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Effect of urolithins A and B on ectopic endometrial growth in a murine model of endometriosis

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Endometriosis is an often painful disease in reproductive-aged women, in which endometrial-like tissue grows outside the uterine cavity. Since the limited current therapeutic alternatives fail in alleviating the symptoms and based on our previous research in *in vitro* models using the same compounds as the ones used in the present study, we aimed to evaluate the effects of urolithins A (UA) and B (UB) on the growth and survival of endometriotic-like lesions in a murine model of endometriosis. Female BALB/C mice were surgically induced with endometriosis and treated with 2.5 mg kg^{-1} day^{-1} intraperitoneal UA or UB. The mice were monitored daily and weighed and the estrous stage was determined. After 28 days of treatment, lesions were counted, measured, excised, and fixed. Both urolithins proved not to affect the estrous cycle or body weight of the mice. UA completely prevented endometriotic-like lesions, while UB diminished the implant volume ($p < 0.05$). Treatment also reduced epithelial and stromal cell proliferation within the implants ($p < 0.001$ and $p < 0.01$, respectively) and apoptosis was enhanced ($p < 0.05$ and $p < 0.01$, respectively). These results are promising and reveal that urolithins A and B, separately, have a beneficial effect on the overall endometriotic growth without affecting the body weight or estrous cycle.

1. Introduction

Endometriosis is a benign gynaecological disease defined by the presence of endometrial tissue outside the uterine cavity that commonly arises during the reproductive age of women.[1,2] As the disease is estrogen-dependent,[3–6] treatment options for endometriosis are combined oral contraceptives and progestins,[7,8] creating a state of iatrogenic menopause or pseudo-pregnancy.[9] The classical treatments have important disadvantages, including suppression of reproductive function, a high rate of recurrence, and other adverse effects that limit their long-term use.[7,10–12] Subsequently, endometriosis has a substantial effect on the quality of life of patients,[13–16] with negative consequences on daily life activities, sexual function and personal relationships.

Over the years, natural compounds have become a valuable resource due to their potential use in the development of treatments for various pathologies.[17–21] Recent reports have demonstrated by *in vivo* and *in vitro* studies that polyphenols, flavonoids and other antioxidants are able to inhibit proliferation, induce apoptosis and cause cytotoxicity in cancer cells without affecting healthy cells.[22–24] This is of particular interest because although endometriosis is a benign disorder, it shares important characteristics with cancer,[25] like the ability of endometriotic cells to invade distant tissues, low levels of apoptosis, and high rates of cell proliferation. Urolithins are a subfamily of metabolites generated by the human intestinal microbiota[32,33] from ellagitannins and EA, which are polyphenols mainly found in fruits such as strawberries, raspberries, blueberries, blackberries, walnuts, pomegranates and muscadine grapes.[34,35] They are dibenzopyran-6-one derivatives with different hydroxyl substitutions, produced through the loss of one of the two lactones present in EA and by successive removal of hydroxyls.[32] The major end products of these metabolic reactions are 3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]-pyran-6-one known as UA and its mono-hydroxy analogue known as UB.[36] In previous *in vitro* studies, it has been demonstrated that they have anti-inflammatory, anticancer, antioxidant, antimicrobial and antiestrogenic effects.[37,38] Moreover, in our most recent work we demonstrated for the first time the anti-proliferative, anti-migratory, anti-invasive and pro-apoptotic effects of UA and UB in a variety of *in vitro* models of endometriosis.[39]

In this sense, several studies have shown that ellagic acid (EA) and especially its metabolites, the urolithins, exert a wide range of beneficial health effects including anti-oxidant, anti-inflammatory, anti-estrogenic and anti-carcinogenic effects.[26–31] However, until now there has been no evidence of

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their systemic effect on endometriotic-like lesion development in an *in vivo* model of endometriosis.

Due to the questioned efficacy of the current therapeutics,[40] and based on previous studies performed by our research group,[39,41–43] we focused our search for alternative therapies towards natural compounds. The aim of our study was to evaluate the effects of urolithins A (UA) and B (UB) *in vivo* on the growth and survival of endometriotic lesions in experimental endometriosis in a BALB/c mouse model.

2. Experimental methods

2.1. Animals

In this study, 40 2-month-old female BALB/c mice were used. All procedures were performed according to the NIH guidelines for the care and use of laboratory animals and approved by the Instituto de Biología y Medicina Experimental (IBYME) Ethics and Research Committee (CE 025-2/2012) and IBYME Institutional Commission for the Care and Use of Laboratory Animals (CICUAL: 031/2016), Buenos Aires, Argentina. A total of 5 animals died or had to be sacrificed between 2 and 3 days after surgery because they did not fully recover from the intervention.

2.2. Surgical induction of endometriosis

Endometriotic-like lesions were induced through transplantation of one of the uterine horns to the bowel mesothelium as previously described.[44] Briefly, animals were deeply anesthetized with an intraperitoneal injection of ketamine (120 mg kg^{-1}) and xylazine (10 mg kg^{-1}). Afterwards, mice underwent laparotomy by a mid-ventral incision to expose the uterus and the intestine. The right uterine horn of each animal was removed, opened longitudinally, and then cut into 4 mm^2 pieces. Three equal pieces of tissue of identical size and characteristics were then sutured onto the mesothelium layer with a single 6–0 nylon suture (Supralon, Ethicon, Somerville, NJ, USA) with the endometrial tissue facing the serosa. Finally, the abdomen was then closed with a 5–0 nylon suture.

Because surgery itself can have effects on the immune system, we also included a group of sham animals. These animals underwent the same surgical procedure, in which the uterine horn was removed, but no tissue was sutured.

2.3. Experimental design

14 days after surgery, animals with induced endometriosis were randomly assigned into three different treatment groups: control (1% DMSO in PBS), UA (Santa Cruz, 2.5 mg kg^{-1} in PBS with 1% DMSO) and UB (Santa Cruz, 2.5 mg kg^{-1} in PBS with 1% DMSO). Sham animals received the vehicle (PBS with 1% DMSO). All treatments were administered daily by intraperitoneal injection, for 28 consecutive days and started on postoperative day 14, a time point at which the endometriotic lesions are considered to be already developed.[45,46] Mice were monitored daily.

Urolithin levels can reach up to micromolar concentrations in human serum depending on the microbiota composition,[36,47,48] which leads to a large inter-individual variability in urolithin levels.[33,49,50] Therefore, in order to bypass the intrinsic individual variation in microbiota,[51] we injected UA and UB directly into the peritoneal cavity, in agreement with the doses and administration route used in previous *in vivo* model studies.[51–53]

The endometriosis induction/treatment protocol was applied as follows:

Day 0: arrival of the animals to the in-house animal facility.

Day 7: endometriosis induction surgery.

Day 14: treatments began to be administered daily.

Day 42: end of the experiment/sacrifice.

2.4. Evaluation of the well-being of mice

Mice were carefully observed to detect any changes in their grooming behavior, activity levels and food consumption from post-surgical day 1 up to the day of sacrifice. They were weighed twice a week starting 14 days after the induction surgery.

2.5. Evaluation of the estrous cycle

To assess the effect of these therapies on the estrous cycle, all groups were sampled once a day by the vaginal smear method[54] during the last 16 days of treatment. Vaginal samples were collected between 8 and 9 a.m., and 40 μl of physiological solution was inserted into the vaginal cavity at room temperature, withdrawn, and smeared on a microscope slide. Estrous cycle stages were determined according to the type, number, and morphology of the cells in the smear. Diestrus index was calculated using the formula:[55]

$$\text{Diestrus index} = \frac{\text{number of days with a clear diestrus smear}}{\text{total duration of cycled days}} \times 100$$

Estrous cyclicity was evaluated from three aspects: (1) the number of cycles observed in 16 consecutive days, counting both complete and incomplete cycles; (2) the cycle length which was calculated by counting the days between two successive estrous stages with both proestrus and diestrus stages occurring in between; and (3) the number of days or time spent in each stage.

2.6. Evaluation of endometriotic-like lesions

After 28 days of treatment, animals were sacrificed by cervical dislocation. The abdomen was opened by a ventral midline incision. Implantation sites were localized by the presence of a lesion or a suture alone. Lesions were counted and measured in two perpendicular diameters (d, D) using a calliper.

The system of classification of the growth of the lesions was used in accordance with the study by Quereda *et al.*[56] with modifications: Grade 0 (the lesion had disappeared, or if it was visible it never became a cyst), Grade 1 (the lesion formed a vesicle whose major diameter was <2 mm or, if larger, it was

solid), Grade 2 (the lesion formed a cyst with fluid, and its major diameter was ≥2 mm, but <4 mm), and Grade 3 (the diameter of the vesicle was ≥4 mm).

Lesion volumes were determined using the following formula (where r and R are the radii, $r < R$):[57]

$$V = \frac{4}{3} \times \pi \times r^2 \times R$$

Then lesions were excised, fixed, embedded in paraffin, and cut into 5 μm serial sections. Several sections from each specimen were stained with hematoxylin–eosin and examined microscopically for the presence of histological hallmarks of endometriosis.

2.7. Immunohistochemistry for proliferating cell nuclear antigen

Serial sections of endometriotic-like lesions were subjected to standard immunohistochemistry procedures for proliferating cell nuclear antigen (PCNA) as described previously.[58] Briefly, the sections were incubated with rabbit anti-mouse PCNA polyclonal antibody (1 : 800, FL-261, Santa Cruz Biotechnology, Santa Cruz, USA) and the corresponding secondary biotinylated antibody (1 : 200, rabbit biotinylated anti IgG antibody, Sigma-Aldrich). They were then incubated with a streptavidin-peroxidase conjugate (Dako) and exposed to diaminobenzidine (DAB, Dako) as the peroxidase substrate. Finally, the sections were counterstained with Gill's hematoxylin. The presence of brown nuclear reactivity indicated PCNA-positive cells. Negative controls were carried out, replacing the primary antibody with a rabbit immunoglobulin G isotype antibody (1 : 800, Roche). The number of PCNA immunopositive cells was established using a standard light microscope at 400× magnification. At least 300 epithelial and stromal cells were counted from representative fields, considering all lesions. The percentage of PCNA positive cells was established per mouse, blinded to the treatment conditions, and the mean value per group was calculated.

2.8. TUNEL assay

For apoptosis quantification, the sections were processed for *in situ* immunolocalization of nuclei exhibiting DNA fragmentation by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique, using the *In Situ* Cell Death Detection Kit, POD (Roche). As a negative control, the tissue samples were subjected to treatment without TdT. At least 300 epithelial and stromal cells were counted, and the percentage of TUNEL positive cells was calculated per mouse, blinded to the treatment conditions, and the mean value per group was calculated.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad PRISM software 6.0 (GraphPad Software Inc.). Statistical comparisons between groups were performed using parametric one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, or nonparametric one-way ANOVA (Kruskal–Wallis) followed by Dunn's multiple comparison test. Student's *t*-test or the nonparametric Mann–Whitney test was used for statistical comparisons between two groups.

Results were expressed as mean ± standard error (SEM); $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of UA and UB on the well-being of mice

Given the chronic nature of this disease, one of the challenges is to find a treatment with few side effects.[11] Therefore, in our study, the mice were monitored daily to examine whether the treatment with urolithins leads to variations in weight. All mice gained weight during the post-surgical period, as expected in young mice. Urolithin-injected mice gained a similar weight per week compared to the control or even sham mice (Fig. 1A). The average body weight of UA/UB-treated mice at 28 days after injection was also not different from those of the controls. These results revealed that neither the disease nor the treatment generated statistically significant modifications in the body weight (Fig. 1B) or the food intake. Besides, the behavior and the activity levels of the animals were also unchanged.

3.2. Effect of UA and UB on the estrous cycle

A further important aspect to be evaluated is the potential effect on the estrous cycle since signs such as persistent diestrus, and non-cyclic and lengthy estrus cycles are considered indicators of the compound's toxicity.[55] Therefore, we next examined the estrous cycle of all animals during the last 16 days of treatment. The normal estrous cycle has a characteristic periodicity. This examination showed that the estrous cycle pattern of all groups remains regular (Fig. 1C). When analyzing the results of all mice involved in this study, no significant differences were observed either in the number of cycles or in their duration between the different groups (Fig. 1D and F).

To quantify the time course of the estrous cycle, we graphed the data per group as days spent in each stage of the cycle. In all cases, a regular estrous cycle pattern was observed. Even though some variations can be observed between groups, the mean time spent in each stage showed no statistically significant difference (Fig. 1F). The same is true even for diestrus indexes (sham: 48.64%, control: 33.06%, UA: 39.60%, and UB: 36.83%; $p > 0.05$ *vs.* control or sham in all cases).

Overall, these data clearly demonstrated that the treatment with urolithins does not generate any alterations of the estrous cycle with respect to the control or the sham group.

3.3. Morphological and histopathological evaluation of the endometriotic-like lesions

After 28 days of treatment with UA or UB, animals were sacrificed and the abdominal cavity was explored to localize and measure the developed lesions. The results of this macroscopic cavity examination revealed that all animals that underwent the induction surgery had developed lesions or, in their

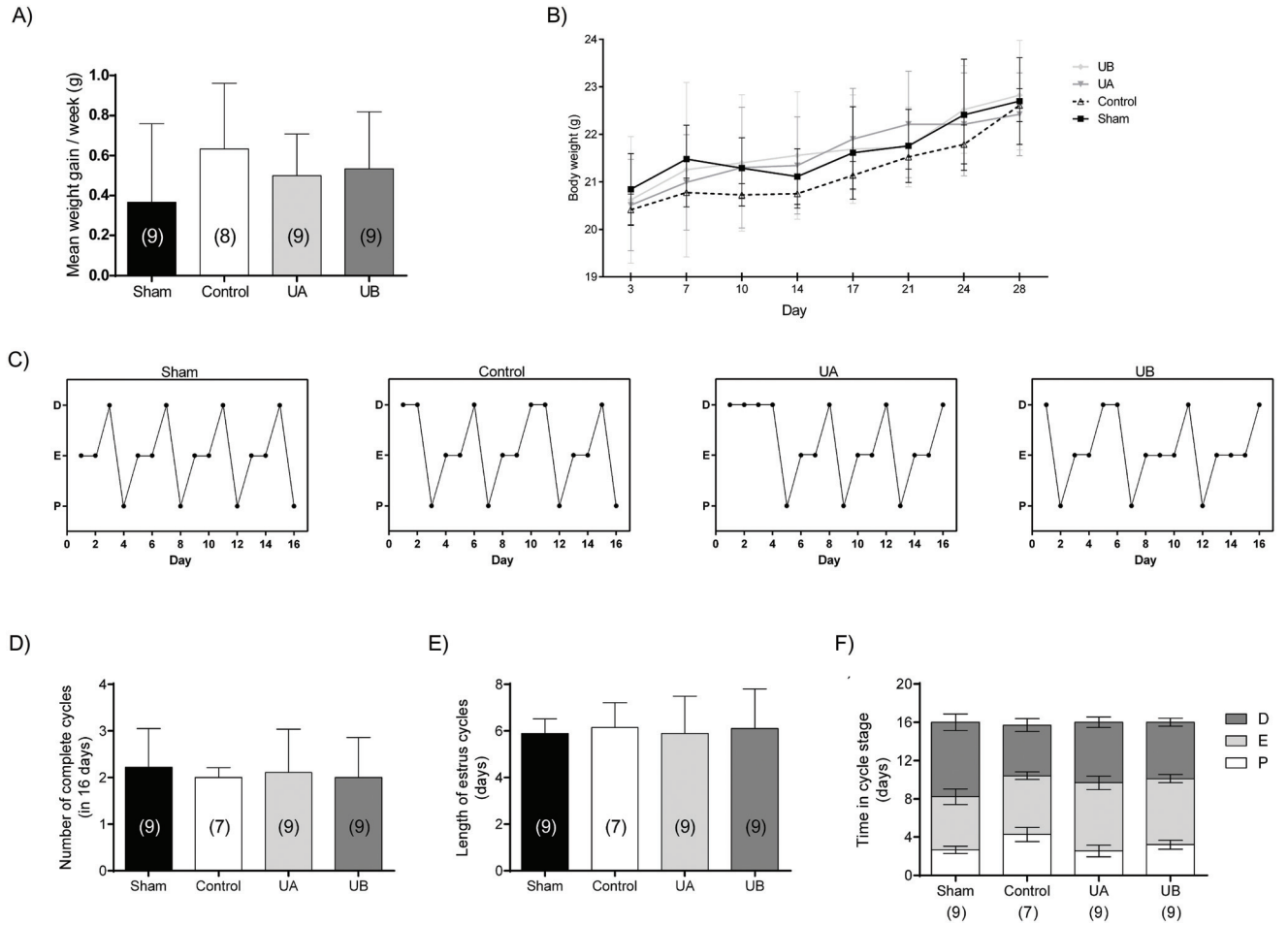

Fig. 1 Weight variations and estrous cyclicity of mice. All mice were weighed twice a week and the mean per week was calculated. (A) Mean weight gain per week per group. (B) Progression of mouse body weight throughout the treatment. The estrous cycle (P: proestrus, E: estrus and D: diestrus) of all the animals was evaluated. (C) Representative graphs of one animal per group showing the evolution of the estrous cycle; (D) number of estrous cycles in 16 days; (E) total duration of the estrous cycle; (F) time spent in each stage of the estrous cycle. Results are expressed as mean ± SEM. *N* is expressed in parentheses in the graphs.

absence, the sutured and undeveloped initial implanted tissue was visualized, confirming the efficiency of the surgical induction of endometriosis. As expected, no lesion or tissue was observed in the sham group.

Regarding the grade of lesion growth, the treatments increased the presence of lesions with growth grade 0, and decreased the incidence of lesions with growth grade 3 (Table 1). Particularly, lesions with growth grade 0 were more frequent in the UA group (95.2% *vs.* 30.4% in the control group).

Moreover, morphological analyses revealed ovoid-shaped lesions, while the histopathological evaluation showed typical endometrial components such as glands and stroma, confirming successful experimental endometriosis (Fig. 2A).

3.4. Effect of UA and UB on endometriotic-like lesion growth

Fig. 2 shows the percentage of lesions developed per animal and their size at the end of the experiment. Both UA and UB caused a reduction in the percentage of developed lesions per mouse compared to the control group (Fig. 2B). UB treated animals developed about 50% of the surgically induced lesions; while in the group treated with UA, only one animal developed a single lesion.

Table 1 Grade of lesion growth reported for lesions on each group

Grade of lesion growth			
	Control *N* (%)	UA *N* (%)	UB *N* (%)
Grade 0	7 (30.4)	20 (95.2)	14 (56)
Grade 1	—	—	—
Grade 2	4 (17.4)	—	5 (20)
Grade 3	12 (52.2)	1 (4.8)	6 (24)
Total	23 (100)	21 (100)	25 (100)

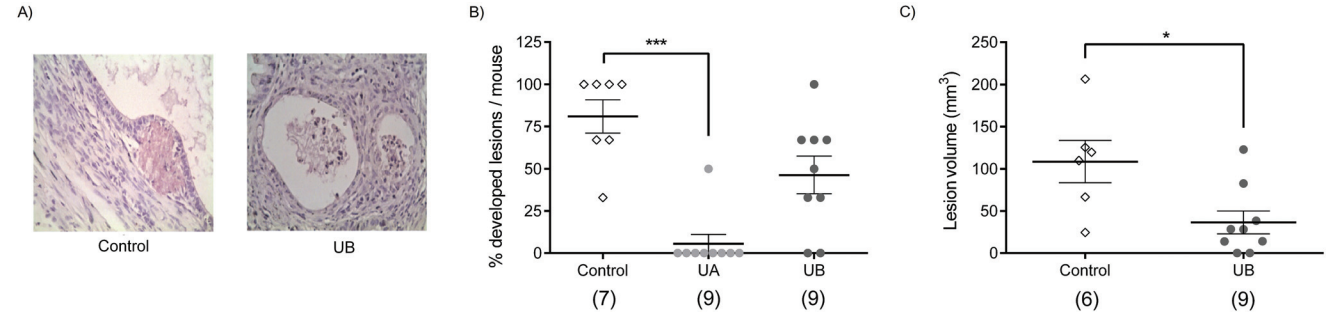

Fig. 2 Endometriotic-like lesion development. After 28 days of treatment, the animals were sacrificed and the peritoneal cavity was examined. (A) Representative images of endometriotic-like lesions: control and UB groups (the UA image is not shown since only one lesion was found). Magnification: 400×. (B) Percentage of lesions developed per mouse and (C) volume of lesions developed in each experimental group. Results are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ *versus* control group. *N* is expressed in parentheses in the graphs.

Moreover, treatment with UB caused a statistically significant decrease in the end-point volume of developed lesions compared to the control group (Fig. 2C) ($p < 0.05$).

3.5. Effect of UB on the cell proliferation of endometriotic-like lesions

Cell proliferation was evaluated in the histological sections of developed endometriotic-like lesions by immunolocalization of PCNA. Cell proliferation in the epithelial fraction (Fig. 3A) of the lesions was significantly diminished compared to the control group when animals were treated with UB (UB $p < 0.001$ *versus* control); similarly, this treatment significantly reduced the stromal (Fig. 3B) proliferating cells compared to the control group (UB $p < 0.01$ *versus* control).

Micrographs show representative histological sections of endometriotic-like lesions (Fig. 3C).

3.6. Effect of UB on cell apoptosis in endometriotic-like lesions

In accordance with the results obtained for cell proliferation, UB significantly increased the apoptotic index in epithelial and stromal cells of endometriotic-like lesions (Fig. 3D: UB $p < 0.05$ *versus* control for epithelial cells, Fig. 3E: UB $p < 0.01$ *versus* control for stromal cells). Micrographs show representative histological sections of endometriotic-like lesions (Fig. 3F).

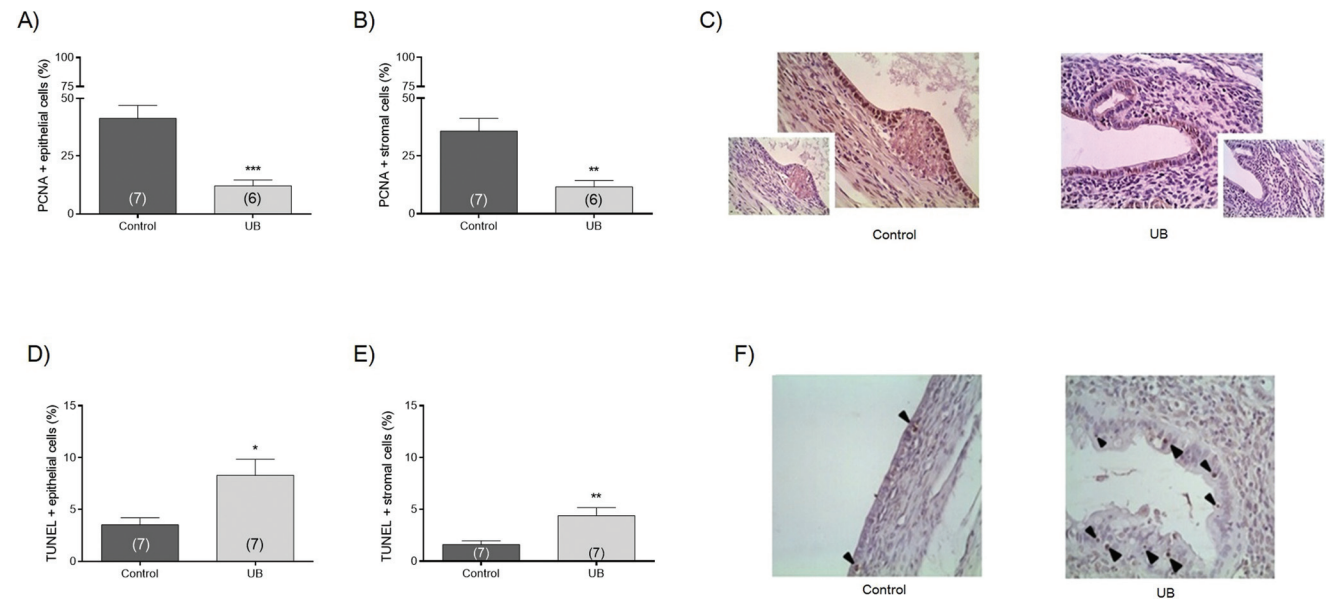

Fig. 3 Immuno-histochemical assessment of proliferation and apoptosis on endometriotic-like lesions. After 28 days of treatment, the developed lesions were removed and fixed. Cell proliferation within the implants was evaluated by immunohistochemistry of PCNA. The percentage of PCNA+ (A) epithelial and (B) stromal cells was quantified. Photomicrographs of PCNA immunostaining are displayed (C). Inset: one section of each slide was incubated with rabbit IgG isotype antibody as a negative control. Magnification: 400×. Apoptosis within the implants was evaluated by a TUNEL assay. The percentage of TUNEL+ (D) epithelial and (E) stromal cells was quantified. Photomicrographs of TUNEL immunostaining are displayed (F). One section of each slide was incubated in the absence of TdT enzyme as a negative control. Magnification: 400×. Results are expressed as mean ± SEM. **$p < 0.01$ and ***$p < 0.001$ with respect to the control group. *N* is expressed in parentheses in each bar.

4. Discussion

Current treatment for endometriosis usually includes surgery and/or prolonged hormonal manipulation, aimed at ameliorating the symptoms of the disease. As stated by de Ziegler *et al.*,[59] it is essential that the relative benefits of each therapeutic option are weighed and that the main reason for their choice does not derive from the main activity of the first consulting professional, since it is a complex disease that intertwines different symptoms depending on each patient. Even though great efforts are being taken by researchers to give better and longer lasting answers to patients, the high recurrence rate and the numerous side effects of the medical treatments[7,10–12] are some of the most challenging problems faced nowadays. This has led investigations to focus on finding new and more effective alternatives for patients. A variety of natural compounds found in food and plants, some specific phytochemicals extracted from them, and multi-component herbal preparations are being tested for the treatment of different diseases such as cancer[60] and even endometriosis.[32–34,61] Given that most dietary polyphenols undergo extensive metabolism by the microbiota of the intestine,[62] and taking into account previous results obtained in our laboratory and earlier promising results obtained in cancer studies, in the present study we focus on urolithins A and B as the major active metabolites of EA.[32,34]

Given the potential impact of endometriosis symptoms on mental well-being and social functioning,[63] the behavioral evaluation of mice subjected to the endometriosis model is an interesting aspect to take into account when we evaluate their well-being. Previously, several behavioral alterations had been observed in rat endometriosis models resembling human depression, such as anxiety, anhedonia, apathy, and despair-like behavior, as well as changes in pain sensitivity.[64] In this sense, our results indicated that there were no differences in the weight gain per week for the different groups (Fig. 1A). Moreover, our findings indicate that urolithins did not alter food consumption, grooming behavior, or activity levels.

Due to the importance of estrogen in this pathology,[3] we decided to evaluate whether the estrous cycle was altered upon treatment. Evaluation of the estrous cycle in experimental animals[65] is a useful indicator of the integrity of the hypothalamic–pituitary–ovarian axis and the state of functioning of the female reproductive system, and it can also be used to investigate the impact of drugs/treatments on reproductive function. Our results indicated that treatment with both urolithins did not disrupt the reproductive cycle. As previously stated by Cooper and Goldman,[66] vaginal cytology samples must be collected over at least 14 consecutive days in order to allow one to identify any cyclicity alterations. Considering this, in our work we took samples of the animals for 16 consecutive days. Usually, the estrous cycle length in mice averages 4–5 days, but occasional 6-day cycles may be observed in some mice.[67,68] Consequently, in this study, the cycle length averages 5–6 days (Fig. 1E). Regarding the time spent in each stage, even though it varies between 6 and 72 h depending on the stage and individual mouse,[68] it has been established that diestrus is the longest with an average duration of 48–72 h.[65] Accordingly, we assessed both the time spent in each stage and the percentage of days in diestrus (diestrus index) over 16 days and concluded that there were no statistically significant differences between groups (Fig. 1F). In addition, by histological analyses we were able to recognize the typical structures of the ovaries and uterus (data not shown), which led us to conclude that the treatments do not affect the morphology and histology of these organs. Overall, our results indicated that after 28 days of experimentation all the groups displayed regular estrous cycles (Fig. 1C–F) characterized by a similar number, length and time spent in each stage. However, more specific assays are needed to determine the effect of the treatment on the ovarian function.

We then evaluated the effect of UA and UB on endometriotic-like lesions. In a previous report using the autologous surgery model, Kizilay *et al.*[69] sacrificed 2 test animals 10 days after induction surgery and confirmed that the endometriosis model had been created macroscopically and microscopically. A first comparison among the groups was made through the grade of lesion growth (Table 1). Based on the results obtained for the control group, the development of experimental endometriosis in our study was satisfactory, since in all the cases at least 1 of the 3 ectopic tissues implanted was found during the induction surgery. In particular, 52.2% of the lesions in the control group belonged to the most advanced grade (grade 3), while almost all the implants in the UA group (95.2%) were of the lowest developmental grade (grade 0). The results demonstrate that UA treatment leads to the non-development of endometriotic-like lesions. This classification of the growth of the implants proposed by Quereda *et al.*[56] allows us to do a macroscopic evaluation of the growing degree of self-transplanted tissues and validates the model.[70] Moreover, the hematoxylin–eosin stained sections of all the lesions confirmed the presence of histological hallmarks (glands and stroma) of endometriosis (Fig. 2A).

In our study, we also found that both UA and UB were able to decrease the number of established lesions per mouse (Fig. 2B), especially UA which undoubtedly completely inhibited endometriotic-like lesions. Moreover, UB exerted a statistically significant reduction of the end-point size of the lesions (Fig. 2C), by diminishing cell proliferation and increasing apoptosis in stromal and epithelial cells (Fig. 3), two characteristics that are known to be dysregulated in the endometriotic lesions and the eutopic endometrium of women with endometriosis.[71–73] It is important to emphasize that the treatments began 14 days after surgery, in order to evaluate the possible effect on the growth, maintenance and regression of already established endometriotic-like lesions rather than just their establishment. This certainly reflects what actually occurs with patients, who consult a specialist once the lesions are already established.

In various *in vivo* and *in vitro* cancer models, urolithins have proven to have anti-proliferative, pro-apoptotic and anti-angiogenic activity and anti-tumor effects.[32,38,53,74,75] Moreover, Fu *et al.*[76] demonstrated that UA significantly inhibited the IL-1β-induced inflammatory response by targeting the

PI3K/Akt/NF-κB signalling pathway in osteoarthritis *in vitro* and *in vivo* models. These findings are promising since recent results from our laboratory[77] confirmed the alteration in the PI3K/AKT pathway regulation in endometriosis patients and demonstrated clear differences between the stages of endometriosis, emphasizing the importance of this pathway in the first stage of the disease.

In summary, we were able to demonstrate the effectiveness of UA and UB in the reduction in the number of endometriotic-like lesions and their size by anti-proliferative and pro-apoptotic effects, without affecting the body weight or estrous cycle. Therefore, taking into account that suppression of hormonal stimulation is one of the currently prescribed pharmacological treatments for endometriosis, our findings suggest that urolithins could be a safe treatment option because of their non-interference with cyclicity and support their use as putative compounds for the treatment of this disease. To the best of our knowledge, this is the first study to describe the inhibitory effects of these two compounds in endometriosis development. A major challenge remains in the identification of accurate doses without affecting fertility or pregnancy in reproductive aged endometriosis patients.

Author contributions

B. M. C. carried out the experimental work, analysed and critically discussed the data, and prepared the manuscript; C. O. helped in performing the experiments, discussed the data, and revised the manuscript; D. M. helped with endometriosis induction surgery, discussed the data, and revised the manuscript; A. G. R. helped with animal handling, discussed the data, and revised the manuscript; M. A. B. helped in designing the study, assisted with general animal handling, discussed the data, and revised the manuscript; R. I. B. devised and elaborated the project, and directed Bárbara Mc Cormack.

Conflicts of interest

The authors declare no conflicts of interest.

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