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 racial predisposition being 2.2 times more frequent among first-degree relatives in families with two or more affected limbs [5-6].

Two models for fibroids outset, growth, and maintenance have been proposed: (1) increased levels of 17β-estradiol (E), progesterone (P), 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.
pregnancy. The purpose of progesterone on the uterine tissues including the endometrium, myometrium, and colonic 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 at trees that are more than 20 meters high; its fruits are rich in tannins and saponins [16]. *E. contortisiliquum* seeds contain a 20 kDa molecular mass trypsin inhibitor, the *Enterolobium contortisiliquum* Trypsin Inhibitor – EcTI, in the concentration of 5.6 mg of inhibitor per gram of seeds, which indicates a potential alternative to commercial inhibitor purified and homogenized in 20 mM Tris/HC1 (1.10, wv). The tertiar 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 kalikrein (hPK), 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 washed and homogenized in 20 mM Tris/HC1 (1.10, wv) solution. The crude extract was centrifuged at 5000 x g at 4°C for 10 min. After centrifugation, the supernatant was supplemented with 50 mM NaCl and applied to a DEAE-Sephadex column (2x25 cm) previously equilibrated with 100 mM Tris/HC1 buffer at pH 8.0. The inhibitor activity was measured by the inhibition of trypsin hydrolysis using 1 mM N-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 Healthcare), equilibrated with 50 mM Tris/HC1 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 (CETP0858/10) 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 and performed in compliance with the Declaration of Helsinki. Patients (N= 8) who volunteered to participate in the study signed and performed in compliance with the Declaration of Helsinki.

**Immunoblot analysis - Western blot analysis**

After cell cultures were treated for 24 h with 2% FBS, E, P, and EcTI, alone or in combination, they were washed twice with PBS (10 mM phosphate buffer saline at pH 7.4). These plates were scraped with a disposable cell scraper and lysis buffer containing 1% Triton X100, 50 mM Tris/HC1 (pH 8.0), 150 mM NaCl, 100 mM glycyrrhizin, 100 nM sodium fluoride, 1 mM sodium dodecylsulfate (SDS), 1 mg/ml aprotinin, 0.5% sodium deoxycholate, 1 mM NaVO3, (sodium orthovanadate), and 10 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/HC1 + 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-Actk rabbit primary antibodies, anti-Erk 1/2 MAPK rabbit and anti-phospho-Erk 1/2 MAPK rabbit, anti-FAK rabbit, anti-phospho-Akt rabbit (Tyr-397), anti-Src rabbit and anti-phospho-Src rabbit (Tyr-416), anti-Erk 1/2 MAPK rabbit and anti-phospho-FAK rabbit, and anti-p-Akt rabbit and anti-p-Src 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).

**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 analyzed according to the manufacturer's instructions. Cells were plated at 5 x 10^4 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% CO2. These cells were subsequently treated with E (100 nM), P (100 nM), and EcTI (50 µM) for 2 h. After 24 h incubation, the BrdU solution (100 µM) was added to the medium and cells were subsequently incubated for 4 h in a humidified atmosphere containing 5% CO2. Thereafter, cells were incubated in a solution of nucleases to fix and denature the DNA for the incorporation of anti-BrdU. Incorporation of 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.
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 ≤ 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), and progesterone (P), and EcTI on cells
Cell proliferation and metabolism assay
Treatment with estradiol (E) 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) 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) 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 or P) or in hormone conjugates (E + P), (Figure 1D and 1F). The use of hormone conjugates associated with EcTI (E + P + 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.

The phosphorylation of the Akt protein in adjacent tissue cells increases significantly with hormone conjugate (E + P) as well as treatment with EcTI, alone or associated with estradiol (E) (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), progesterone (P), and hormone conjugate (E + P) 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 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 (100 nmoL/L), P (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<0.05; **p<0.01; ***p<0.001.

Figure 2. Detection of signaling phosphoproteins by immunoblot analysis in leiomyoma and miometrial adjacent cells. Cells were treated with E (100 nmoL/L), P (100 nmoL/L), and...
Cells were treated with E (100 nmol/L), P (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) or hormone conjugate (E + P), the association of progesterone with EcTI (P + EcTI), or hormone conjugate associated with EcTI (E + P + 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 process induced by Anoikis [33], unlike that observed in leiomyoma cells (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) associated with EcTI and hormone conjugate associated with EcTI (E + P + EcTI) (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.

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) 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) or hormone conjugate associated with EcTI (E + P + 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) and hormone conjugate (E + P) 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].
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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, restricts 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), hormone conjugate (E + P), and hormone conjugate associated with EcTI (E_i + P_i + 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) 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 conjugated treatment. This effect is corroborated by 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 (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.

REFERENCES


