**Abstract**

**Study question:** What are the effects of plant-derived antioxidant compounds urolithin A (UA) and B (UB) on the growth and pathogenetic properties of an in vitro endometriosis model?

**Summary answer:** Both urolithins showed inhibitory effects on cell behavior related to the development of endometriosis by differentially affecting growth, adhesion, motility, and invasion of endometriotic cells in vitro.

**What is known already:** Endometriosis is one of the most common benign gynecological diseases in women of reproductive age and is defined by the presence of endometrial tissue outside the uterine cavity. As current pharmacological therapies are associated with side effects interfering with fertility, we aimed at finding alternative therapeutics using natural compounds that can be administered for prolonged periods with a favorable side effects profile.

**Study design, size, duration:** In vitro cultures of primary endometriotic stromal cells from 6 patients subjected to laparoscopy for benign pathologies with histologically confirmed endometriosis; and immortalized endometrial stromal (St-T1b) and endometriotic epithelial cells (12Z) were utilized to assess the effects of UA and UB on endometriotic cell properties. Results were validated in three-dimensional (3D) in vitro co-culture spheroids of 12Z and primary endometriotic stroma cells of one patient, and organoids from 3 independent donors with endometriosis.

**Participants/materials, setting, methods:** The effects on cell growth were measured by non-radioactive colorimetric assay to measure cellular metabolic activity as an indicator of cell viability (MTT assay) and flow cytometric cell cycle assay on primary cultures, St-T1b, and 12Z. Apoptosis analyses, the impact on in vitro adhesion, migration, and invasion were evaluated in the cell lines. Moreover, Real-Time Quantitative Reverse Transcription polymerase chain reaction (RT-qPCR) assays were performed on primary cultures, St- T1b and 12Z to evaluate a plausible mechanistic contribution by factors related to proteolysis (matrix metalloproteinase 2, 3 and 9 -MMP2, MMP3, MMP9-, and tissue inhibitor of metalloproteinases -TIMP-1-), cytoskeletal regulators (Ras-related C3 botulinum toxin substrate 1 -RAC1-, Rho-associated coiled-coil containing protein kinase 2 -ROCK2-), and cell adhesion molecules (Syndecan 1 -SDC1-, Integrin alpha V-ITGAV-). Finally, the urolithins effects were evaluated on spheroids and organoids by formation, viability, and drug screen assays.

**Main results and the role of chance:** 40 µM UA and 20 µM UB produced a significant decrease in cell proliferation in the primary endometriotic cell cultures (P < 0.001 and P < 0.01, respectively) and in the St-T1b cell line (P < 0.001 and P < 0.05, respectively). In St-T1b, UA exhibited a mean half-maximum inhibitory concentration (IC50) of 39.88 µM, while UB exhibited a mean IC50 of 79.92 µM. Both 40 µM UA and 20 µM UB produced an increase in cells in the S phase of the cell cycle (P < 0.01 and P < 0.05, respectively). The same concentration of UA also increased the percentage of apoptotic ST-t1b cells (P < 0.05), while both urolithins decreased cell migration after 24 h (P < 0.001 both). Only the addition of 5 µM UB decreased the number of St-T1b adherent cells. TIMP-1 expression was upregulated in response to treating the cells with 40 µM UA (P < 0.05). Regarding the 12Z endometriotic cell line, only 40 µM UA decreased proliferation (P < 0.01); while both 40 µM UA and 20 µM UB produced an increase in cells in the G2/M phase (P < 0.05 and P < 0.01, respectively). In this cell line, UA exhibited a mean IC50 of 40.46 µM, while UB exhibited a mean IC50 of 54.79 µM. UB decreased cell migration (P < 0.05), and decreased the number of adherent cells (P < 0.05). Both 40 µM UA and 20 µM UB significantly decreased the cellular invasion of these cells; and several genes were altered when treating the cells with 40 µM UA and 10 µM UB. The expression of MMP2 was downregulated by UA (P < 0.001), and expression of MMP3 (UA P < 0.001 and UB P < 0.05) and MMP9 (P < 0.05, both) were downregulated by both urolithins. Moreover, UA significantly downregulated ROCK2 (P < 0.05), whereas UB treatment was associated with RAC1 downregulation (P < 0.05). Finally, the matrix adhesion receptors and signaling (co)receptors SDC1 and ITGAV were downregulated upon treatment with either UA or UB (P < 0.01 and P < 0.05, respectively in both cases). Regarding the effects of urolithins on 3D models, we have seen that they significantly decrease the viability of endometriosis spheroids (80 µM UA and UB: P < 0.05 both) as well as affecting their area (40 µM UA: P < 0.05, and 80 µM UA: P < 0.01) and integrity (40 µM UA and UB: P < 0.05, 80 µM UA and UB: P < 0.01). On the other hand, UA and UB significantly inhibited organoid development/outgrowth (40 and 80 µM UA: P < 0.0001 both; 40 µM UB: P < ns-0.05-0.001, and 80 µM UB: P < 0.01-0.001-0.001), and all organoid lines show urolithins sensitivity resulting in decreasing viability (UA exhibited a mean IC50 of 33.93 µM, while UB exhibited a mean IC50 of 52.60 µM).