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Berberine inhibits the proliferation of human uterine leiomyoma cells

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Objective: To determine whether berberine (BBR), a naturally occurring plant-derived alkaloid, inhibits the proliferation of human uterine leiomyoma (UtLM) cells.
Design: Laboratory research.
Setting: Laboratory.
Patient(s): UtLM and normal human uterine smooth muscle (UtSMC) cell lines.
Intervention(s): Treatment with [1] BBR (10, 20, and 50 μM), [2] BBR (20 and 50 μM) and/or 17β-estradiol (E_2; 10 and 100 nM), and [3] BBR (20 and 50 μM) and/or progesterone (P_4; 10 and 100 nM) for 24 or 72 hours.
Main Outcome Measure(s): Cell proliferation, cell cycle, apoptosis, and related genes expression were determined.
Result(s): BBR inhibited UtLM cell proliferation by inducing G2/M cell cycle arrest and apoptosis. Cell cycle G2/M phase-related genes were altered by BBR treatment: the expression of cyclin A1, cyclin B1, and Cdk1 were down-regulated, while Cdk4, p21, and p53 were up-regulated. BBR-treated cells stained positively for annexin V and manifested increased BAX expression. E_2- and P_4-induced UtLM cell proliferation was blocked by BBR treatment. In marked contrast, even the highest concentration of BBR (50 μM) did not influence cell proliferation in UtSMC cells.
Conclusion(s): BBR selectively inhibits cellular proliferation and blocks E_2- and P_4-induced cell proliferation in UtLM but not in normal UtSMC cells. In addition, BBR did not demonstrate cytotoxicity effects in normal human UtSMCs. Our results suggest BBR could be a potential therapeutic agent for the treatment of uterine leiomyoma. (Fertil Steril® 2015;■: ■–■. ©2015 by American Society for Reproductive Medicine.)
Key Words: Berberine, uterus, leiomyomas, fibroids, anti-tumorigenic, antineoplastic, treatment

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Uterine leiomyomas are benign smooth muscle cell tumors of the myometrium and are the most common pelvic tumors in women (1, 2). Leiomyomas affect up to 50% of women ages 35–49 years (3). Symptoms of uterine leiomyomas include acute and chronic pelvic pain, excessive vaginal bleeding, dyspareunia, iron-deficiency anemia, miscarriage, and infertility (4, 5). The estimated economic burden for uterine leiomyomas in the United States is large, with estimates ranging from $5.9 to 34.4 billion yearly (6). Currently, there are no approved effective long-term medicinal treatments for these tumors.

Berberine (BBR), a natural alkaloid isolated from a number of important medicinal plant species such as Berberis aristata and Berberis aquifolium, is a traditional Chinese herb with antibacterial (7), antihypertensive (8), anti-inflammatory (9), antidiabetic (10), and antihyperlipidemic (11) effects. BBR has also been used for many years in North American folk medicine to treat subacute and chronic inflammatory conditions including gastric disorders, respiratory diseases, and cancer (12). Recently, BBR has been shown to be effective in inhibiting the growth of a variety of human cancers, including melanoma, lung cancer, neuroblastoma, colonic carcinoma, breast cancer, and hepatocellular carcinoma (13–18).

The antineoplastic effects of BBR are manifested both in vitro and in vivo, as assessed by suppression of tumor cell proliferation, induction of tumor cell apoptosis, and inhibition of both tumor invasion and metastasis (19). Molecular mechanisms for the antineoplastic properties of BBR involve [1] p53 dependent cell-cycle arrests at G0/G1, G1, and/or G2/M and

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suppressed expression of cyclins (e.g., cyclin B, D, E) and cyclin-dependent kinases (e.g., CDK 2, 4, 6); [2] modulation of the mitochondria/caspase-dependent and/or Fas/FasL signaling pathways, resulting in alterations in the ratio of anti-apoptotic (Bcl-2 proper, Bcl-XL) and proapoptotic (Bax, Bid) members of the Bcl-2 family proteins; [3] changes in other cell signaling pathways including the Ros, JNK, PKC, ERK, and ATF3 pathways; and [4] inducing apoptosis via positive or negative regulation of various cytokines functioning in the cellular network, including the up-regulation of GADD153, the inhibition of cyclooxygenase-2 (COX-2) and Mcl-1, and the down-regulation of nucleolar phosphoprotein nucleophosmin/B23 and telomerase (see review 12).

Collectively, these mechanisms suggest that BBR may be a promising candidate for clinical use in certain neoplastic growths. Consequently, we have hypothesized that BBR will have a similar effect on normal and leiomyomatous myometrial cells. To test this hypothesis, human uterine leiomyoma (UtLM) and normal uterine smooth muscle cell (UtSMC) lines were treated with BBR, and their proliferation, apoptosis, and expression of related genes was determined.

MATERIALS AND METHODS

Cell Culture

Immortalized UtLM and normal (UtSMC) human myometrial cell lines were provided by Dr. Ayman Al-Hendy, from cells originally generated by Dr. Darlene Dixon (20) via transfection with human telomerase gene. Cells were maintained in smooth muscle growth medium-2 (SmBM; catalog no. CC-3181, Lonza) containing 5% fetal bovine serum (FBS) and supplemented with SmBM singlequots (catalog no. CC-4149).This SmBM singlequot contains hEGF, insulin, hFGF-B, and gentamicin/amphotericin-B. For the BBR stimulation experiments, BBR was directly added to maintain medium.

For the 17β-estradiol (E_2; Sigma, catalog no. E2758) stimulation experiments, cells were grown in serum-free SmBM for 24 hours and then treated with E_2 and/or BBR in SmBM containing 1% FBS. For the progesterone (P_4; Sigma, catalog no. P8783) stimulation experiments, cells were grown in serum-free Dulbecco's modified Eagle medium (DMEM) for 48 hours and then treated with P_4 and/or BBR in the same medium.

Cell Proliferation (MTS) Assay

Cell proliferation was determined by using the CellTiter 96 Cell Proliferation MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] Assay kit (Promega). Experiments were conducted in 96-well plates with 5,000 cells/well initially. After treatment for 72 hours, cells were washed twice using phosphate-buffered saline (PBS) and incubated in 100 μL per well of SmBM or DMEM (in P_4 stimulation experiment). Twenty microliters of CellTiter 96 solution was added to each well. Absorbance was determined with a microplate reader at 490 nm.

Real-time Quantitative PCR (qPCR)

Total RNA was extracted using the miRACLE Isolation Kit (Jinfiniti Biosciences). First-strand cDNA of mRNA was synthesized using the High Capacity cDNA Reverse transcription Kit (Applied Biosystems). Real-time quantitative PCR was performed by using an iTag Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc.) on an Applied Biosystems 7300 real-time PCR system. Primers for two-cell proliferation markers (*MKI67* [21] and *PCNA* [22]), six G2/M phase-related genes (*cyclin A1*, *cyclin B1*, *P21*, *P53*, *cyclin-dependent kinase 1* [*CDK1*], and *cyclin-dependent kinase 4* [*CDK4*]) (23–25), and three genes that are typically overexpressed and play important roles in the pathogenesis of uterine leiomyomas (*pituitary tumor-transforming gene-1* [*PTTG-1*], *E2F transcription factor 1* [*E2F1*], and *cyclooxygenase-2* [*COX-2*]) (26–28) were purchased from www.realtimeprimers.com. *β-Actin* was used as an internal control. Relative fold change of targets genes expression was calculated by using the $2^{-\Delta\Delta Ct}$ method.

Cell Cycle Analysis

UtLM cells were plated in six-well plates with culture medium. Cells were treated with various concentrations (0, 10, 20, and 50 μM) of BBR (catalog no. B3251, Sigma-Aldrich) for 24 hours. The cells were then collected and fixed in cold 70% ethanol at 4°C. After washing, the cells were subsequently treated with 50 mg/mL propidium iodide (PI) and 100 mg/mL RNaseA for 30 minutes in the dark and subjected to flow-cytometric analysis to determine the percentage of cells in specific phases of the cell cycle (subG1, G0/G1, S, and G2/M). Flow-cytometry was performed in the Georgia Regents University campus flow-cytometry core facility by using FACSCalibur Analyzers (Becton Dickinson) equipped with a 488-nm argon laser.

Annexin V Staining

The ability of annexin V to specifically bind phosphatidylserine (PS) is widely used in cellular biology as a method to detect apoptotic cells (29). In normal viable cells, PS is located on the cytoplasmic surface of the cell membrane. However, PS will translocate from the inner to the outer leaflet of the membrane in the intermediate stages of apoptosis (30). This process exposes PS to the external cellular environment, where it can be detected.

To detect apoptosis, UtLM cells were plated on eight-well chamber slides incubated in culture medium overnight. Cells were then treated with 20 μM BBR for 24 hours. After incubation, cells were washed with cold PBS and incubated in annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 5 μL of annexin V Alexa Fluor 488 conjugate (catalog no. A13201, Life Technologies) for 15 minutes at room temperature. After incubation, cells were washed once with annexin V binding buffer. The slide was mounted with Aqueous Mounting Medium with anti-Fading agents (catalog no. M01, Biomeda Corporation) and examined with fluorescent inverted microscopy (catalog no. CKX41, Olympus) at 488 nM.

Statistical Analysis

Comparisons of multiple groups were carried out by analysis of variance (ANOVA) followed by a post-test by using the