mg/mL MTT solution (Calbiochem) in PBS and incubated for 3 h at 37°C and 5% CO_2. After incubation, the solution was removed, formazan crystals were dissolved in DMSO (dimethyl sulphoxide) (Sigma), and absorbance was measured at 540 nm using a microplate reader (Spectra max Plus 384, Molecular Devices, CA, USA).

The Bromodeoxyuridine (BrdU) cell proliferation assay

Cell proliferation was assessed by the incorporation of BrdU (Kit III, Roche, Mannheim, Germany), a synthetic analog of the thymide nucleoside incorporated into the DNA of cells at the time of proliferation [24], and according to the manufacturer's instructions. Cells were plated at 5 × 10^3 density on 96-well microplates (Corning Inc.), the medium was removed after cell attachment and replaced with DMEM-F12 with 2% FBS; plates were incubated for 24 h at 37°C and 5% CO_2. These cells were subsequently treated with E_2 (100 nM), P_4 (100 nM), and EcTI (50 µM) for 24 h. After 24 h incubation, the BrdU solution (100 µM) was added to the medium and cells were incubated for 4 h at 37°C in a humidified atmosphere containing 5% CO_2. Thereafter, cells were incubated in a solution of nucleases to fix and denature the DNA for the incorporation of anti-BrdU. Incorporated DNA was detected by the addition of the anti-BrdU POD antibody. The immune complex was detected by a subsequent substrate colorimetric reaction and quantified by measuring absorbance at 405 nm in the FlexStation Multi-Mode Microplate Reader (Molecular Devices). All experiments were performed in triplicate.

Immunoblot analysis - Western blot analysis

After cell cultures were treated for 24 h with 2% FBS, E_2, P_4, and EcTI, alone or in combination, they were washed twice with PBS (10 mM phosphate buffered saline at pH 7.4). These plates were scraped with a disposable cell scraper and lysis buffer containing 1% Triton X100, 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 100 mg/mL phenylmethylsulfonyl fluoride (PMSF), 0.1% sodium dodecylsulfate (SDS), 1 mg/ml aprotinin, 0.5% sodium deoxycholate, 1 mM Na_3VO_4 (sodium orthovanadate), and 100 mM NaF (sodium fluoride) at pH 7.5 and subsequently frozen at -80°C. Proteins were quantified through the Micro BCA Protein Assay kit from Pierce (Rockford, IL, USA). Protein extracts (100 µg) were electrophoresed in a 10% and 12% SDS-polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% BSA in trizma base saline by 25 mM Tris/HCl + 192 mM glycine, pH 7.5 (TBS) at 4°C overnight and incubated at room temperature for 2 h with the corresponding anti-Akt rabbit and anti-phospho-Akt rabbit primary antibodies, anti-Erk 1/2 MAPK rabbit and anti-phospho-Erk 1/2 MAPK rabbit, anti-FAK rabbit, anti-phospho-FAK rabbit (Tyr-397), anti-Src rabbit and anti-phospho-Src rabbit (Tyr-416), anti-p130cas rabbit (Y165), anti-pRb rabbit, anti-p27 rabbit, anti-p53 rabbit, anti-BcL-2 rabbit, anti-Bax rabbit, and anti-β-actin rabbit antibodies. All antibodies were diluted 1:1000 in TBS containing 0.1% Tween20 (TBST), 1 % BSA. Membranes were further incubated for one hour at room temperature with the appropriate secondary antibodies (anti-rabbit IgG, HRP linked antibody, Cell Signaling Technology) diluted in 1% BSA in TBST. Membranes were washed three times after each step with TBS in moderate agitation for five minutes. Antigen–antibody complexes were detected using the ECL chemiluminescence detection system (Amersham, Arlington Heights, IL, USA). All antibodies were acquired from Cell Signaling Technology (Beverly, MA, USA).

Statistical analysis

All experiments were performed in triplicate and results were averaged. The statistical analyses were performed using GraphPad PRISM5.0 (La Jolla, CA). The Tukey's post-test was used to compare means between two or more independent groups. Two-way ANOVA was used to compare group means influenced by two independent factors. The error bars represent the SD. The level of $p \leq 0.05$ was accepted as significant.

Results and Discussion

Determination of the EcTI dose for an effective treatment

The fact that EcTI did not interfere in the viability of adjacent tissue cells (Figure 1A) is an interesting result because the developmental characteristic of these cells is not modified. In leiomyoma cells, a significant decrease in viability was observed with increased inhibitor concentration (Figure 1B). It is possible to note that collagen plays an important role in the viability of these cells because their proliferation resembles that of cells from adjacent tissues in the experiment carried out in the presence of collagen type I. Moreover, the concentration of 50 µM was used in further experiments since the cell response was similar using 100 µM.

The effects of hormones, estradiol (E_2) and progesterone (P_4), and EcTI on cells

Cell proliferation and metabolism assay

Treatment with estradiol (E_2) caused a significant decrease in the proliferation of adjacent tissue cells (Figure 1C), although their cellular metabolism increased (Figure 1E). These results corroborate those reported by Ruiz-Cortés [14] showing that hormonal treatment (E_2) alone may lead to decreased proliferation in non-leiomyomatous cells. This effect was neutralized by EcTI, which per se improves proliferation in this cell type. The treatment with Progesterone (P_4) did not alter cell proliferation and cellular metabolism in all conditions in which this hormone was present.

In leiomyoma cells, an increase in proliferation and the metabolic process by hormonal treatments were observed, either isolated (E_2 or P_4) or in hormone conjugates ($E_2 + P_4$), (Figure 1D and 1F). The use of hormone conjugates associated with EcTI (E_2+P_4+EcTI) showed a significant decrease in cell proliferation and metabolism (Figure 1D and 1F). Although our results show increased proliferation by hormone treatment (Figure 1D), the cell viability experiments indicated that cells have their metabolism decreased (Figure 1F), corroborating clinical treatments [25-26] using combined hormone therapy to block tumor growth. The efficacy of the EcTI protein in these processes was very clear because the treatment with protease inhibitor alone decreased proliferation and metabolic process.

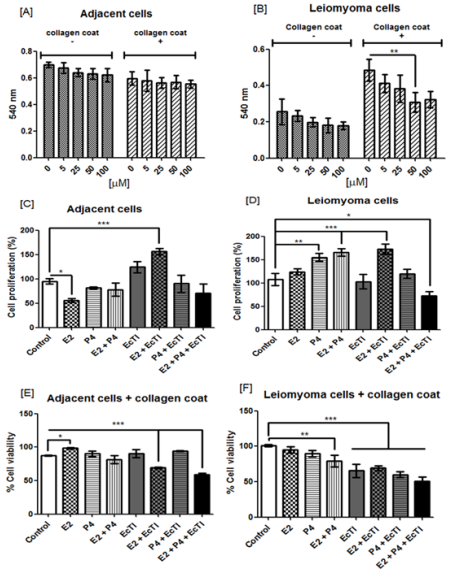


Figure 1. Effects of EcTI on the viability, proliferation of leiomyoma, non-leiomyomatous (adjacent) cells. Cells were treated with increasing concentrations of EcTI (5, 25, 50, and 100 µM) for 24 h and viability was determined by the MTT assay. **(A)** adjacent cells and **(B)** leiomyoma cells. Cells were treated with E_2 (100 nmol/L), P_4 (100 nmol/L), and EcTI (50 µM) for 24 h. Proliferation and viability was determined using the MTT assay. **(C and E)** adjacent cells and **(D and F)** leiomyoma cells. Absorbance values were measured at 540 nm and normalized to the non-treated cells control. Data from 8 patients. The statistical significance was evaluated using one-way ANOVA followed by the Tukey's test. The error bars represent the SD. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

The effects of hormones estradiol (E_2), progesterone (P_4), and EcTI on cells signaling

An undeniable feature of uterine tissues and leiomyomas is their responsiveness to steroid hormones [27]. Thus, we decided to study the expression of cell signaling proteins after exposing leiomyoma and adjacent tissue cells to hormone treatment only and in combination with the EcTI treatment.

Survival pathway analysis (Akt protein)

The phosphorylation of the Akt protein in adjacent tissue cells increases significantly with hormone conjugate ($E_2 + P_4$) as well as treatment with EcTI, alone or associated with estradiol (E_2) (Figure 2A). Conversely, decreased phosphorylation was observed in all treatments in leiomyoma cells, being more effective when cells were treated with the association of hormones and EcTI (Figure 2B) indicating that the hormone-associated protease inhibitor decreases the survival of leiomyoma cells but not that of adjacent tissue cells. To clarify the mechanism involved in this process, we evaluated the effects of these treatments on cell signaling pathways.

Analysis of the FAK/Src signaling pathways

The phosphorylation analyses of FAK/Src proteins are associated with several other signaling pathways [28]. It is interesting to note that there was an increase in the FAK/Src phosphorylation by estradiol (E_2), progesterone (P_4), and hormone conjugate ($E_2 + P_4$) in adjacent tissue cells (Figure 2C). The fact that EcTI associated with hormones leads to phosphorylation at a level lower than that observed in the control (Figure 2C) may be relevant because these adjacent tissue cells present the normal characteristics of myometrial cells. The phosphorylation of FAK/Src in leiomyoma cells was significantly decreased in all treatments (Figure 2D). The loss of focal adhesion, stimulating the Anoikis process, might be a consequence of the decreased FAK/Src phosphorylation. In the specific case of EcTI, its effect blocking cell adhesion mediated by collagen I, which is present in large qualities in leiomyoma, has been reported in studies by de Paula et al. [20].

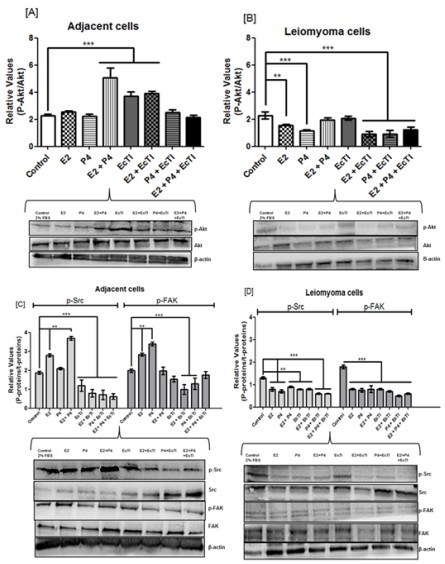


Figure 2. Detection of signaling phosphoproteins by immunoblot analysis in leiomyoma and miometrial adjacent cells. Cells were treated with E_2(100 nmol/L), P_4 (100 nmol/L), and

EcTI (50 µM) for 24 h. After the SDS-PAGE separation of cell lysates and electrotransfer to PVDF membranes, membranes were blocked and incubated with the rabbit primary antibodies anti-phospho-Akt, anti-Akt, anti-phospho-Src (Tyr-416), anti-Src, anti-phospho-FAK (Tyr-397), anti-FAK and anti-β-actin. Graph bars represent the densitometric analyses of the immunoblotting results. The results are represented as band intensities in arbitrary units relative to the respective total phospho-proteins loads and total proteins loads. Antibody binding was visualized by chemiluminescence, and the quantification of the relative Akt, Src and FAK respectively expression in adjacent cells (A and C) and leiomyoma cells (B and D) in each sample was performed by densitometry analysis in the Image J software with β-actin normalization. The error bars represent the SD. **p≤0.01; ***p≤0.001.

Analysis of the proliferation pathway (Erk protein)

In non-leiomyomatous cells, treatments with hormones and EcTI lead to increased Erk expression (Figure 3A), a protein that is related to cell proliferation. The increase of Erk (phospho-p44/42) MAPK in leiomyoma has already been reported [29] and discussed considering the contribution of this pathway to the establishment of pathophysiological conditions [30].

Although we observed a significant increase in Erk phosphorylation in leiomyoma cells in the treatment with progesterone (P_4) or hormone conjugate (E_2+P_4), the association of progesterone with EcTI (P_4+EcTI), or hormone conjugate associated with EcTI (E_2+P_4+EcTI), neutralized this effect because the Erk phosphorylation remains similar to that observed in the control (Figure 3B).

Analysis of focal adhesion through p130cas (Y165)

The p130cas protein is closely associated with several functions in cell survival, proliferation, and migration pathways [31]. Although the FAK/Src phosphorylation impairment in adjacent cells may reflect on cell adhesion blockade, the marked increase in the phosphorylation of p130cas, a protein involved in the focal adhesion pathway [32], by EcTI associated with steroid hormones (Figure 3C) might have a compensatory effect. In leiomyoma cells, it is possible to observe an increase in p130cas phosphorylation, however, this phosphorylation is much more effective when the cells are exposed to progesterone (P_4) associated with EcTI and hormone conjugate associated with EcTI (E_2 + P_4) (Figure 3D). Indeed, it is clear that the effect on p130cas phosphorylation was higher in adjacent cells, suggesting protection from the apoptosis process induced by Anoikis [33], unlike that observed in leiomyomatous cells, indicating that they could be in the apoptotic process.

The effect of the treatments leads us to question how these treatments influence cell adhesion. In this case, the adhesion process is not impaired by the decreased phosphorylation in this pathway because the treatment increased the phosphorylation of another protein involved.

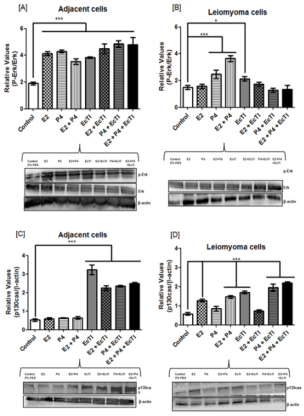


Figure 3. Detection of signaling phosphoproteins by immunoblot analysis in leiomyoma and miometrial adjacent cells. Cells were treated with E_2(100 nmol/L), P_4 (100 nmol/L), and EcTI (50 µM) for 24 h. After the SDS-PAGE separation of cell lysates and electrotransfer to PVDF membranes, membranes were blocked and incubated with the rabbit primary antibodies anti-phospho-Erk 1/2 MAPK, anti-Erk 1/2 MAPK, anti-p130cas (Y165) and anti-β-actin. Graph bars represent the densitometric analyses of the immunoblotting results. The results are represented as band intensities in arbitrary units relative to the respective total phospho-proteins loads and total proteins loads. Antibody binding was visualized by chemiluminescence, and the quantification of the relative Erk and p130cas respectively expression in adjacent cells (**A** and **C**) and leiomyoma cells (**B** and **D**) in each sample was performed by densitometry analysis in the Image J software with β-actin normalization. The error bars represent the SD. *p≤0.05; ***p≤0.001.

Analysis of tumor suppressor proteins: Rb, p27 and p53

The retinoblastoma protein (Rb) is a tumor suppressor protein that regulates cell cycle by controlling its progression, especially in the G1 phase [34]. We observed an expressive increase of this protein in the treatment with progesterone (P_4) when analyzing adjacent tissue cells, suggesting that progesterone recruits Rb signaling cell cycle arrest in these cells. On the other hand, the decrease in protein expression in the other treatments may indicate cellular normalization for cycle continuity (Figure 4A). In leiomyoma cells, regardless of the treatment, this protein is at a lower level than that observed in the control, indicating that cells are progressing in their normal cycle (Figure 4B).

The p27 protein is also considered a potent tumor suppressor; there is a direct relationship between low levels of p27 and fast proliferation in benign and very malignant conditions [35]. In the analysis of adjacent tissue cells, a slight increase in the level of this protein caused by hormones alone (P_4) or hormone conjugate associated with EcTI (E_2 + P_4 + EcTI) was observed (Figure 4A). An increase in this protein was observed in leiomyoma cells submitted to treatments, suggesting cell cycle arrest and indicating the efficacy of the treatment proposed since this protein induces apoptosis in leiomyoma, reducing the viability and proliferation of leiomyomatous cells [36].

The p53 tumor suppressor protein plays an essential role in the regulation of cell cycle, especially in the transition from G0 to G1. It is also considered one of the most important proteins to maintain the stability and integrity of the genome [37-38]. It is evident that the progesterone (P_4) and hormone conjugate (E_2 + P_4) treatments signalized abnormality leading to increased expression of this protein in adjacent tissue cells (Figure 4A); it is interesting that the protease inhibitor neutralizes this effect. Another interesting result is the fact that the expression of this protein did not increase in leiomyoma (Figure 4B) indicating that these cells somehow overcome the protein information that would result in cell cycle arrest. This analysis corroborates the suggestion that leiomyomatous cells are in apoptotic process because a decrease in p53 protein is directly related to the process of cell death induced by Anoikis [33].

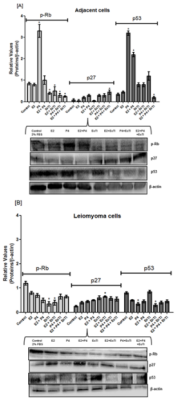


Figure 4. Detection of signaling phosphoproteins by immunoblot analysis in leiomyoma and miometrial adjacent cells. Cells were treated with E_2(100 nmol/L), P_4 (100 nmol/L), and

EcTI (50 µM) for 24 h. After the SDS-PAGE separation of cell lysates and electrotransfer to PVDF membranes, membranes were blocked and incubated with the rabbit primary antibodies anti-pRb, anti-p27, anti-p53, and anti-β-actin (control). Graph bars represent the densitometric analyses of the immunoblotting results. The results are represented as band intensities in arbitrary units relative to the respective total phospho-proteins loads and total proteins loads. Antibody binding was visualized by chemiluminescence, and the quantification of the relative pRb, p27 and p53 respectively expression in adjacent cells (A) and leiomyoma cells (B) in each sample was performed by densitometry analysis in the Image J software with β-actin normalization. The error bars represent the SD. *p≤0.05 (Treatments vs Control).

Analysis of cell death regulatory proteins: Bax (pro-apoptotic) and BcL-2 (anti-apoptotic)

BcL superfamily proteins are important regulators of programmed cell death. BcL-2, when expressed, results in prolonged cell survival, restricting the activation of caspases [39]. The Bax protein, when overexpressed, induces accelerated programmed cell death [40]. The analysis of these proteins in adjacent tissue cells showed decreased Bax in all treatments; the most significant decreases were observed in the estradiol (E_2), hormone conjugate (E_2 + P_4), and hormone conjugate associated with EcTI (E_2 + P_4 + EcTI) treatments (Figure 5A).

In uterine leiomyoma, the role of cell proliferation and apoptosis is closely related to the expression of these two regulatory proteins [41]. These authors showed decreased expression of the Bax protein in relation to non-leiomyomatous cells correlating the fact that there was no increase in mitotic activity in these cells. However, increased inhibition of apoptosis was observed in response to decreased protein expression and our results corroborate these associations. Interestingly, all treatments lead to increased expression of this protein (Figure 5B), however, the most significant result was obtained with the association of progesterone (P_4) with EcTI, suggesting the induction of death by this conjugated treatment. This effect is corroborated by the decreased anti-apoptotic protein BcL-2 caused by this same treatment (Figure 5B). Therefore, these results indicate that treatment with progesterone (P_4) associated with EcTI induces cell death by Anoikis.

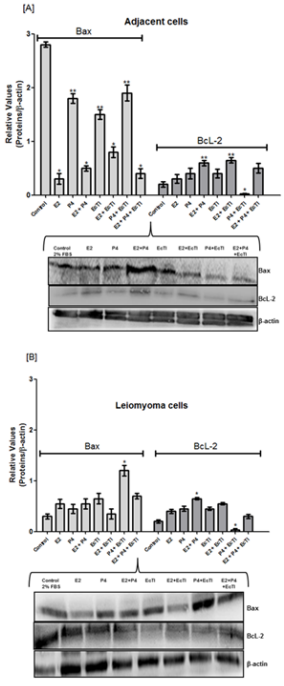
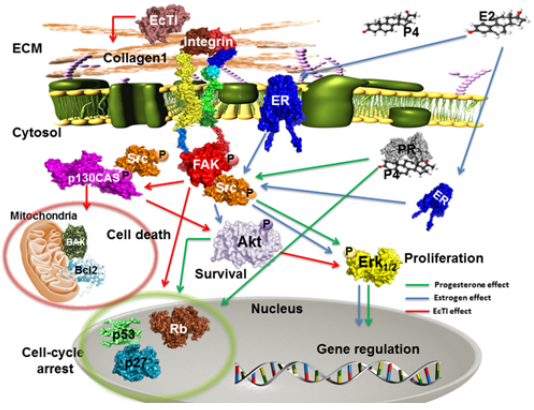


Figure 5. Detection of signaling phosphoproteins by immunoblot analysis in leiomyoma and miometrial adjacent cells. Cells were treated with E_2 (100 nmol/L), P_4 (100 nmol/L), and EcTI (50 µM) for 24 h. After the SDS-PAGE separation of cell lysates and electrotransfer to PVDF membranes, membranes were blocked and incubated with the rabbit primary antibodies anti-Bax (pro-apoptotic), anti-BcL-2 (anti-apoptotic), and anti-β-actin (control). Graph bars represent the densitometric analyses of the immunoblotting results. The results are represented as band intensities in arbitrary units relative to the respective total phospho-proteins loads and total proteins loads. Antibody binding was visualized by chemiluminescence, and the quantification of the relative Bax and BcL-2 respectively expression in adjacent cells (A) and leiomyoma cells (B) in each sample was performed by densitometry analysis in the Image J software with β-actin normalization. The error bars represent the SD. *p≤0.05; **p≤0.01 (Treatments vs Control).

Conclusion

In conclusion, the results suggest that EcTI, by recruiting the p130cas protein, activates the signaling process for Anoikis that is established by the relation of Bax (pro-apoptotic) and BcL-2 (anti-apoptotic) cell death proteins (Figure 6). It is relevant to mention that non-leiomyomatous cells were less sensitive to the Anoikis process established by this inhibitor, which is essential for the maintenance of the physiological developments of these cells. This study contributes toward relevant future implications in using alternative therapy in the treatment of uterine leiomyoma.



Figures 6. Schematic mechanism of action of EcTI and steroid hormones on leiomyomatous cells. The binding of cells to extracellular matrix proteins is mediated primarily by transmembrane receptors, the integrins which, through focal adhesion, connect the extracellular matrix to the cellular cytoskeleton. Focal adhesion acts as a signaling center that mediates multiple other pathways of protein signaling and activation that are part of focal adhesion including FAK, Src and p130cas. The EcTI uses the matrix collagen to potentiate its effect interfering on several signaling pathways such as MAPK and p130cas, inducing leiomyomatous cells to the Anoikis process through the activation of the cell death pathway.

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