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FULL TITLE: Quercetin and indole-3-carbinol inhibit extracellular matrix expression in human primary uterine leiomyoma cells

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ABSTRACT

Research question: Uterine leiomyomas are characterized by the excessive accumulation of extracellular matrix (ECM) and abnormal cell proliferation. Dietary phytochemicals are plant based chemical compounds with disease-preventive properties and they are able to interrupt tumorigenic processes. Quercetin (3,3',4',5,7-pentahydroxyflavone) is a flavonol found in a lot of edible fruits and vegetables, such as tea, lemon, tomato, onion leaves, and strawberry. Indole-3-Carbinol (I3C; 1H-indole-3-ylmethanol) is produced from naturally occurring glucosinolates contained in a wide variety of plants, including members of the family Cruciferae and particularly members of the genus Brassica.
Do Quercetin or Indole-3-Carbinol have *in vitro* effect on myometrial and leiomyoma cells?

Design: This study was designed to investigate if quercetin and I3C can regulate ECM expression, cell migration and proliferation of primary human leiomyoma and myometrial cells. Myometrial and leiomyoma cells were treated with quercetin or I3C at different concentrations (10 µg/ml; 50 µg/ml; 100 µg/ml; and 250 µg/ml) for 48 h to measure mRNA and protein expressions of ECM, as well as cell migration and the proliferation rate.

Main results: Quercetin and I3C significantly decreased collagen1A1 and fibronectin mRNA expression, and protein expression of fibronectin in myometrial and leiomyoma cells. We also observed a significant reduction of the migration of both cell types, and a reduction of proliferation of myometrial cells in response to quercetin and I3C treatments.

Conclusions: This study demonstrates the *in vitro* anti-fibrotic, anti-migratory and anti-proliferative effects of quercetin and I3C laying the scientific bases for the development of new therapeutic and/or preventive agents for the uterine leiomyomas.

KEYWORDS: Quercetin, indole-3-carbinol, uterine fibroid, antifibrotic; dietary phytochemicals; extracellular matrix

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Key Message:

This study demonstrates the *in vitro* anti-fibrotic (reduction of collagen1A1 and fibronectin) and anti-migratory effects of quercetin and I3C in myometrial and leiomyoma cells, as wells the anti-proliferative effect in myometrial cells. This laying the scientific bases for the development of new therapeutic and/or preventive agents for the uterine leiomyomas.

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INTRODUCTION

Uterine leiomyoma, or uterine fibroid, is the most common benign tumor in gynecology that affects many women especially in fertile age. This tumor develops in the myometrium or muscular layer of the uterus (Walker and Stewart 2005). Fibroids cause pelvic pain, abnormal vaginal bleeding, pressure on the bladder, infertility and obstetric complications (Ciavattini *et al.* 2015, Kashani *et al.* 2016). Surgery (such as hysterectomy, and myomectomy) has been the definitive treatment for symptomatic fibroids. Gonadotrophin-releasing hormone agonist (GnRHa) (Friedman *et al.* 1989, Stovall *et al.* 1995) and ulipristal acetate (Donnez *et al.* 2015) are playing an important role as presurgical treatments in reducing fibroid size as well as its associated symptoms (such as abnormal bleeding). However, the long-term use of GnRHa is associated with menopausal symptoms and bone loss (Friedman *et al.* 1991, Leather *et al.* 1993). Recently, the concern about the risk of rare but serious liver injury with ulipristal acetate treatment has been raised (Donnez 2018).

Uterine fibroids consist of the excessive amount of extracellular matrix (ECM) proteins including collagen, fibronectin, and versican (Islam *et al.* 2017a). The growth of fibroids is primarily influenced by cell-ECM interaction, and the rigid structure of ECM is believed to be the cause of abnormal bleeding in the uterus (Islam *et al.* 2017a). Therefore, compounds that can regulate ECM production could be an ideal way to control fibroid growth.

Dietary phytochemicals are plant origin chemical compounds that have disease controlling properties (Islam *et al.* 2014a, Islam *et al.* 2017c). Quercetin is a flavonoid, found in the most of edible fruits and vegetables, such as tea, lemon, tomato, onion leaves, and strawberry. This flavonoid is known to exert antifibrotic effects in hepatic fibrosis (Lee *et al.* 2003), pulmonary fibrosis (Baowen *et al.* 2010) and kidney fibroblasts (Hu *et al.* 2009). Quercetin suppresses TGF-β-induced collagen production in lung fibroblasts (Nakamura *et al.* 2011), as well as collagen and fibronectin production, and TGF-β/Smad-signaling pathway in keloid fibroblasts (Phan *et al.* 2003a, Phan *et al.* 2003b).

Epidemiological studies report that intake of quercetin rich foods reduced the risk of gastric cancer by 43%

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and colon cancer by 32%. Consumption of quercetin was also reported to reduce lung cancer risk by 51% and even in heavy smokers by 65% (Dunnick and Hailey 1992, Pereira *et al.* 1996).

Indole-3-carbinol (I3C) is produced from naturally occurring glucosinolates contained in a wide variety of plants, including members of the family Cruciferae and particularly members of the genus Brassica. I3C has been reported to inhibit cell proliferation as well as expression levels of α-SMA, type I collagen in hepatic stellate cells (Ping *et al.* 2011). The combination of epidemiological and experimental data provides suggestive evidence that a high intake of cruciferous vegetables protects against some cancers at various sites. In a nationwide study of postmenopausal women in Sweden, consumption of cruciferous vegetables was inversely associated with breast cancer risk. Although cruciferous vegetables have a number of cancer-preventing compounds, I3C alone showed efficacy for the prevention of breast, endometrial and cervical cancers in animal models. Importantly, I3C showed efficacy for treatment of precancerous lesions of the cervix in translational human studies (Auborn *et al.* 2003).

In the present study, we aimed to investigate the effects of quercetin and I3C on extracellular matrix expression as well as the cell migration and proliferation of myometrial and leiomyoma cells.

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MATERIALS AND METHODS

Primary cell cultures

The study included samples of myometrial and usual type of leiomyoma tissue excised from women undergoing hysterectomy for symptomatic fibroids. We included the most homogeneous sample possible considering the high variability, for example, the different age, race, hormonal milieu, tumor size, and location of tumors. In fact, all patients were Caucasian and in proliferative phase of the menstrual cycle. The location of leiomyomas was intramural, and their size was 7-10 cm in diameter. We considered all patients who has not received exogenous hormones for the previous 3 months. All patients gave their informed consent and the permission of the Human Investigation Committee was granted.

After surgery, the myometrial and leiomyoma samples were collected in Hank's Balanced Salt Solution (HBSS, Euroclone, Milan, Italy), and immediately transported to the laboratory. The samples were washed several times with Dulbecco's PBS (Invitrogen, ThermoFischer, Carlsbad, CA, USA) to remove excess blood. After cutting tissue into small pieces, sample were mixed in 0.1% collagenase type 8 (Serva Electrophoresis GmbH, Heidelburg, Germany) in serum free Dulbecco Modified Eagle Medium (DMEM) (Lonza, Walkersville, MD, USA), and incubated at 37°C for 3-5 hours in water bath with manual shaking. After having digested the cells suspension it was centrifuged at 1200 rpm for 10 minutes, and the collagenase was inactivated with fetal bovine serum (FBS) (Gibco, ThermoFischer).

Finally, the cell pellet was dispersed in DMEM containing 10% fetal bovine serum (FBS; Gibco, ThermoFischer), 1% antibiotic (penicillin-streptomycin; Euroclone), 1% fungizone (Amphotericin B; Lonza), and 1% glutamine (Gibco, ThermoFischer) in plastic dishes, and incubated at 37°C in 95% air, 5% CO_2. The growth medium was changed after 24 or 48 hours to remove unattached cells and subsequently twice a week. The purity of cells was assessed by staining with a specific smooth muscle cells marker (anti-α-smooth muscle actin) (Sigma-Aldrich, Milan, Italy). Almost all cells were strongly positive for α-sma.

The lower passage number (P0-P4) of cells was used for experiments to avoid changes in phenotype and gene expression. Myometrial and leiomyoma cells were treated with quercetin and I3C (10 µg/ml; 50 µg/ml; 100 µg/ml; and 250 µg/ml) (Sigma-Aldrich) for 48 h. After treatment, the cells were detached from the petri dish by the TRIzol® reagent (Invitrogen, ThermoFisher). This reagent produces 3 phases: the upper white transparent phase containing the RNA, a white disk with the DNA, and the a lower pink phase containing the proteins.

RNA Extraction and Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted using the white transparent phase from TRIzol® reagent (Invitrogen, ThermoFisher), according to the manufacturer's instructions. Samples were digested with a ribonuclease-free deoxyribonuclease (Promega Corp., Madison, WI, USA), and the RNA was cleaned up and concentrated

using ReliaPrep™ RNA Cell Miniprep System (Promega Corp.). We performed the reverse transcriptase (RT) using the high-capacity cDNA reverse transcriptase kit (Applied Biosystems, ThermoFischer, Foster, CA, USA) with 1μg RNA, and we performed the TaqMan real-time PCR for all the genes analyzed. We used the TaqMan gene expression assays (Applied Biosystems, ThermoFischer): collagen1A1 (Hs00164004_m1), fibronectin (Hs00365052_m1), versican (Hs00171642_m1) and the housekeeping genes, hypoxanthine phosphoribosyltransferase 1 (HPRT1), (Hs99999909_m1) and β-actin (ACTB) (Hs99999903_m1), performing the following thermal cycle protocol (initial denaturation at 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds) using 100 ng cDNA in a final reaction volume of 10 μL. The blank for each reaction, consisting of amplifications performed in the absences of reverse transcriptase enzyme, was performed.

Western Blotting

Proteins were extracted using the pink phase from TRIzol® reagent (Invitrogen, ThermoFisher), following the manufacturer's instructions. Soluble protein was quantified using a Bradford protein assay (Bio-Rad, Milan, Italy), and equal amounts of proteins were loaded to 4-12% NuPAGE gels (Invitrogen, ThermoFischer), and resolved by SDS-PAGE under reducing conditions. Proteins were transferred to 0.2 μm nitrocellulose membranes in an X-cell II apparatus (Invitrogen, ThermoFischer) according to the manufacturer's instructions. Ponceau S solution (Euroclone) was used for the detection of proteins on nitrocellulose membranes. After blocking the membrane with 5% (wt/vol) nonfat milk powder in Tris-buffered saline with Tween 20 (TBST; 50 mm Tris-HCl, pH 7.4, 150 mm NaCl, 0.05% Tween 20), the membrane was incubated overnight with a primary antibody 1:30000 dilution for monoclonal mouse Anti-human fibronectin (Sigma-Aldrich), and 1:3000 dilution for monoclonal mouse tubulin (Sigma-Aldrich). On the next day, a membrane was washed 4 times in TBST and incubated with 1:10000 horseradish peroxidase linked antimouse (Amershan) against Anti-human fibronectin and tubulin for 2 hours. The membrane was washed 4 times in TBST, and immunoreactive proteins were visualized using Clarity ™ western ECL

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substrate (Bio-Rad). Protein levels were measured using Image J 1.49n software (National Institutes of Health, http://imagej.nih.gov/ij).

Immunocytochemistry

Myometrial and Leiomyoma cells were seeded in chamber tissue culture slides and allowed to divide. At the end of treatment, cells were washed 3 times with PBS, treated with 0.2% Triton X-100 in PBS for 5 minutes, and washed 3 times with PBS. To inhibit endogenous peroxidase activity, cells were incubated for 10 minutes with 3% hydrogen peroxide in deionized water. Cells were washed 3 times with PBS, and to block nonspecific background, cells were incubated for 20 minutes at RT with NHS (normal horse serum) diluted 1:75 in 1% bovine serum albumin in PBS. Cells were then incubated with fibronectin (Sigma-Aldrich) monoclonal mouse antibody at 1:600 and monoclonal mouse anti-α-smooth muscle actin (α-sma) at 1:000 (Dako, Agilent Tecnologies, Santa Clara, California, USA) for 1 hour at room temperature. After washing with PBS, cells were incubated with biotinylated anti-mouse IgG made in horse diluted 1:200 against fibronectin and α-sma. The peroxidase ABC method was performed for 1 hour at room temperature using DAB as chromogen. Sections were counterstained in Mayer's hematoxylin, dehydrated, and mounted with Eukitt solution.

Wound closure assay

Myometrial and leiomyomas cells were grown to confluence and then scratch wounded with a sterile plastic micropipette tip. Cells were rinsed three times with warm media to wash away scraped off cells in the wound and then kept in serum-free media in the absence or presence of quercetin or I3C (250 µg/ml) for 48 hours. Digital images were taken of the initial wound and at 12 hours, 24 hours and 48 hours (Rodriguez *et al.* 2005, Liang *et al.* 2007, Wright *et al.* 2012). The area (mm^2) of the wound not occupied by cells was measured using a morphological imaging system Image J 1.49n software (National Institutes of Health, http://imagej.nih.gov/ij). Closure percentage was calculated as:

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$$\left[1-\frac{area\,of\ remmaing\ wound}{area\,of\ initial\ wound}\right]\times 100$$

Cell proliferation assay

Cellular growth curves were measured using the CyQuant cell proliferation assay kit according to the manufacturer's instructions (Invitrogen, Life Technologies). Myometrial and leiomyoma cells were seeded in 96-well plates at various initial density of cells: 150 cells/well; 100 cells/well; 50 cells/well in a total volume of 300 µl DMEM supplemented with 10% FBS. Cells were treated with quercetin or I3C at 250 µg/ml, and left untreated (Invitrogen, Life Technologies), and allowed to divide for the number of days indicated (days 1, 2 and 4). At the indicated times, media were discarded, and plates were frozen at -80°C. At the day of the assay, plates were thawed, cells were lysed, and total cellular nucleic acid was measured using fluorescence at 520 nm emission after excitation at 480 nm (Ciarmela *et al.* 2008, Ciarmela *et al.* 2009, Islam *et al.* 2014b).

Data analysis

Statistical analyses were performed using GraphPad Prism version 6.01 for Windows (GraphPad, San Diego, CA). The data were analyzed using non-parametric 'Kruskal-Wallis' ANOVA, followed by post hoc 'Dunn' test for multiple comparisons. Differences were considered significant when $p < 0.05$. All experiments were done in triplicate, except for the cell proliferation assay in which n=6.

RESULTS

Effects of quercetin and indole-3-carbinol on extracellular matrix mRNA expression in primary myometrial and leiomyoma cells

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To determine the antifibrotic effect of quercetin and I3C, primary myometrial and leiomyoma cells were treated with either compounds at different concentrations: 10 μg/ml; 50 μg/ml; 100 μg/ml; 250 μg/ml for 48 hours. The mRNA expression was measured by real-time PCR. Quercetin reduced collagen1A1 mRNA in primary myometrial and leiomyoma cells and fibronectin mRNA in primary myometrial cells at the highest dose (250 μg/ml). The versican mRNA expression levels were affected by neither of these compounds (Figure 1).

Effects of quercetin and indole-3-carbinol on protein expression of fibronectin in primary myometrial and leiomyoma cells

Data from western blotting showed that protein expression levels of fibronectin were reduced after treatments with quercetin or I3C, in primary leiomyoma and myometrial cells compared to untreated controls (Figure 2).

Data from immunocytochemistry also showed decreased expression levels of fibronectin in primary leiomyoma and myometrial cells in response to quercetin or I3C treatment (Figure 3).

Effect of quercetin and indole-3-carbinol on migration of primary myometrial and leiomyoma cells

To evaluate the effect of quercetin and I3C (250 μg/ml) on cell migration, myometrial and leiomyoma cells were treated with these compounds at 250 μg/ml or left untreated for 48 hour, and digital images were captured from the initial wound and after 12 hour, 24 hour (data non show) and 48 hour that were analyzed through Image J software. Results showed that quercetin and I3C significantly reduced the migration of both myometrial and leiomyoma cells (Figures 4a - b).

Effect of quercetin and indole-3-carbinol on proliferation of primary myometrial and leiomyoma cells

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To demonstrate the antiproliferative effect of quercetin and I3C, myometrial and leiomyoma cells were treated with these two compounds at 250 μg/ml or left untreated for different days of interval (days 1, 2 and 4). Cell proliferation was measured using the CyQuant cell proliferation assay kit. Results showed that leiomyoma cell proliferation was not affected by quercetin and I3C, but they reduced myometrial cell proliferation (Figure 5).

DISCUSSION

Uterine leiomyoma is not a malignant disease; however it is associated with several reproductive and gynecological problems, including menorrhagia, dysmenorrhea, chronic pelvic pain, infertility, recurrent abortion, premature birth and postpartum hemorrhage. In comparison with the burden of disease to women health, medical treatments are very limited, so our present study focuses on seeking preventive therapies.

The role of cell proliferation and of ECM proteins, in producing the leiomyoma bulk structure and growth, provides an important way to control this tumor by developing anti-fibrotic and anti-proliferative agents.

Recent studies are reporting the importance of the role of phytochemical agents on uterine leiomyomas. The present results suggest that quercetin the main active compound of the onion, may be a potential adjuvant for the treatment of uterine disorders, in line with previous studies have conducted in vivo on rats (Wu *et al.* 2015). Isoliquiritigenin, a licorice flavonoid, induced antiproliferative effects on uterine leiomyoma ELT3 cells (Lin *et al.* 2019a). Resveratrol, a polyphenol which was also found to be a potent anti-proliferative agent against human leiomyoma cells in in vitro and in murine models (Chen *et al.* 2019). In addition, Adlay seeds anti-proliferative effect was demonstrated on rat uterine leiomyoma ELT3 cells (Lin *et al.* 2019b).

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We evaluated in this study, the *in vitro* anti-fibrotic, anti-migratory and anti-proliferative effects of two phytochemicals (quercetin and I3C) in myometrial and leiomyoma cells. We demonstrated anti-fibrotic effects of quercetin and I3C by assessing the expression levels of three major ECM components (collagen1A1, fibronectin, and versican). Collagen acts as a central structural component of the ECM that maintains cellular morphology. It plays important roles in regulating proliferation, migration, differentiation, survival and wound healing and the fibrotic processes (Pickering 2001). Leiomyoma cells demonstrates an overexpression of collagen types I mRNA in leiomyomas compared to the adjacent myometrium (Leppert *et al.* 2006). Fibronectin, a glycoprotein of the ECM, plays important roles in cell adhesion, migration, growth and differentiation as well as in fibrosis. Leiomyoma cells expresses elevated levels of fibronectin compared to myometrial cells (Arici and Sozen 2000). Versican, a large chondroitin sulphate proteoglycan, plays important roles in cell migration, cell adhesion, cell proliferation, tissue stabilization, tissue homeostasis and inflammation (Islam *et al.* 2015). The up-regulation of versican expression has been reported in leiomyoma cells compared to healthy counterparts (Norian *et al.* 2009).

We found that quercetin and I3C reduced collagen1A1 mRNA expression in both myometrial and leiomyoma cells, as well as fibronectin mRNA expression in myometrial cells. Quercetin was effective at all doses used (10 µg/ml; 50 µg/ml; 100 µg/ml; 250 µg/ml) while I3C was effective only at 250 µg/ml. Although fibronectin mRNA expression levels were not changed in leiomyoma cells after quercetin or I3C treatment, western blotting analysis showed a significant reduction of fibronectin protein expression in both myometrial and leiomyoma cells. The visible reduction of fibronectin protein expression was also observed in both myometrial and leiomyoma cells, compared to untreated controls, as measured by immunocytochemistry. Beside antifibrotic effects of quercetin and I3C, they also induced a reduction of cell migration of both myometrial and leiomyoma cells. Both compounds were unable to induce cell growth inhibition of leiomyoma cells while myometrial cells were affected.

The anti-fibrotic effects of quercetin and I3C have been demonstrated in different cell types (Phan *et al.* 2003a, Phan *et al.* 2003b, Hu *et al.* 2009, Nakamura *et al.* 2011, Ping *et al.* 2011). Our present study further confirms their anti-fibrotic potential. In our previous study, we demonstrated that strawberry

extracts were able to inhibit expression of ECM components, including collagen1A1, fibronectin, and versican in leiomyoma cells (Islam *et al.* 2017b, Giampieri *et al.* 2019). Strawberry contains a number of bioactive compounds such as, ellagic acid, kaempferol, catechins, anthocyanins, including quercetin. As expected, similar to strawberry extract, quercetin also inhibited expression of ECM components, collagen1A1 and fibronectin in leiomyoma cells, demonstrating that quercetin is one of the active antifibrotic compounds of strawberry fruit.

Because of their therapeutic potential, quercetin and I3C are now under clinical trial for different pathologies. The phase I, and II clinical trials of quercetin have been completed or now recruiting for multiple conditions, including squamous cell carcinoma (NCT03476330). Quercetin was administered twice daily at an adjusted dose based on weight for a maximum total daily dose of 4000 mg/day. If the patient is 70 kg or more, the dose is automatically assigned at the maximum dose of 4000 mg/day. For the patients with type 2 diabetes (NCT01839344) the dose maximum of quercetin is 250 mg capsules; while the oral single dose of 2000 mg for the patients with chemotherapy induced oral mucositis (NCT01732393). Patients in the intervention group were administered two, 250 mg quercetin capsules daily for 3 weeks. Patients in the placebo group received two placebo capsules containing lactose. Patients were examined every other day for evaluation of initiation and severity of oral mucositis. In addition, phase III, randomized, double blind clinical trial of quercetin for coronary artery disease progression has recently been listed (not yet recruiting) (NCT03943459). In a 60-day trial, 60 postmenopausal women with CAD, divided into three groups with 20 women each: Group 1 - Quercetin (500 mg / day). Furthermore, phase IV clinical trial of quercetin containing onion peel extract has been completed on endothelial function (NCT02180022). Regarding I3C, the phase I clinical trial has been completed for preventing cancers in healthy participants (NCT00100958 and NCT00033345). In addition, clinical trial phases II and III of I3C on prostatectomy patients with PSA (prostate-specific antigen) recurrence have also been completed (NCT00579332). The advance clinical trials of quercetin and I3C support the possible use of these two compounds or their dietary sources also for uterine fibroids as preventive and/or therapeutic agents. In particular, patients could benefit most from these therapeutic agents while seeking pregnancy or during the pregnancy itself,

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when a significant growth of fibroids has been described, with possible onset of adverse obstetric outcomes (Ciavattini *et al.* 2016), also considering the impossibility to use other pharmacological agents. Moreover, since the severity of fibroids-related symptoms is reduced after menopause, these compounds may be used in perimenopausal women, potentially avoiding the need for surgery.

Overall, the present data support the hypothesis that these two natural compounds can be developed as therapeutics and preventive dietary or locally administrated phytochemicals for uterine fibroids.

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REFERENCES

Arici, A., Sozen, I., 2000. Transforming growth factor-beta3 is expressed at high levels in leiomyoma where it stimulates fibronectin expression and cell proliferation. Fertility and Sterility. 73, 1006-1011

Auborn, K.J., Fan, S., Rosen, E.M., Goodwin, L., Chandraskaren, A., Williams, D.E., Chen, D., Carter, T.H., 2003. Indole-3-carbinol is a negative regulator of estrogen. J Nutr. 133, 2470S-2475S

Baowen, Q., Yulin, Z., Xin, W., Wenjing, X., Hao, Z., Zhizhi, C., Xingmei, D., Xia, Z., Yuquan, W., Lijuan, C., 2010. A further investigation concerning correlation between anti-fibrotic effect of liposomal quercetin and inflammatory cytokines in pulmonary fibrosis. European journal of pharmacology. 642, 134-139

Chen, H.Y., Lin, P.H., Shih, Y.H., Wang, K.L., Hong, Y.H., Shieh, T.M., Huang, T.C., Hsia, S.M., 2019. Natural antioxidant resveratrol suppresses uterine fibroid cell growth and extracellular matrix formation in vitro and in vivo. Antioxidants (Basel). 8,

Ciarmela, P., Wiater, E., Smith, S.M., Vale, W., 2009. Presence, actions, and regulation of myostatin in rat uterus and myometrial cells. Endocrinology. 150, 906-14

Ciarmela, P., Wiater, E., Vale, W., 2008. Activin-a in myometrium: Characterization of the actions on myometrial cells. Endocrinology. 149, 2506-16

Ciavattini, A., Clemente, N., Delli Carpini, G., Di Giuseppe, J., Giannubilo, S.R., Tranquilli, A.L., 2015. Number and size of uterine fibroids and obstetric outcomes. J Matern Fetal Neonatal Med. 28, 484-8

Ciavattini, A., Delli Carpini, G., Clemente, N., Moriconi, L., Gentili, C., Di Giuseppe, J., 2016. Growth trend of small uterine fibroids and human chorionic gonadotropin serum levels in early pregnancy: An observational study. Fertil Steril. 105, 1255-1260

Donnez, J., 2018. Liver injury and ulipristal acetate: An overstated tragedy? Fertility and sterility. 110, 593-595

Donnez, J., Hudecek, R., Donnez, O., Matule, D., Arhendt, H.J., Zatik, J., Kasilovskiene, Z., Dumitrascu, M.C., Fernandez, H., Barlow, D.H., Bouchard, P., Fauser, B.C., Bestel, E., Terrill, P., Osterloh, I., Loumaye, E., 2015. Efficacy and safety of repeated use of ulipristal acetate in uterine fibroids. Fertil Steril. 103, 519-27 e3

Dunnick, J.K., Hailey, J.R., 1992. Toxicity and carcinogenicity studies of quercetin, a natural component of foods. Fundam Appl Toxicol. 19, 423-31

Friedman, A.J., Hoffman, D.I., Comite, F., Browneller, R.W., Miller, J.D., 1991. Treatment of leiomyomata uteri with leuprolide acetate depot: A double-blind, placebo-controlled, multicenter study. The leuprolide study group. Obstetrics and Gynecology. 77, 720-725

Journal Pre-proof

Friedman, A.J., Rein, M.S., Harrison-Atlas, D., Garfield, J.M., Doubilet, P.M., 1989. A randomized, placebo-controlled, double-blind study evaluating leuprolide acetate depot treatment before myomectomy. Fertility and Sterility. 52, 728-33

Giampieri, F., Islam, M.S., Greco, S., Gasparrini, M., Forbes Hernandez, T.Y., Delli Carpini, G., Giannubilo, S.R., Ciavattini, A., Mezzetti, B., Mazzoni, L., Capocasa, F., Castellucci, M., Battino, M., Ciarmela, P., 2019. Romina: A powerful strawberry with in vitro efficacy against uterine leiomyoma cells. J Cell Physiol. 234, 7622-7633

Hu, Q., Noor, M., Wong, Y.F., Hylands, P.J., Simmonds, M.S.J., Xu, Q., Jiang, D., Hendry, B.M., Xu, Q., 2009. In vitro anti-fibrotic activities of herbal compounds and herbs. Nephrology Dialysis Transplantation. 24, 3033-3041

Islam, M.S., Akhtar, M.M., Ciavattini, A., Giannubilo, S.R., Protic, O., Janjusevic, M., Procopio, A.D., Segars, J.H., Castellucci, M., Ciarmela, P., 2014a. Use of dietary phytochemicals to target inflammation, fibrosis, proliferation, and angiogenesis in uterine tissues: Promising options for prevention and treatment of uterine fibroids? Mol Nutr Food Res. 58, 1667-84

Islam, M.S., Catherino, W.H., Protic, O., Janjusevic, M., Gray, P.C., Giannubilo, S.R., Ciavattini, A., Lamanna, P., Tranquilli, A.L., Petraglia, F., Castellucci, M., Ciarmela, P., 2014b. Role of activin-a and myostatin and their signaling pathway in human myometrial and leiomyoma cell function. J Clin Endocrinol Metab. 99, E775-85

Islam, M.S., Ciavattini, A., Petraglia, F., Castellucci, M., Ciarmela, P., 2017a. Extracellular matrix in uterine leiomyoma pathogenesis: A potential target for future therapeutics. Human reproduction update. 24, 59-85

Islam, M.S., Giampieri, F., Janjusevic, M., Gasparrini, M., Forbes-Hernandez, T.Y., Mazzoni, L., Greco, S., Giannubilo, S.R., Ciavattini, A., Mezzetti, B., Capocasa, F., Castellucci, M., Battino, M., Ciarmela, P., 2017b. An anthocyanin rich strawberry extract induces apoptosis and ros while decreases glycolysis and fibrosis in human uterine leiomyoma cells. Oncotarget. 8, 23575-23587

Islam, M.S., Greco, S., Janjusevic, M., Ciavattini, A., Giannubilo, S.R., Dâ€™Adderio, A., Biagini, A., Fiorini, R., Castellucci, M., Ciarmela, P., 2015. Growth factors and pathogenesis. Best Practice & Research Clinical Obstetrics & Gynaecology. In Press

Islam, M.S., Segars, J.H., Castellucci, M., Ciarmela, P., 2017c. Dietary phytochemicals for possible preventive and therapeutic option of uterine fibroids: Signaling pathways as target. Pharmacological Reports. 69, 57-70

Kashani, B.N., Centini, G., Morelli, S.S., Weiss, G., Petraglia, F., 2016. Role of medical management for uterine leiomyomas. Best Pract Res Clin Obstet Gynaecol. 34, 85-103

Journal Pre-proof

Leather, A.T., Studd, J.W.W., Watson, N.R., Holland, E.F.N., 1993. The prevention of bone loss in young women treated with gnrh analogues with" add-back" estrogen therapy. Obstetrics & Gynecology. 81, 104-107

Lee, E.S., Lee, H.E., Shin, J.Y., Yoon, S., Moon, J.O., 2003. The flavonoid quercetin inhibits dimethylnitrosamine-induced liver damage in rats. Journal of pharmacy and pharmacology. 55, 1169-1174

Leppert, P.C., Catherino, W.H., Segars, J.H., 2006. A new hypothesis about the origin of uterine fibroids based on gene expression profiling with microarrays. Am J Obstet Gynecol. 195, 415-420

Liang, C.C., Park, A.Y., Guan, J.L., 2007. In vitro scratch assay: A convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc. 2, 329-33

Lin, P.H., Kung, H.L., Chen, H.Y., Huang, K.C., Hsia, S.M., 2019a. Isoliquiritigenin suppresses e2-induced uterine leiomyoma growth through the modulation of cell death program and the repression of ecm accumulation. Cancers (Basel). 11,

Lin, P.H., Shih, C.K., Yen, Y.T., Chiang, W., Hsia, S.M., 2019b. Adlay (coix lachryma-jobi l. Var. Ma-yuen stapf.) hull extract and active compounds inhibit proliferation of primary human leiomyoma cells and protect against sexual hormone-induced mice smooth muscle hyperproliferation. Molecules. 24,

Nakamura, T., Matsushima, M., Hayashi, Y., Shibasaki, M., Imaizumi, K., Hashimoto, N., Shimokata, K., Hasegawa, Y., Kawabe, T., 2011. Attenuation of transforming growth factor-beta-stimulated collagen production in fibroblasts by quercetin-induced heme oxygenase-1. American journal of respiratory cell and molecular biology. 44, 614-620

Norian, J.M., Malik, M., Parker, C.Y., Joseph, D., Leppert, P.C., Segars, J.H., Catherino, W.H., 2009. Transforming growth factor beta3 regulates the versican variants in the extracellular matrix-rich uterine leiomyomas. Reproductive Sciences. 16, 1153-1164

Pereira, M.A., Grubbs, C.J., Barnes, L.H., Li, H., Olson, G.R., Eto, I., Juliana, M., Whitaker, L.M., Kelloff, G.J., Steele, V.E., Lubet, R.A., 1996. Effects of the phytochemicals, curcumin and quercetin, upon azoxymethane-induced colon cancer and 7,12-dimethylbenz[a]anthracene-induced mammary cancer in rats. Carcinogenesis. 17, 1305-11

Phan, T.T., Lim, I.J., Sun, L., Chan, S.Y., Bay, B.H., Tan, E.K., Lee, S.T., 2003a. Quercetin inhibits fibronectin production by keloid-derived fibroblasts. Implication for the treatment of excessive scars. Journal of dermatological science. 33, 192-194

Phan, T.T., See, P., Tran, E., Nguyen, T.T.T., Chan, S.Y., Lee, S.T., Huynh, H., 2003b. Suppression of insulin-like growth factor signalling pathway and collagen expression in keloid-derived fibroblasts by quercetin: Its therapeutic potential use in the treatment and/or prevention of keloids. British Journal of Dermatology. 148, 544-552

Journal Pre-proof

Pickering, J.G., 2001. Regulation of vascular cell behavior by collagen form is function. Circulation research. 88, 458-459

Ping, J., Li, J.-T., Liao, Z.-X., Shang, L., Wang, H., 2011. Indole-3-carbinol inhibits hepatic stellate cells proliferation by blocking nadph oxidase/reactive oxygen species/p38 mapk pathway. European journal of pharmacology. 650, 656-662

Rodriguez, L.G., Wu, X., Guan, J.L., 2005. Wound-healing assay. Methods Mol Biol. 294, 23-9

Stovall, T.G., Muneyyirci-Delale, O., Summitt Jr, R.L., Scialli, A.R., 1995. Gnrh agonist and iron versus placebo and iron in the anemic patient before surgery for leiomyomas: A randomized controlled trial. The leuprolide study group. Obstetrics & Gynecology. 86, 65-71

Walker, C.L., Stewart, E.A., 2005. Uterine fibroids: The elephant in the room. Science. 308, 1589-1592

Wright, C.S., Pollok, S., Flint, D.J., Brandner, J.M., Martin, P.E., 2012. The connexin mimetic peptide gap27 increases human dermal fibroblast migration in hyperglycemic and hyperinsulinemic conditions in vitro. J Cell Physiol. 227, 77-87

Wu, C.-H., Shieh, T.-M., Wang, K.-L., Huang, T.-C., Hsia, S.-M., 2015. Quercetin, a main flavonoid in onion, inhibitsthe pgf2α-induced uterine contraction in vitroand in vivo. Journal of Functional Foods. Volume 19, Part A, , Pages 495-504

Journal Pre-proof

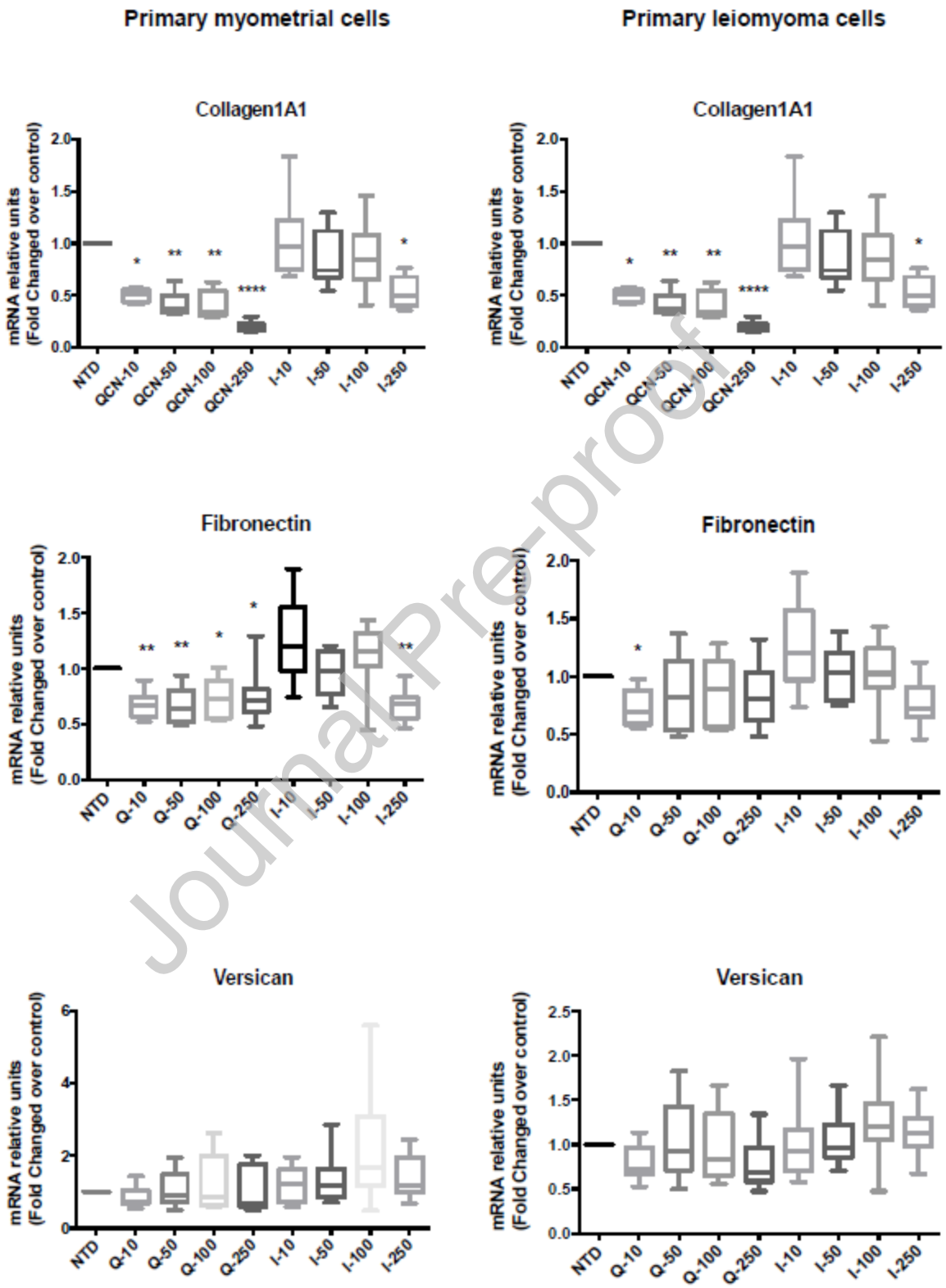
BIOGRAPHY



Dr. Ciarmela is an Associate Professor of Human Anatomy at Università Politecnica delle Marche, Italy. She focused her research investigation in uterine physiology and pathophysiology and explored the role of growth factors. The studies conducted are aimed at understanding the pathogenesis of uterine fibroids and at developing potential therapeutic agents.

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FIGURE LEGENDS

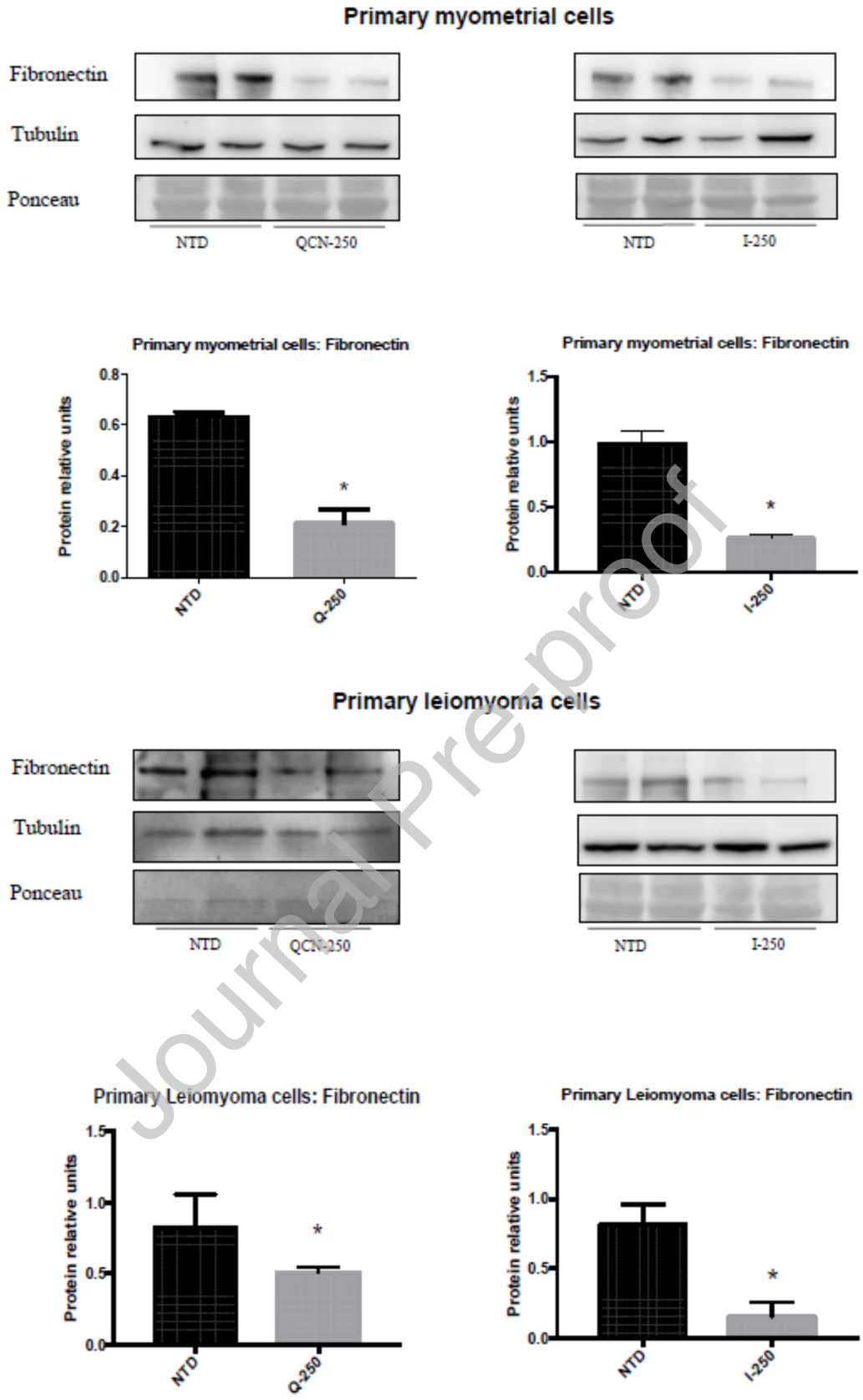


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Figure 1: Effect of quercetin and indole-3-carbinol on extracellular matrix expression in myometrial and leiomyoma cells.

Results of real-time PCR for relative amounts of collagen1A1, fibronectin and versican in myometrial and leiomyoma cells in response to different concentration of quercetin at 10 µg/ml (Q-10); 50 µg/ml (Q-50); 100 µg/ml (Q-100); 250 µg/ml (Q-250); or indole-3-carbinol at 10 µg/ml (I-10); 50 µg/ml (I-50); 100 µg/ml (I-100); 250 µg/ml (I-250). Data are expressed as median, first quartiles, third quartiles and minimum and maximum value (n=6 for RNA) *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001

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Figure 2: Effect of quercetin and indole-3-carbinol on fibronectin protein expression in myometrial and leiomyoma cells.

Western blotting showing the protein expression of fibronectin in myometrial and leiomyoma cells in response to treatment with quercetin at 250 µg/ml (Q-250) and indole-3-carbinol at 250 µg/ml (I-250) or left untreated. Representative gels and data analysis showing the decrease expression of fibronectin after treatment with quercetin or indole-3-carbinol compared with negative control (untreated cells).

Data are expressed as mean ± SD (n=3 for protein) *P<0.05.

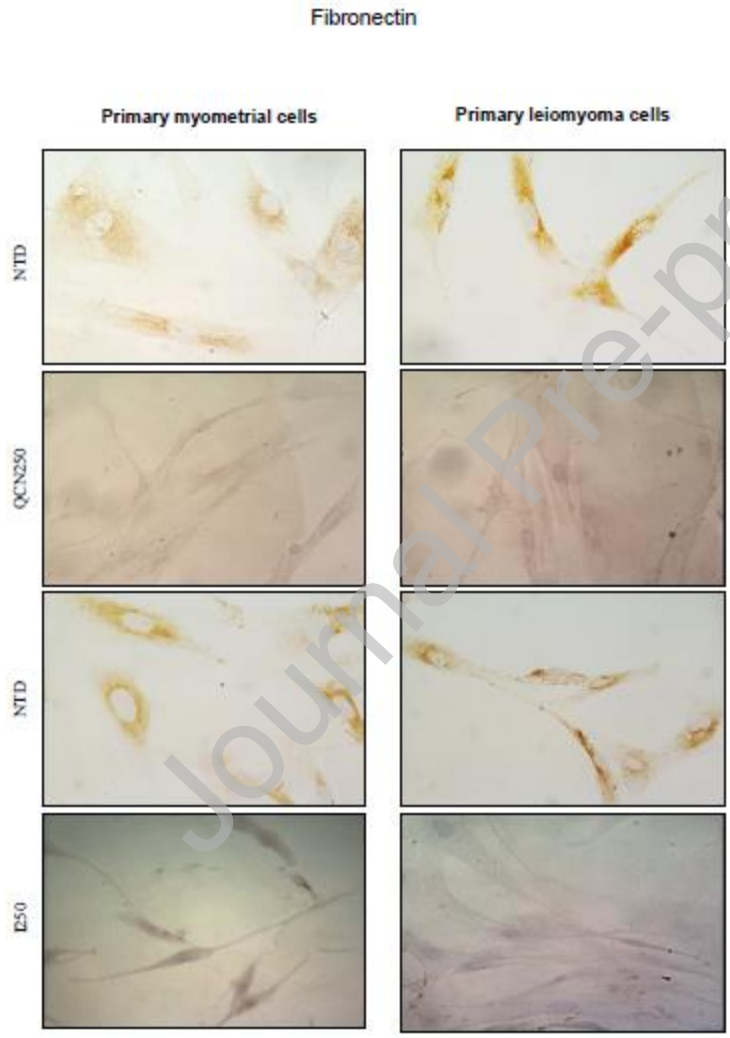


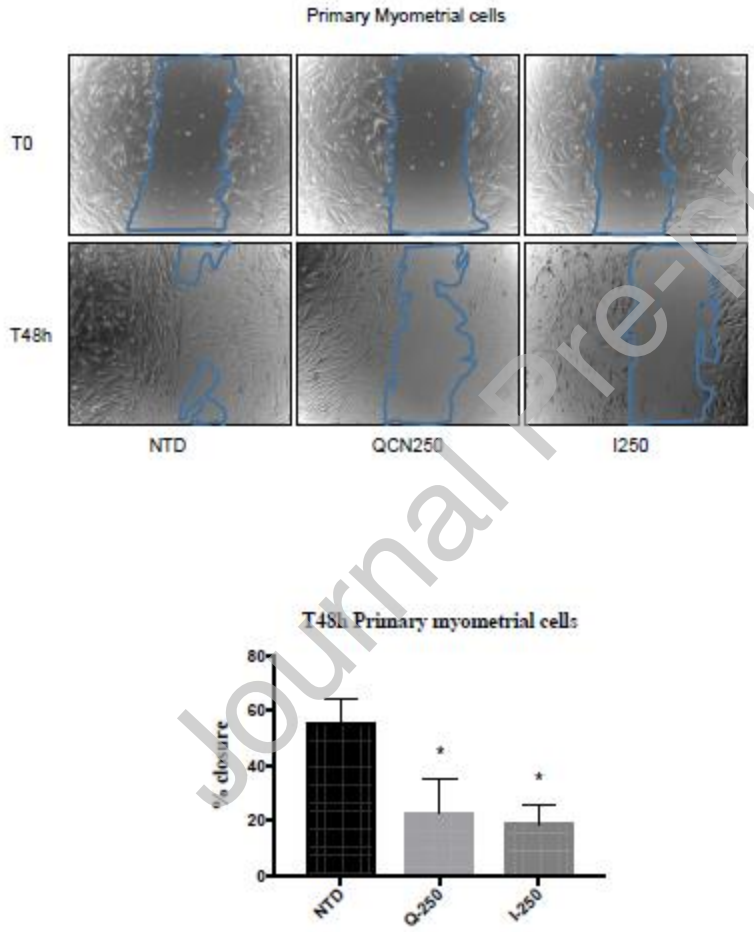
Figure 3: Effect of quercetin and indole-3-carbinol on fibronectin expression in myometrial and leiomyoma cells.

Immunocytochemistry shows the effects of quercetin and indole 3-carbinol on fibronectin expression in

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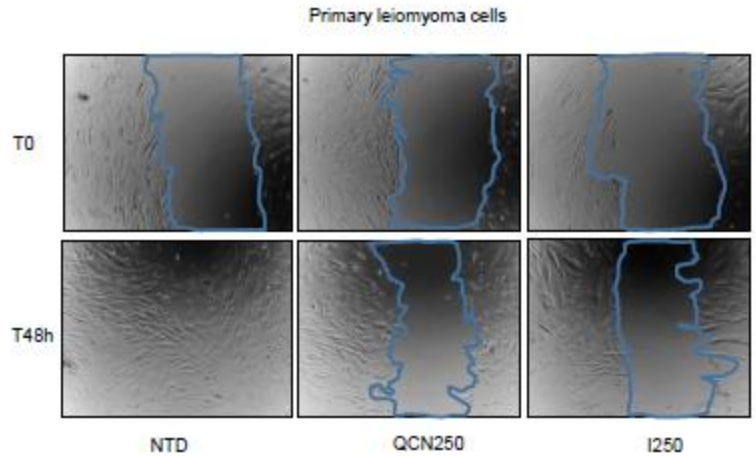
myometrial and leiomyoma cells in response to treatments of quercetin at 250 µg/ml (Q-250) and indole-3-carbinol at 250 µg/ml (I-250) compared with untreated cells. Microscope observation showing the decrease expression levels of fibronectin after treatments with quercetin and indole 3-carbinol compared with negative control (untreated cells).

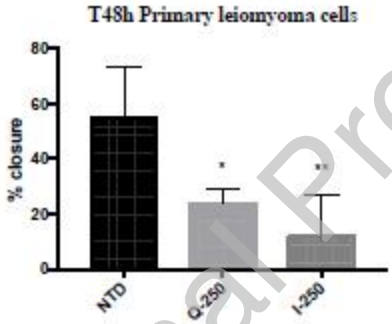
Figure 4a



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Figure 4b





Figures 4a-b: Effect of quercetin and indole-3-carbinol on migration of myometrial and leiomyoma cells.

Wound closure experiments. Representative photographs, and graphs showing the effects of quercetin (250 μg/ml) or indole-3-carbinol (250 μg/ml) treatments on wound closure; both treatments significantly inhibit wound closure in myometrial and leiomyoma cells. Data are expressed as mean ± SD (n=3). *P<0.05; **P<0.01

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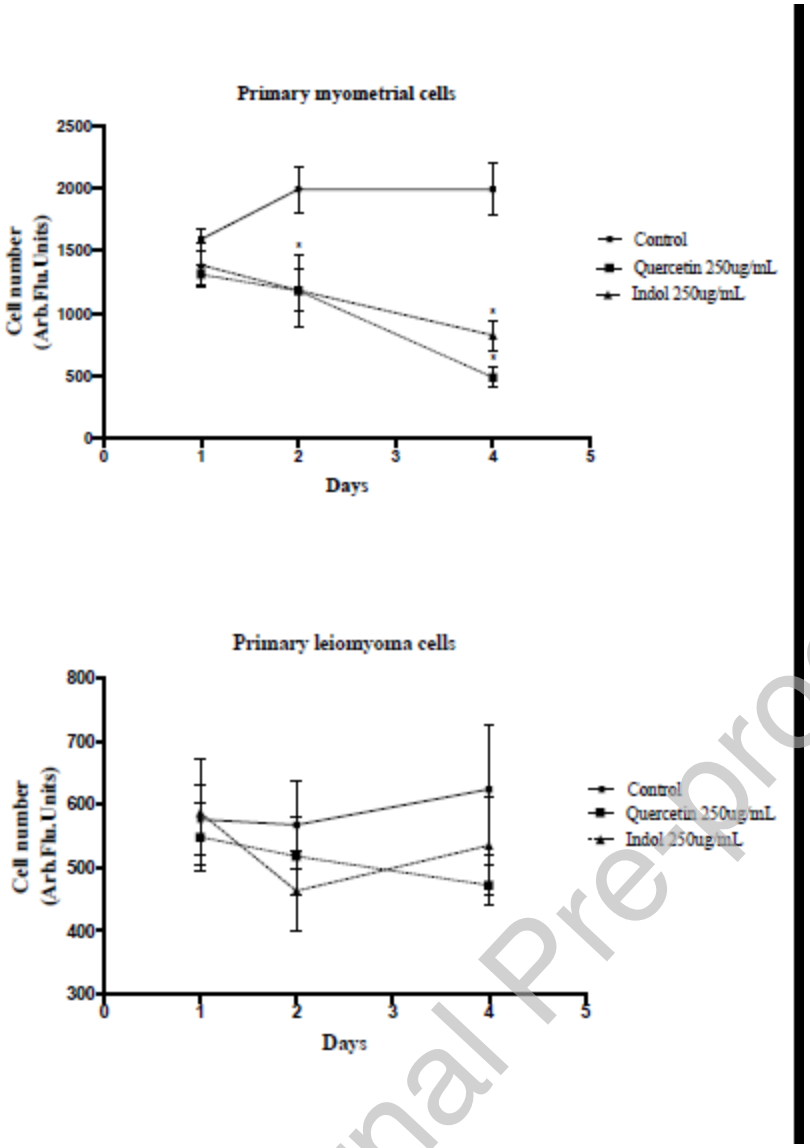


Figure 5: The effect of quercetin and indole-3-carbinol on proliferation of myometrial and leiomyoma cells.

Cell proliferation assay. Representative graphs showing the effects of quercetin (250 μg/ml) or indole-3-carbinol (250 μg/ml) treatments. Both compounds significantly inhibit proliferation of myometrial cells while leiomyoma cells were not affected. Data are expressed as mean ± SD (n=6).

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