A combination of Vitamin D3 and Epigallocatechin Gallate significantly inhibits leiomyoma growth in an experimental animal model

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ABSTRACT — OBJECTIVE: *Leiomyoma, or uterine fibroid, is the most common benign gynecological neoplasm. It can cause abnormal uterine bleeding, pain, urinary incontinence, constipation and dyspareunia. Hysterectomy is the most used treatment. Currently great efforts are carried out to find alternative therapeutic strategies. Among them, vitamin D and epigallocatechin gallate administration gave promising results. We developed a rat leiomyoma model to investigate their mechanisms of action and evaluate their effects.*

MATERIALS AND METHODS: *Leiomyoma was induced in thirty-five female Wistar rats by diethylstilbestrol and progesterone according to a standardized animal model. Control group (no Leiomyoma) received only distilled water. Vitamin D3 and Epigallocatechin Gallate were administered orally for 3 weeks to all rats, with the exception of controls. Treatment groups: Leiomyoma + Saline, Leiomyoma + Vitamin D3, Leiomyoma + Epigallocatechin Gallate, Leiomyoma + Vitamin D3 + Epigallocatechin Gallate. The evaluation was made by histological, immunohistochemical and biochemical examination.*

RESULTS: *Leiomyoma rats displayed all the typical features of the pathology. Histological analysis demonstrated that the treatments allowed to reach the same condition of control rats. All parameters detected by immunohistochemical investigation (PCNA, Ki67, Bcl-2, Caspase-3, α-SMA, Type 1 Collagen, estradiol and progesterone) were found significantly increased in Leiomyoma rats respect to controls. Vitamin D and Epigallocatechin Gallate significantly reduced them all, with the exclusion of Caspase-3. Of note, their combined administration achieved a greater decrease in PCNA than both the single treatments.*

CONCLUSIONS: *This experimental study confirmed the therapeutic efficacy of vitamin D and Epigallocatechin Gallate, separately and together, and allowed to highlight their effects at molecular level.*

KEYWORDS
Leiomyoma, Epigallocatechin Gallate, Vitamin D3, Estradiol, Progesterone, Rat experimental model.

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INTRODUCTION

Leiomyoma is defined as a benign tumor of muscle and fibrous connective tissue and it is one of the most frequent gynecological pathologies[1,2]. Also called uterine fibroid, this monoclonal tumor may be defined as disordered myofibroblasts. Leiomyoma tissue contains abundant extracellular matrix (ECM) with large amounts of glycosaminoglycans and disordered, highly cross-linked interstitial collagens[3,4].

The most recent epidemiologic study on uterine fibroids reported that the prevalence ranges from 4.5% to 68.6% and the incidence ranges from 217 to 3745 cases per 100000 women/year[5].

Although asymptomatic in many cases, abnormal uterine bleeding is the most common sign of leiomyoma in patients. Other symptoms include pain due to necrosis, urinary incontinence due to bladder pressure, constipation due to rectal pressure and dyspareunia[6-8]. This condition usually leads to an impairment in quality of life, as reported from 1756 European women in the study by Downes et al[9]. Decrease in fertility is another important issue, especially in younger women affected[10].

Somatic mutations of myometrial cells, local growth factors and sex steroids are thought to be involved in the ethiopathogenesis of leiomyoma[11]. A significant increase of PCNA and Ki67 (two proliferation markers), Bcl-2 (an apoptosis regulator), active-Caspase-3 (necessary for progression of apoptosis), α-SMA (marker for a subset of activated fibrogenic cells, myofibroblasts) and type I collagen (the predominant type of collagen in leiomyoma) characterizes uterine fibroids[12]. Arrest in apoptosis is a typical feature of leiomyoma cells[13]. Leiomyomas display high levels of estrogen, progesterone[14,15], epidermal growth factor (EGF)[16], insulin like growth factor (IGF-I, II)[17] and vascular endothelial growth factor (VEGF)[18]. Estrogen is reputed the most important factor for leiomyoma growth because leiomyomas are diagnosed after menarche, grow during pregnancy and usually spontaneously shrink after menopause[19]. Women with anovulatory cycles and obese subjects with higher aromatase activity are examples of an increase in estrogen that leads to fibroid growth[20].

In case of abnormal uterine bleeding as the leading symptom, levonorgestrel intrauterine system, gonadotropin-releasing hormone analogues, selective progesterone receptor modulators, oral contraceptives, progestins and danazol are typical medical therapies[21].

Until today, hysterectomy is the most common therapeutic intervention when the leading symptoms are leg and back pain; pelvic pressure, heaviness or discomfort; abdominal bloating, urinary frequency or incontinence, and constipation. Annually around 200000 hysterectomy cases are performed in the United States and the cost is reported as more than 4 billion dollars[22]. According to size, number and proximity to the endometrium, myomectomy may be chosen as a surgical alternative, especially in women who wish to preserve their fertility[23].

Hysterectomy not only irreparably compromises reproductive function, but it is also associated to morbidity and, even though infrequent, mortality. Therefore, alternative therapeutic strategies are always under investigation. Ongoing researches focus on alternative therapies for this frequent pathology that deteriorates the quality of life and leads to loss of workforce. Nutritional support has gained importance in the recent years. Vitamin D, Epigallocatechin Gallate, Paricalcitol, aromatase inhibitors, Cabergoline and Curcumin are the most popular ingredients and medications investigated[24,25].

The aim of our study was to investigate the mechanisms of action activated by vitamin D3 and EGCG, together or separately, and evaluate their effects in a rat leiomyoma model, to contribute in offering alternative therapeutic options other than surgery.

MATERIALS AND METHODS

Animals

This study was performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Dokuz Eylül University, School of Medicine, Animal Laboratory, Izmir Turkey (03/2019 Protocol Number). Thirty-five female Wistar (200-220 gr) rats were included in the study; they were kept at 20–22°C with free access to water and pellet food and housed in 12-h light/dark cycle. The study groups were the following: **Group 1:** Leiomyoma + Saline (L), **Group 2:** Leiomyoma+Vitamin D3 (L+D), **Group 3:** Leiomyoma+Epigallocatechin Gallate (L+E), **Group 4:** Leiomyoma+Vitamin D3+Epigallocatechin Gallate (L+D+E), **Group 5:** no Leiomyoma-no treatment (control).

Experimental model of leiomyoma and treatment

Diethylstilbestrol (1.35 mg/kg) was given via oral gavage on Mondays, Wednesdays and Fridays and 1.0 mg progesterone was injected in the lateral side of the lower extremity every Sunday for 20 weeks to accomplish the long-term leiomyoma model for 5 weeks[26].

Control groups received 2 ml distilled water on Mondays, Wednesdays, Fridays via oral gavage and were injected with 0.05 mL saline on lateral side of lower extremity every Sunday.

Vitamin D3 was given at a dosage of 1750 IU/kg[27] and Epigallocatechin Gallate at a dosage of 6.6 mg/kg/day[28]. Both compounds were administered via oral route for 3 weeks. Dosage and length of treatment were the same for the group of rats taking these compounds together.

Histological examination

At the end of the experiments, the rats were sacrificed under anaesthesia and right uterine horns were fixed in 10% formalin solution. After routine histological procedures, uterus specimens were blocked in paraffin. From these 5 µm sections were taken on poly-L-lysin coated slides using a rotary microtome (RM 2135, Leica, Nussloch, Germany) with disposable metal microtome blades (Type N35, Feather Company, Osaka, Japan). Sections were stained with hematoxylin and eosin (H&E) and Masson's trichrom for histomorphological evaluation under light microscope. The images were analyzed using a computer assisted image analyzer system consisting of a microscope (BX-51, Olympus, Tokyo, Japan) equipped with a high-resolution video camera (DP-71, Olympus, Tokyo, Japan). Uterine diameter was measured after staining with H&E stain and analysed using FIJI (Imaje J) software.

In agreement with literature data, uterine tissues with experimentally induced leiomyoma were asymmetrical, pale and contain prominent cysts, nodules and swelling. Control groups without leiomyoma induction showed regular and bright uterine tissues.

Immunohistochemical staining

Antibodies against PCNA, Ki67, Bcl-2, active-Caspase-3, α-SMA and Collagen type-1 were used for immunohistochemistry at a dilution of 1:100. After deparaffinization and rehydration, the sections were treated with 10 mM citrate buffer (AP-9003-125, Lab vision, Fisher Scientific, Pittsburgh, PA, USA) for 5 min (for Caspase-3) or tripsin (TA-015-TR, Lab Vision, Fisher Scientific, Pittsburgh, PA, USA). Sections were incubated in a solution of 3% H_2O_2 for 5 min to inhibit endogenous peroxidase activity; and then with normal serum blocking solution. They were again incubated in a humid chamber for 18 h at 4°C with antibody, thereafter with biotinylated IgG, and then with streptavidin conjugated to horseradish peroxidase (HRP) for 30 min each prepared according to kit instructions (85-9043, Invitrogen, Dover, DE, USA). Sections were finally stained with DAB (diaminobenzidine) (1718096, Roche, Mannheim, Germany) and counterstained with Mayer hematoxylin and analyzed by using a light microscope[29].

Immunohistochemical Scoring

Immunostaining intensity was categorized into the following scores: 0 (no staining), 1 (weak, but detectable staining), 2 (moderate staining), and 3 (intense staining). The H-score value was derived for each specimen by calculating the sum of the percentage of cells. Categorized by intensity of staining, multiplied by its respective score, by means of the formula: H-score = $\sum$Pi (i+1). For each slide, five different fields were evaluated microscopically at 200 X magnification. H-score evaluation was performed by at least two investigators independently, blinded to the source of the samples as well as to each other's results and the average score was utilized[30].

Biochemical Assay

Determination of Rat Estradiol (E2) and Progesterone Levels

Left uterine horn homogenates were evaluated for estradiol and progesterone using ELISA method. All tissues were weighed and recorded. Tissues were cut into small pieces 800 µL soft cold PBS (pH 7.4) was added. They were homogenized on a Qiagen Tissue Lyser II instrument for 2 minutes and centrifuged at Qiagen Tissue Lyser II at 3000 g for 40 minutes and the supernatants were collected. Protein levels in supernatants were detected using bicinchoninic acid protein (BCA) assay kit (Thermo, Cat No: BCA, Protein assay kits, 23225, Rockford, IL, USA). All homogenates were stored at −80° C until the day of analysis. After the optimization study, Enzyme-Linked Immunosorbent Assay (ELISA) kits were used to evaluate YL BIONT (Cat No: YLA0205RA) Rat Estradiol (E2) ELISA Kit and YL BIONT (Cat No: YLA0207RA) Rat Progesterone ELISA Kit. Measurements were taken using BioTek Elisa Reader at 450 nm wavelength. All raw and processed data are available in excel workbook.

Statistical analysis

All values were expressed as mean ± standard deviation. The Kruskal-Wallis (between all groups) and Mann-Whitney U-test (for two groups) were used to compare staining intensity values between groups. All statistical analyses were performed using the SPSS software for Windows, Version 15.0 (SPSS, Chicago, IL, USA). The significance was accepted when the p-value was lower than 0.05 ($p<0.05$). For biochemical results, all statistical analyses were performed using the statistical package Graph Pad software (Graph Pad for Mac, Prism 8, La Jolla, CA, USA).

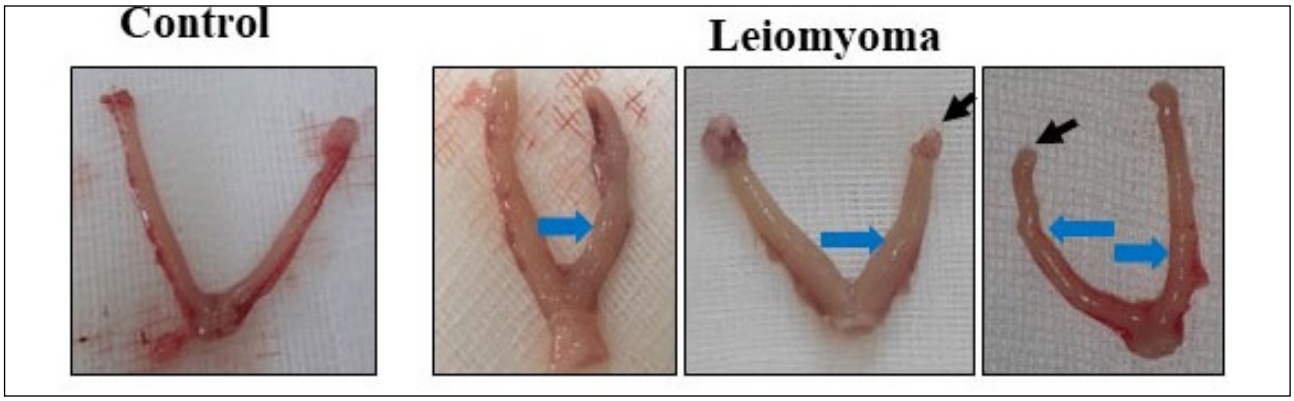

Figure 1. Uterine structures from control and leiomyoma group. Normal uterine structure was observed in control group. In the leiomyoma group, cystic nodules, swelling (blue arrows) and asymmetrical uterus (black arrows) were observed.

RESULTS

Success of the experimental rat model of uterine leiomyoma

At the end of the experiment, the control group revealed normal uterine macroscopic appearance with thin and equal horns, instead the uteri of leiomyoma group were asymmetric with cystic nodules and swelling (Figure 1).

Histological Results

Supplementation with Vitamin D3 and EGCG successfully decreased the size of leiomyomas
As shown in Figure 2, H&E staining showed that the uterine wall thickness increased in leiomyoma group. Masson's trichrome staining showed that the extracellular collagen content increased in leiomyoma group whereas all treated groups displayed a similar significant recovery that allowed to reach the same condition of control rats (Figure 2).

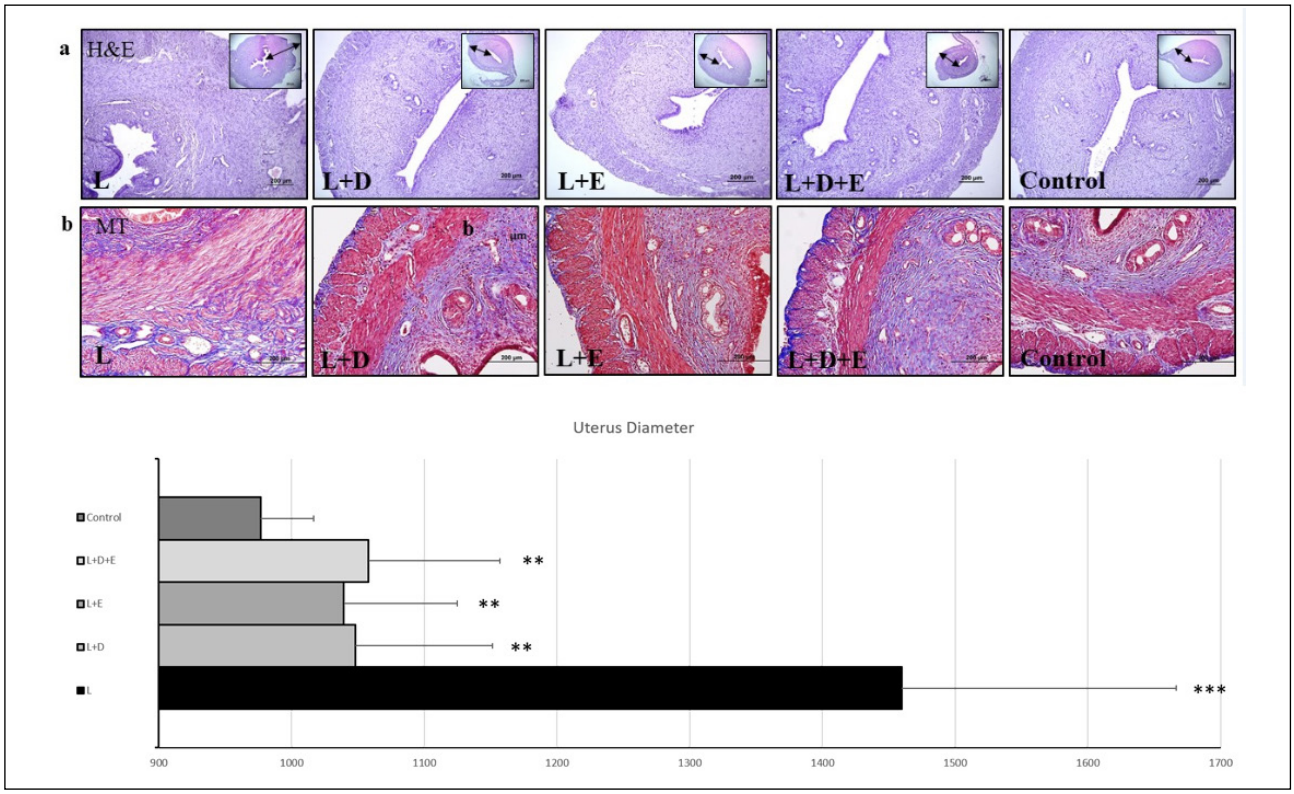

Figure 2. Representative hematoxylin and eosin-stained (pictures a) and Masson's trichrom stained (pictures b) histologic sections from all groups of rats: control, leiomyoma (L) and leiomyoma treated with Vitamin D3 (L+D), EGCG (L+E) and both compounds (L+D+E). Control: no leiomyoma-no treatment. The panel c shows uterin wall diameter of all groups. All images were captured at original magnification X40. In all treatment groups, connective tissue content and uterine diameter were decreased when compared to the leiomyoma group. The leiomyoma group showed significantly increased uterine diameter respect to control (***p<0.001). All the treatments significantly reduced the diameter respect to leiomyoma group **(p<0.005). Data were analyzed via Mann-Whitney U-test.

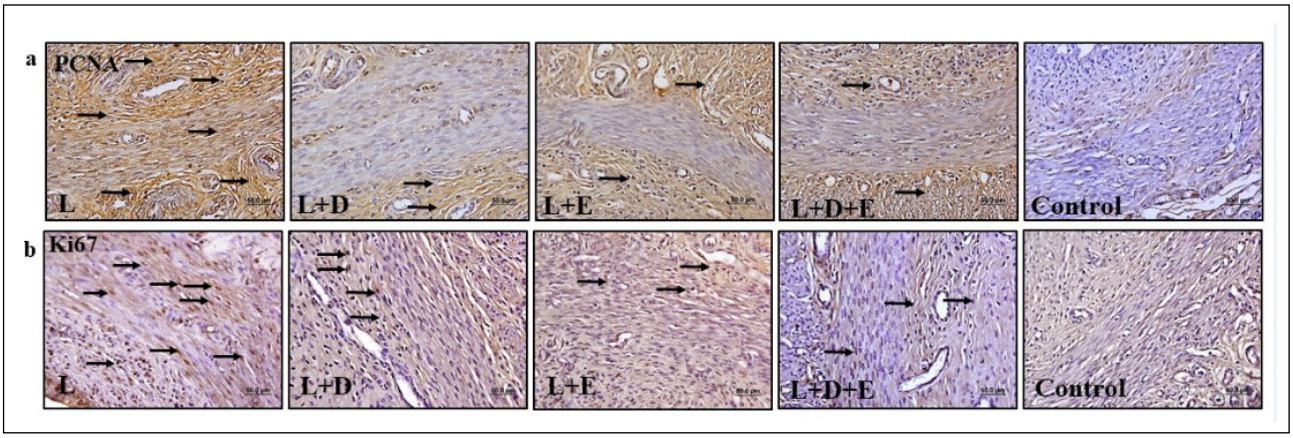

Figure 3. Effects of Vitamin D3 (L+D) and EGCG (L+E) alone or together (L+D+E), on PCNA and Ki67 expression in leiomyoma (L). Control: no leiomyoma-no treatment.

Immunohistochemical results

Proliferation and apoptosis significantly decreased with Vitamin D3 and EGCG treatment

To show the expression and localization of cell proliferation markers, such as PCNA and Ki67, we performed immunohistochemical analyses on leiomyoma. PCNA and Ki67 were expressed in the nucleus of the cells. The expression of PCNA and Ki67 was increased in leiomyoma group. However, whereas Ki67 expression was decreased in the same way by Vitamin D3 and EGCG, given separately or together, their combined administration resulted in a further reduction in PCNA expression, significantly greater than that obtained with their single administration (Figure 3 and Table 1).

To further understand the effects of Vitamin D3 and EGCG on apoptosis signaling, we performed immunochemistry tests with active Caspase-3 and Bcl-2 antibody. Significantly higher levels of active-Caspase-3 immunoreactivity were observed in leiomyoma group compared to the control group. The treatments further increase Caspase3, thus increasing apoptosis. On the other hand, Bcl-2 was significantly increased in leiomyoma group compared to the treatment and control groups (Figure 4 and Table 1). These results suggest that Vitamin D3 and EGCG heals uterine leiomyoma by reducing PCNA and Ki67, and by restoring physiological pathways of apoptosis.

To indicate the expression and localization of cell extracellular matrix and smooth muscle actin changes we performed the immunohistochemical analyses on leiomyoma with α-SMA and Collagen type-1, a main component of extracellular matrix. The expression of α-SMA and Collagen type-1 increased in leiomyoma group, whereas their expression decreased in the treated groups (Figure 5 and Table 1).

Biochemical Results

Estradiol and progesterone levels significantly decreased after Vitamin D3 and EGCG treatment

Estradiol Levels: In the uterus of L group rats, estradiol level was significantly increased with respect to control group (p=0.026) and with respect to L+D and L+D+E groups (p=0.002, p=0.002). Estradiol was also lower in L+E group than in L group, even if the result found was not significant (*Figure 6a*).

Table I. Immunohistochemical scoring of uterus tissues.

	PCNA	Ki67	Bcl-2	Caspase-3	α-SMA	Collagen1
L	354.0±1.52	345.5±5.56	342±3.69	223.8±3.43	429.5±6.55	353.8±2.41
L+D	258.4±4.57[a,b]	252.7±2.13[a]	254.5±3.35[a]	256.4±1.27[a]	255.7±1.60[a]	257.5±2.99[a]
L+E	255.8±1.46[a,b]	252.8±1.95[a]	255.4±1.39[a]	257.2±1.79[a]	255.1±2.47[a]	256±2.16[a]
L+D+E	253±2.94[a]	252.8±1.77[a]	254.2±1.49[a]	256.8±1.34[a]	252.1±4.67[a]	255.5±1.39[a]
Control	191.6±2.30	163±2.30	207.1±4.01	186.4±0.97	166.1±1.57	204.4±3.15
Overall p-value*	0.00001	0.00003	0.00002	0.00003	0.00002	0.00002

Mean ± standard deviation.
L: leiomyoma, L+D: leiomyoma treated with Vitamin D3, L+E: leiomyoma treated with EGCG, L+D+E: leiomyoma treated with both compounds.
*calculated via Kruskal-Wallis test; [a]statistically significant *vs.* group L; [b] statistically significant *vs.* group L+D+E, calculated via Mann-Whitney U-test.

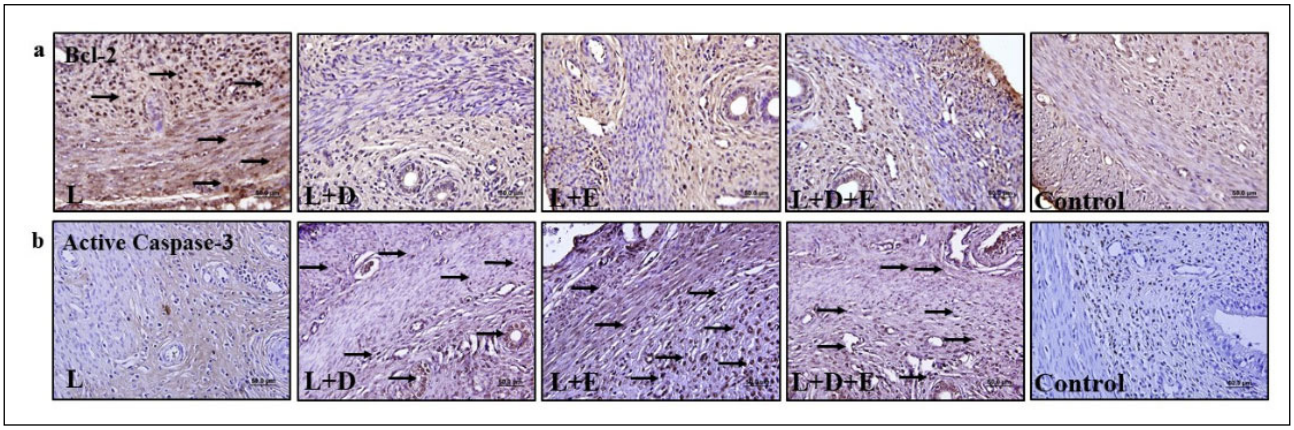

Figure 4. Effects of Vitamin D3 (L+D) and EGCG (L+E) alone or together (L+D+E), on Bcl-2 and active-Caspase-3 expression in leiomyoma (L). Control: no leiomyoma-no treatment.

Progesterone Levels: In the uterus of L group rats, progesterone level was significantly increased when compared with control group (p=0.0152). Progesterone level of L+D, L+E and L+D+E group in the uterus was significantly decreased compared to L group (p=0.0152, p=0.0012, p=0.0047) (*Figure 6b*).

In both cases, estradiol and progesterone levels in all treated rats (with the exception of L+E group) were very similar to controls.

DISCUSSION

Vitamin D is an immunomodulator with hormonal features extensively studied in chronic and malign diseases; it is known to be an important regulator in cell proliferation and differentiation, angiogenesis inhibition and apoptosis stimulation[25]. The receptor of vitamin D is expressed in a large number of tissues and cells. Among them, we mention adipose, adrenal, bone, brain, breast, cartilage, colon, epididymis, intestine, kidney, lung, muscle, lymphocytes (B&T), ovary, uterus (myometrium and endometrium) and also cancer cells. Of note, low levels of vitamin D strongly relate to high leiomyoma occurrence[25]. *In vitro* experiments showed that vitamin D exerts several therapeutic effects in uterine fibroid. It downregulates proliferating cell nuclear antigen (PCNA), cyclin-dependent kinase 1 (CDK1) and B-cell lymphoma-2 (Bcl-2) and reduces the expression catechol-O-methyltransferase (COMT), stimulating apoptosis[31,32].

Vitamin D is known to be a growth inhibitor that induces apoptosis in breast cancer cells[33]. Several studies confirmed its effect on leiomyoma. Halder demonstrated that 1,25-Dihydroxyvitamin D3 treatment exhibits antiestrogenic effects in human uterine leiomyoma cells[34] and is effective in decreasing leiomyoma volume in an Eker rat model[35]. Other studies demonstrated that vitamin D inhibits the cell growth and reduces the size of human uterine leiomyoma, without side effects or toxicity[31,36]. All these molecular pathways involved in the onset and development of uterine fibroids may be useful to get substantial improvements in their treatment[37]. Finally, in agreement with the above evidence, low vitamin D serum levels have been reported to be inversely correlated with the severity of uterine fibroids and to increase their incidence[38].

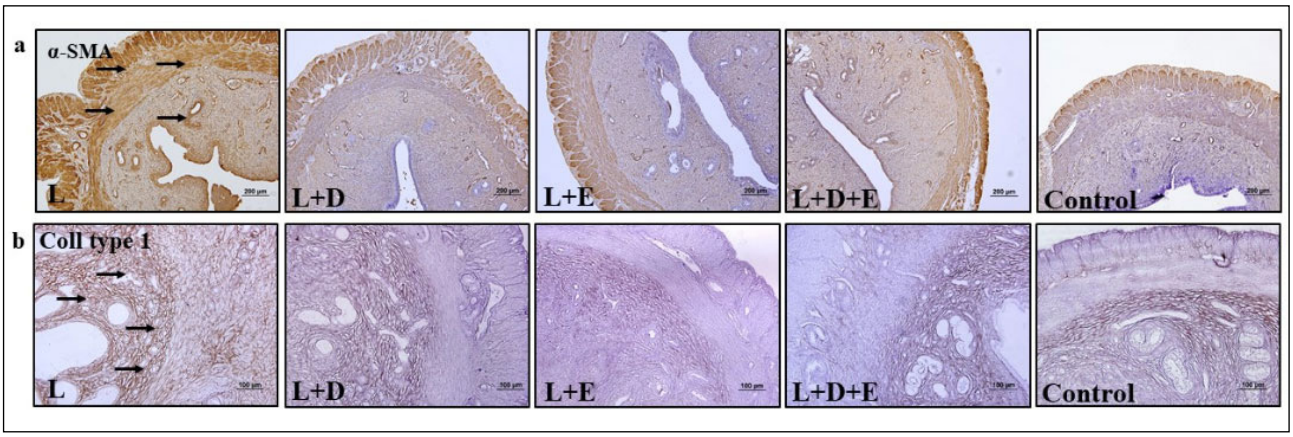

Figure 5. Effects of Vitamin D3 (L+D) and EGCG (L+E) alone or together (L+D+E), on α-SMA and Collagen type-1 expression in leiomyoma (L). Control: no leiomyoma-no treatment.

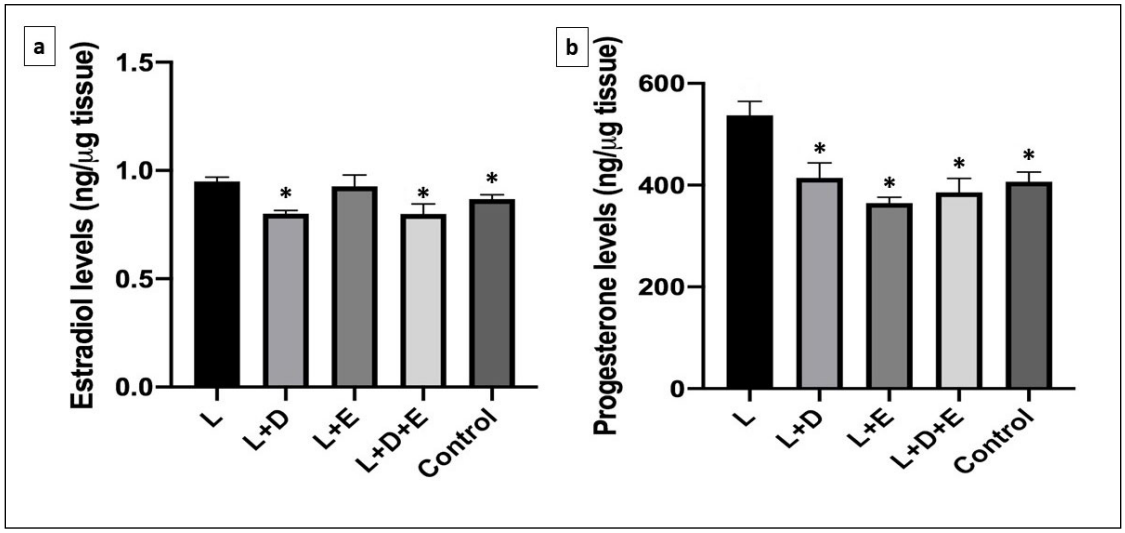

Figure 6. Effects of Vitamin D3 (L+D) and EGCG (L+E) alone or together (L+D+E), on rat estradiol and progesterone levels using ELISA method (leiomyoma: L). Control: no leiomyoma-no treatment. The reduction of estradiol and progesterone level in the different groups analyzed via Mann-Whitney U-test was statistically significant (*= $p<0.005$).

EGCG is another molecule that gained interest in research for its potential role in contrasting the leiomyoma growth. EGCG is a plant compound, the most abundant catechin in green tea. Catechins bioflavonoids are well known for their antioxidant and anti-inflammatory activity[39-41]. EGCG has multiple targets and exert its action in a pleiotropic manner. Its property for improving the quality of life in patients with inflammatory diseases has been extensively studied. EGCG seems to protect cells from damage associated with oxidative stress, by scavenging reactive oxygen species. The anti-inflammatory effect of EGCG may be attributed to the downregulation of nuclear factor kappa B (NF-κB)[40,42]. *In vitro* studies showed that EGCG induces apoptosis and promotes cell growth arrest[43]. In 2013 for the first time, a double-blinded, placebo-controlled randomized clinical trial was carried out treating premenopausal women with leiomyoma ≥2 cm^3 daily with EGCG for 4 months[44]. Interestingly, after this supplementation, a significant reduction by an average of 32.6% of fibroid volume was observed. Furthermore, the uterine fibroid-specific symptom severity significantly decreased and the health-related quality of life improved in these patients. Moreover, pro-EGCG analogues were shown to be antiproliferative, antiangiogenic and antifibrotic in cell cultures[25]. Of note, EGCG is able to decrease leiomyoma volume also in animal experiments[25].

Furthermore, it was found that the combined supplementation of vitamin D and EGCG decreased fibroid volume, increasing the quality of life in women[1,2].

In addition, it is important to underscore that both vitamin D and EGCG are safe molecules at the therapeutic dose used.

Our results are perfectly in agreement with the scientific evidence on this subject. The current study demonstrated that PCNA and Ki67 expression decreases in the groups treated with vitamin D3 and/or EGCG. It is interesting to highlight that the single administration of Vitamin D or EGCG obtained a lesser effect on PCNA than their combined administration. Moreover, we found that caspase-3 growths and Bcl-2 goes down in the leiomyoma groups when treated with vitamin D3 and/or EGCG. Therefore, these two dietary supplements can restore physiological pathways of apoptosis. Furthermore, we found that they reduce typical markers of fibroid as α-SMA and collagen, increased in leiomyoma group.

A substantial increase of estradiol and progesterone levels was detected also in leiomyoma group. Such increment was significantly inhibited by the two treatments, except for estradiol in EGCG treated rats. These results strongly suggest to take advantage from the potential role of vitamin D and EGCG for an effective, safe non-surgical medical treatment option for leiomyomas.

CONCLUSIONS

Obviously, this is a preclinical study in rats, and for this reason our findings need to be supported by further researches and clinical trials. However, this study is the first to show the effects of vitamin D3 alone and together with EGCG on leiomyoma mass, taking in consideration histological, immunohistochemical and biochemical data. Also, this work provides the first evidence that the combination of EGCG and vitamin D has a superior efficiency rather than the single molecules on a molecular level. If further clinical trials will confirm our preclinical results, our findings could pave the way for an improvement in the management of leiomyoma in women.

Conflict of Interest:
The authors declare that they have no conflict of interests with the present publication.

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