View Article Online
View Journal

Food & Function

Linking the chemistry and physics of food with health and nutrition

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: B. Mc Cormack, C. N. Olivares, D. Madanes, A. Ricci, M. Bilotas and I. Barañao, *Food Funct.*, 2021, DOI: 10.1039/D1FO01702K.

This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.

View Article Online
DOI: 10.1039/D1FO01702K

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

Bárbara Andrea Mc Cormack, (ORCID ID: 0000-0002-9246-5766)*[a] Carla Noemí Olivares, [b] Daniela Madanes, [a] Analía Gabriela Ricci, (ORCID ID: 0000-0002-7260-0836) [a] Mariela Bilotas, [a] Rosa Inés Barañao, [a]

a. Laboratorio de Inmunología de la Reproducción, Instituto de Biología y Medicina Experimental (IBYME-CONICET), Vuelta de Obligado 2490, Buenos Aires C1428ADN, Argentina.

b. Laboratorio de Fisiopatología Endometrial, Instituto de Biología y Medicina Experimental (IBYME-CONICET), Vuelta de Obligado 2490, Buenos Aires C1428ADN, Argentina.

Correspondence: Bárbara A. Mc Cormack. Vuelta de Obligado 2490, Buenos Aires C1428ADN, Argentina. Phone number: 011 4783-2869 E-mail: barbymccormack@gmail.com

View Article Online
DOI: 10.1039/D1FO01702K

Abstract

Endometriosis is an often painful disease of 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 like 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 2.5mg/kg/day intraperitoneal UA or UB. Mice were monitored daily, 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 mice. UA completely avoided 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 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 overall endometriotic growth without affecting the body weight or estrous cycle.

Key words: Endometriosis – Urolithins – Cell Proliferation – Apoptosis – Estrous cycle

View Article Online
DOI: 10.1039/D1FO01702K

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 ages 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 removals of hydroxyls [32]. The major end products of these metabolic reactions are the 3,8-dihydroxy-6H-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 the ellagic acid (EA) and specially its metabolites, the urolithins, exert a wide range of beneficial health effects including anti-

View Article Online
DOI: 10.1039/D1FO01702K

oxidant, anti-inflammatory, anti-estrogenic and anti-carcinogenic effects [26-31]. However, until now there is no evidence of 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 made by our research group [39, 41-43], we focused our search of 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 the 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) and xylazine (10 mg/kg). 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 immune system, we also included a group of sham animals. These animals underwent the same surgical procedure, the uterine horn was removed, but no tissue was sutured.

View Article Online
DOI: 10.1039/D1FO01702K

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 in PBS with 1% DMSO) and UB (Santa Cruz, 2.5 mg/kg in PBS with 1% DMSO). Sham animals received vehicle (PBS with 1% DMSO). All treatments were administered daily by intraperitoneal injection, during 28 consecutive days and started in post-operative day 14, time point at which the endometriotic lesions are considered to be already developed [45,46]. Mice were monitored daily.

Urolithins 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 urolithins 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 reports [51-53].

The endometriosis induction/treatment protocol was applied as follows:

Day -7: arrival of the animals to the in-house animal facility.
Day 0: endometriosis induction surgery.
Day 14: treatments began to be administered daily.
Day 42: end of the experiment / Sacrifice.

2.4 Evaluation of mice´s wellbeing

Mice were carefully observed to detect any changes in their grooming behavior, activity levels and food consumption from post-surgical day 1 up to 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., 40µl of physiological solution at room temperature was inserted into the vaginal cavity, withdrawn, and smeared on a microscope slide. Estrous cycle stages were determined according to the type, number, and morphology of cells in the smear. Diestrus index was calculated using the formula [55]:

View Article Online
DOI: 10.1039/D1FO01702K

$$Diestrus\ index = \frac{Numbers\ of\ days\ with\ clear\ diestrus\ smear}{Total\ duration\ of\ cycled\ (Days)} \times 100$$

Estrous cyclicity was evaluated from three aspects: 1) the number of cycles observed in 16 consecutive days, counting either 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 to 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 radiuses, 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, 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 streptavidin-peroxidase conjugate (Dako) and exposed with 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 X 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 condition, and the mean value per group was calculated.

2.8 TUNEL assay

For apoptosis quantification, sections were processed for in situ immune-localization of nuclei exhibiting DNA fragmentation by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique, using of In Situ Cell Death Detection Kit, POD (ROCHE). As a negative control, 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 condition, 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 t-test or nonparametric Mann Whitney were used for statistical comparisons between two groups.

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

3. Results

View Article Online
DOI: 10.1039/D1FO01702K

3.1 Effect of UA and UB on mice´s wellbeing

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 daily monitored to examine whether the treatment with urolithins leads to variations in weight. All mice gained weight during the postsurgical period, as expected in young mice. Urolithins-injected mice gained similar weight per week than control or even sham mice (Figure 1A). The average body weight of UA/UB-treated mice at 28 days after injection was also not different than controls. These results exposed that neither the disease nor the treatment generated statistically significant modifications in the body weight (Figure 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, 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 (Figure 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 (Figure 1D and 1F).

To quantify the time course of 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 (Figure 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 regarding the control or the Sham group.

3.3 Morphologic and histopathologic evaluation of the endometriotic-like lesions

View Article Online
DOI: 10.1039/D1FO01702K

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 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 (Figure 2A).

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

Figure 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 mice compared to the control group (Figure 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.

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

3.5 Effect of UB on endometriotic-like lesions cell proliferation

Cell proliferation was evaluated in histological sections of developed endometriotic-like lesions by immunolocalization of PCNA. Cell proliferation in the epithelial fraction (Figure 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

View Article Online
DOI: 10.1039/D1FO01702K

treatment significantly reduced the stromal (Figure 3B) proliferating cells compared to the Control group (UB $p<0.01$ versus Control).

Micrographs show representative histological sections of endometriotic-like lesions (Figure 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 (Figure 3D: UB $p<0.05$ versus Control for epithelial cells, Figure 3E: UB $p<0.01$ versus Control for stromal cells). Micrographs show representative histological sections of endometriotic-like lesions (Figure 3F).

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 led to focus investigations 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, in the present study we focus on urolithins A and B as the majoritarian active metabolites of EA [32, 34].

View Article Online
DOI: 10.1039/D1FO01702K

Given the potential impact of endometriosis symptoms on mental wellbeing and social functioning [63], the behavioral evaluation of mice submitted to the endometriosis model is an interesting aspect to take into account when we evaluate wellbeing. 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 disorders in the weight gain per week of the different groups (Figure 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 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, 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, estrous cycle length in mice averages 4–5 days; but occasional 6-day cycles may be observed in some individuals [67, 68]. Consequently, in this study, the cycle length averages 5-6 days (Figure 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 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 (Figure 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 does 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 (Figure 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.

View Article Online
DOI: 10.1039/D1FO01702K

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 lesions 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 there were found at least 1 of the 3 ectopic tissues implanted during 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 macroscopically 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 (Figure 2A).

In our study, we also found that both UA and UB were able to decrease the number of established lesions per mouse (Figure 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 (Figure 2C), by diminishing cell proliferation and increasing apoptosis in stromal and epithelial cells (Figure 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 stress out that the treatments began 14 days after surgery, in order to evaluate the possible effect on 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 antiproliferative, proapoptotic, antiangiogenic 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

View Article Online
DOI: 10.1039/D1FO01702K

stages of endometriosis, emphasizing the importance of this pathway in the first stage of the disease.

In summary, we were able to demonstrate UA and UB effectiveness on 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, and 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 option treatment regarding the non-interference with cyclicity and support its use as a putative compound for the treatment of this disease. To the best of our knowledge, this is the first study to denote 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 age endometriosis patients.

Author contributions

BMC, carried out experimental work, analysed and critical discussed the data, and prepared the manuscript; CO, helped to perform the experiments, discussed data, and revised the manuscript; DM, helped with endometriosis induction surgery, discussed data, and revised the manuscript; AGR, helped with animal handling, discussed data, and revised the manuscript; MAB, helped to design the study, assisted with general animal handling, discussed data, and revised the manuscript; RIB, devised and elaborated the project, and directed Bárbara Mc Cormack.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by grants from the National Agency for Promotion of Science and Technology (ANPCYT) BID-PICT 2012-1056, CONICET PIP 1122015, Fundación Instituto de Biología y Medicina Experimental, and Fundación René Barón.

5. References

1. L. C. Giudice and L. C. Kao, Endometriosis, *Lancet*, 2004, **364**, 1789.
2. B. Eskenazi and M. L. Warner, Epidemiology of endometriosis, *Obstet Gynecol Clin North Am*, 1997, **24**, 235.
3. J. Kitawaki, N. Kado, H. Ishihara, H. Koshiba, Y. Kitaoka and H. Honjo, Endometriosis: the pathophysiology as an estrogen-dependent disease, *J Steroid Biochem Mol Biol*, 2002, **83**, 149.
4. R. O. Burney and L. C. Giudice, Pathogenesis and pathophysiology of endometriosis, *Fertil Steril*, 2012, **98**, 511.
5. L. C. Giudice, Clinical practice. Endometriosis, *N Engl J Med*, 2010, **362**, 2389.
6. P. Vercellini, P. Viganò, E. Somigliana and L. Fedele, Endometriosis: pathogenesis and treatment, *Nat Rev Endocrinol*, 2014, **10**, 261.
7. S. Ferrero, F. Barra and U. Leone Roberti Maggiore, Current and Emerging Therapeutics for the Management of Endometriosis, *Drugs*, 2018, **78**, 995.
8. P. Vercellini, L. Buggio, M. P. Frattaruolo, A. Borghi, D. Dridi and E. Somigliana, Medical treatment of endometriosis-related pain, *Best Pract Res Clin Obstet Gynaecol*, 2018, **51**,68.
9. A. L. Rocha, F. M. Reis and F. Petraglia, New trends for the medical treatment of endometriosis, *Expert Opin Investig Drugs.,* 2012, **21**(7), 905
10. H. Abu Hashim, Gonadotrophin-releasing hormone analogues and endometriosis: current strategies and new insights, *Gynecol Endocrinol*, 2012, **28**, 314.
11. M. Rotondi, D. Labriola, M. Rotondi, F. P. Ammaturo, G. Amato, C. Carella, A. Izzo and S. Panariello, Depot leuprorelin acetate versus danazol in the treatment of infertile women with symptomatic endometriosis, *Eur J Gynaecol Oncol*, 2002, **23,** 523.
12. A. M. Quaas, E. A. Weedin and K. R. Hansen, On-label and off-label drug use in the treatment of endometriosis, *Fertil Steril*, 2015, **103**, 612.
13. P. Rde, V. F. do Amaral, Depressive symptoms, anxiety, and quality of life in women with pelvic endometriosis, *Eur J Obstet Gynecol Reprod Biol,* 2009, **142**, 53.

14. F. Siedentopf, N. Tariverdian, M. Rücke, H. Kentenich and P. C. Arck, Immune status, psychosocial distress and reduced quality of life in infertile patients with endometriosis, *Am J Reprod Immunol*, 2008, **60**, 449.
15. S. Z. Jia, J. H. Leng, J. H. Shi, P. R. Sun and J. H. Lang, Health-related quality of life in women with endometriosis: a systematic review, *J Ovarian Res*, 2012, **5**, 29.
16. M. C. P. Marinho, T. F. Magalhaes, L. F. C. Fernandes, K. L. Augusto, A. V. M. Brilhante and L. R. P. S. Bezerra, Quality of Life in Women with Endometriosis: An Integrative Review, *J Womens Health (Larchmt)*, 2018, **27**, 399.
17. E. Ernst, The role of complementary and alternative medicine in cancer, *Lancet Oncol*, 2000, **1**, 176.
18. F. Bina, S. Soleymani, T. Toliat, M. Hajimahmoodi, M. Tabarrai, M. Abdollahi and R. Rahimi, Plant-derived medicines for treatment of endometriosis: A comprehensive review of molecular mechanisms, *Pharmacol Res*, 2019, **139**, 76.
19. M. Mehriardestani, A. Aliahmadi, T. Toliat and R. Rahimi, Medicinal plants and their isolated compounds showing anti-Trichomonas vaginalis- activity, *Biomed Pharmacother*, 2017, **88**, 885.
20. M. Mobli, M. Qaraaty, G. Amin, I. Haririan, M. Hajimahmoodi and R. Rahimi, Scientific evaluation of medicinal plants used for the treatment of abnormal uterine bleeding by Avicenna, *Arch Gynecol Obstet*, 2015, **292**, 21.
21. T. N. Chinembiri, L. H. du Plessis, M. Gerber, J. H. Hamman and J. du Plessis, Review of natural compounds for potential skin cancer treatment, *Molecules*, 2014, **19**, 11679.
22. R. H. Liu, Potential synergy of phytochemicals in cancer prevention: mechanism of action, *J Nutr*, 2004, **134**, 3479S.
23. M. A. Moga, O. G. Dimienescu, C. A. Arvatescu, A. Mironescu, L. Dracea and L. Ples, The Role of Natural Polyphenols in the Prevention and Treatment of Cervical Cancer-An Overview, *Molecules*, 2016, **21**, 1055.
24. P. Ovadje, A. Roma, M. Steckle, L. Nicoletti, J. T. Arnason and S. Pandey, Advances in the research and development of natural health products as main stream cancer therapeutics, *Evid Based Complement Alternat Med*, 2015, **2015**, 751348.

25. R. Varma, T. Rollason, J. K. Gupta and E. R. Maher, Endometriosis and the neoplastic process, *Reproduction*, 2004, **127**, 293.
26. I. Kang, T. Buckner, N. F. Shay, L. Gu and S. Chung, Improvements in Metabolic Health with Consumption of Ellagic Acid and Subsequent Conversion into Urolithins: Evidence and Mechanisms, *Adv Nutr*, 2016, **7,** 961.
27. D. Gramec Skledar, T. Tomašič, M. Sollner Dolenc, L. Peterlin Mašič and A. Zega, Evaluation of endocrine activities of ellagic acid and urolithins using reporter gene assays, *Chemosphere*, 2019, **220,** 706.
28. L. Mele, P. Mena, A. Piemontese, V. Marino, N. López-Gutiérrez, F. Bernini, I. Zanotti and D. Del Rio, Antiatherogenic effects of ellagic acid and urolithins in vitro, *Arch Biochem Biophys*, 2016, **599**, 42.
29. R. Vicinanza, Y. Zhang, S. M. Henning and D. Heber, Pomegranate Juice Metabolites, Ellagic Acid and Urolithin A, Synergistically Inhibit Androgen-Independent Prostate Cancer Cell Growth via Distinct Effects on Cell Cycle Control and Apoptosis, *Evid Based Complement Alternat Med*, 2013, **2013**, 247504.
30. L. S. Adams, Y. Zhang, N. P. Seeram, D. Heber and S. Chen, Pomegranate ellagitannin-derived compounds exhibit antiproliferative and antiaromatase activity in breast cancer cells in vitro, *Cancer Prev Res (Phila)*, 2010, **3**, 108.
31. H. S. Aiyer and R. C. Gupta, Berries and ellagic acid prevent estrogen-induced mammary tumorigenesis by modulating enzymes of estrogen metabolism, *Cancer Prev Res (Phila)*, 2010, **3**, 727.
32. J. C. Espín, M. Larrosa, M. T. García-Conesa and F. Tomás-Barberán, Biological significance of urolithins, the gut microbial ellagic Acid-derived metabolites: the evidence so far, *Evid Based Complement Alternat Med*, 2013, **2013,** 270418.
33. F. A. Tomás-Barberán, A. González-Sarrías, R. García-Villalba, M. A. Núñez-Sánchez, M. V. Selma, M. T. García-Conesa and J. C. Espín, Urolithins, the rescue of "old" metabolites to understand a "new" concept: Metabotypes as a nexus among phenolic metabolism, microbiota dysbiosis, and host health status, *Mol Nutr Food Res*., 2017, **61** (1), 10.1002/mnfr.201500901.
34. J. M. Landete, Ellagitannins, ellagic acid and their derived metabolites: A review about source, metabolism, functions and health, *Food Res Int*, 2011, **44**, 1150.

35. J. L. Ríos, R. Giner, M. Marín and M. Recio, A Pharmacological Update of Ellagic Acid, *Planta Medica*, 2018, **84**(15), 1068.
36. B. Cerdá, P. Periago, J. C. Espín and F. A. Tomás-Barberán, Identification of urolithin a as a metabolite produced by human colon microflora from ellagic acid and related compounds, *J Agric Food Chem*, 2005, **53**, 5571.
37. D. Heber, Multitargeted therapy of cancer by ellagitannins, *Cancer Lett*, 2008, **269**, 262.
38. A. González-Sarrías, M. A. Núñez-Sánchez, J. Tomé-Carneiro, F. A. Tomás-Barberán, M. T. García-Conesa and J. C. Espín, Comprehensive characterization of the effects of ellagic acid and urolithins on colorectal cancer and key-associated molecular hallmarks: MicroRNA cell specific induction of CDKN1A (p21) as a common mechanism involved, *Mol Nutr Food Res*, 2016, **60**, 701.
39. B. Mc Cormack, N. Maenhoudt, V. Fincke, A. Stejskalova, B. Greve, L. Kiesel, G. F. Meresman, H. Vankelecom, M. Götte and R. I. Barañao, The ellagic acid metabolites urolithin A and B differentially affect growth, adhesion, motility, and invasion of endometriotic cells in vitro, *Hum Reprod.*, 2021, deab053.
40. F. Barra, C. Scala, V. Mais, S. Guerriero and S. Ferrero, Investigational drugs for the treatment of endometriosis, an update on recent developments, *Expert Opin Investig Drugs*, 2018, **27**, 445.
41. B. A. Mc Cormack, M. A. Bilotas, D. Madanes, A. G. Ricci, J. J. Singla and R. I. Barañao, Potential use of ellagic acid for endometriosis treatment: its effect on a human endometrial cell cycle, adhesion and migration, *Food Funct*, 2020, **11**, 4605.
42. L. Ferella, J. I. Bastón, M. A. Bilotas, J. J. Singla, A. M. González, C. N. Olivares and G. F. Meresman, Active compounds present in Rosmarinus officinalis leaves and Scutellaria baicalensis root evaluated as new therapeutic agents for endometriosis, *Reprod Biomed Online*, 2018, **37**, 769.
43. A. G. Ricci, C. N. Olivares, M. A. Bilotas, J. I. Bastón, J. J. Singla, G. F. Meresman and R. I. Barañao, Natural therapies assessment for the treatment of endometriosis, *Hum Reprod,* 2013, **28**, 178.
44. M. Bilotas, G. Meresman, I. Stella, C. Sueldo and R. I. Barañao, Effect of aromatase inhibitors on ectopic endometrial growth and peritoneal environment in a mouse model of endometriosis, *Fertil Steril*, 2010, **93**, 2513.

45. M. Nisolle, F. Casanas-Roux and J. Donnez, Early-stage endometriosis: adhesion and growth of human menstrual endometrium in nude mice, *Fertil Steril*, 2000, **74**, 306.
46. G. S. Ersoy, M. M. Zolbin, E. Cosar, R. Mamillapalli and H. S. Taylor, Medical Therapies for Endometriosis Differentially Inhibit Stem Cell Recruitment, *Reprod Sci*., 2017, **24**(6), 818.
47. M. V. Selma, D. Beltrán, R. García-Villalba, J. C. Espín and F. A. Tomás-Barberán, Description of urolithin production capacity from ellagic acid of two human intestinal Gordonibacter species, *Food Funct*, 2014, **5**, 1779.
48. P. Gaya, A. Peirotén, M. Medinaa, I. Álvarez and J. M. Landete, *Bifidobacterium pseudocatenulatum* INIA P815: The first bacterium able to produce urolithins A and B from ellagic acid, *J Funct Foods*, 2018, **45**, 95.
49. F. A. Tomás-Barberán, R. García-Villalba, A. González-Sarrías, M. V. Selma and J. C. Espín, Ellagic acid metabolism by human gut microbiota: consistent observation of three urolithin phenotypes in intervention trials, independent of food source, age, and health status, *J Agric Food Chem*, 2014, **62**, 6535.
50. A. Cortés-Martín, M. V. Selma, F. A. Tomás-Barberán, A. González-Sarrías and J. C. Espín, Where to Look into the Puzzle of Polyphenols and Health? The Postbiotics and Gut Microbiota Associated with Human Metabotypes, *Mol Nutr Food Res*, 2020, **64** (9), e1900952.
51. M. Savi, L. Bocchi, P. Mena, M. Dall'Asta, A. Crozier, F. Brighenti, D. Stilli and D. Del Rio, In vivo administration of urolithin A and B prevents the occurrence of cardiac dysfunction in streptozotocin-induced diabetic rats, *Cardiovasc Diabetol*, 2017, **16**, 80.
52. G. Lee, J. S. Park, E. J. Lee, J. H. Ahn and H. S. Kim, Anti-inflammatory and antioxidant mechanisms of urolithin B in activated microglia, *Phytomedicine*, 2019, **55**, 50.
53. M. Larrosa, A. González-Sarrías, M. J. Yáñez-Gascón, M. V. Selma, M. Azorín-Ortuño, S. Toti, F. Tomás-Barberán, P. Dolara and J. C. Espín, Anti-inflammatory properties of a pomegranate extract and its metabolite urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism, *J Nutr Biochem*, 2010, **21,** 717.
54. S. L. Byers, M. V. Wiles, S. L. Dunn and R. A. Taft, Mouse estrous cycle identification tool and images, *PLoS One*, 2012, **7** (4), e35538.

55. T. Auta and A. T. Hassan, Alteration in oestrus cycle and implantation in Mus musculus administered aqueous wood ash extract of Azadirachta indica (neem), *Asian Pac J Reprod*, 2016, **5**, 188.
56. F. Quereda, J. Barroso and P. Acién, Individual and combined effects of triptoreline and gestrinone on experimental endometriosis in rats, *Eur J Obstet Gynecol Reprod Biol*, 1996, **67**, 35.
57. A. G. Ricci, C. N. Olivares, M. A. Bilotas, G. F. Meresman and R. I. Barañao, Effect of vascular endothelial growth factor inhibition on endometrial implant development in a murine model of endometriosis, *Reprod Sci*, 2011, **18**, 614.
58. C. Olivares, A. Ricci, M. Bilotas, R. I. Barañao and G. Meresman, The inhibitory effect of celecoxib and rosiglitazone on experimental endometriosis, *Fertil Steril*, 2011, **96**, 428.
59. D. de Ziegler, B. Borghese and C. Chapron, Endometriosis and infertility: pathophysiology and management, *Lancet.*, 2010, **376** (9742), 730
60. A. Crozier, I. B. Jaganath and M. N. Clifford, Dietary phenolics: chemistry, bioavailability and effects on health, *Nat Prod Rep*, 2009, **26**, 1001.
61. L. Della Corte, M. Noventa, M. Ciebiera, M. Magliarditi, Z. Sleiman, E. Karaman, U. Catena, C. Salvaggio, G. Falzone and S. Garzon, Phytotherapy in endometriosis: an up-to-date review, *J Complement Integr Med.*, 2020, **17**(3), 10.1515/jcim-2019-0084.
62. L. Marín, E. M. Miguélez, C. J. Villar and F. Lombó, Bioavailability of dietary polyphenols and gut microbiota metabolism: antimicrobial properties, *Biomed Res Int*, 2015, **2015,** 905215.
63. L. Culley, C. Law, N. Hudson, E. Denny, H. Mitchell, M. Baumgarten and N. Raine-Fenning, The social and psychological impact of endometriosis on women's lives: a critical narrative review, *Hum Reprod Update,* 2013, **19**(6), 625.
64. P. W. L. L. Filho, A. J. M. Chaves Filho, C. F. X. Vieira, T. Q. Oliveira, M. V. R. Soares, P. M. Jucá, J. Quevedo, T. Barichello, D. Macedo and F. das Chagas Medeiros, Peritoneal endometriosis induces time-related depressive- and anxiety-like alterations in female rats: involvement of hippocampal pro-oxidative and BDNF alterations, *Metab Brain Dis*, 2019, **34**(3), 909.
65. A. F. Ajayi and R. E. Akhigbe, Staging of the estrous cycle and induction of estrus in experimental rodents: an update, *Fertil Res Pract*, 2020, **6**, 5.

66. R. L. Cooper and J. M. Goldman, Vaginal cytology. (In: Daston G, Kimmel C, eds.) An evaluation and interpretation of reproductive endpoints for human health risk assessment, *Washington DC: ILSI Pres*, 1999, 42.
67. J. M. Goldman, A. S. Murr and R. L. Cooper, The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies, *Birth Defects Res B Dev Reprod Toxicol*, 2007, **80**, 84.
68. M. C. Cora, L. Kooistra and G. Travlos, Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears, *Toxicol Pathol*, 2015, **43**, 776.
69. G. Kizilay, Y. H. Uz, G. Seren, E. Ulucam, A. Yilmaz, Z. Cukur and U. A. Kayisli. In vivo effects of curcumin and deferoxamine in experimental endometriosis, *Adv Clin Exp Med,* 2017, **26(2)**, 207.
70. J. Nogueira Neto, O. J. Torres, T. M. Coelho, J. N. Jr Nunes, G. C. Aguiar and L. K. Costa, Evaluation of the macroscopic growth degree of experimental endometriosis in rats, *Acta Cir Bras*, 2007, **22,** 8.
71. S. H. Ahn, K. Khalaj, S. L. Young, B. A. Lessey, M. Koti and C. Tayade, Immune-inflammation gene signatures in endometriosis patients, *Fertil Steril*, 2016, **106,** 1420.
72. K.L. Sharpe-Timms, Endometrial anomalies in women with endometriosis, *Ann N Y Acad Sci*, 2001, **943**, 131.
73. G. F. Meresman, S. Vighi, R. A. Buquet, O. Contreras-Ortiz, M. Tesone and L. S. Rumi, Apoptosis and expression of Bcl-2 and Bax in eutopic endometrium from women with endometriosis, *Fertil Steril*, 2000, **74**, 760.
74. D. Ryu, L. Mouchiroud, P. A. Andreux, E. Katsyuba, N. Moullan, A. A. Nicolet-Dit-Félix, E. G. Williams, P. Jha, G. Lo Sasso, D. Huzard, P. Aebischer, C. Sandi, C. Rinsch and J. Auwerx, Urolithin A induces mitophagy and prolongs lifespan in C. elegans and increases muscle function in rodents, *Nat Med*, 2016, **22**, 879.
75. S. G. Kasimsetty, D. Bialonska, M. K. Reddy, G. Ma, S. I. Khan and D. Ferreira, Colon cancer chemopreventive activities of pomegranate ellagitannins and urolithins, *J Agric Food Chem*, 2010, **58**, 2180.
76. X. Fu, L. F. Gong, Y. F. Wu, Z. Lin, B. J. Jiang, L. Wu and K. H. Yu, Urolithin A targets the PI3K/Akt/NF-κB pathways and prevents IL-1β-induced

620 inflammatory response in human osteoarthritis: in vitro and in vivo studies,
621 *Food Funct*, 2019, **10**, 6135.
622 77. D. Madanes, M. A. Bilotas, J. I. Bastón, J. J. Singla, G. F. Meresman, R. I.
623 Barañao and A. G. Ricci, PI3K/AKT pathway is altered in the endometriosis
624 patient's endometrium and presents differences according to severity stage,
625 *Gynecol Endocrinol*, 2020, **36**, 436.

Figure 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 mice body weight throughout the treatment. Estrous cycle (P: proestrus, E: estrus and D: diestrus) of all the animals were 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) estrous cycle total duration; (F) time spent in each stage of the estrous cycle.
Results are expressed as a mean ± SEM. N expressed in parenthesis in the graphs.

Figure 2: Endometriotic-like lesions development. After 28 days of treatment the animals were sacrificed and the peritoneal cavity was examined. Representative images of endometriotic-like lesions: Control (A) and UB (B) groups (UA image is not shown since only one lesion was found). Magnification 400x. (C) Percentage of lesions developed per mice and (D) 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 expressed in parenthesis in the graphs.

Figure 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 400x. Apoptosis within the implants was evaluated by 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 400x.
Results are expressed as mean ± SEM. **p<0.01 and ***p<0.001 with respect to the Control group. N expressed in parenthesis in each bar.

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

View Article Online
DOI: 10.1039/D1FO01702K

Table 1

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)

View Article Online
DOI: 10.1039/D1FO01702K

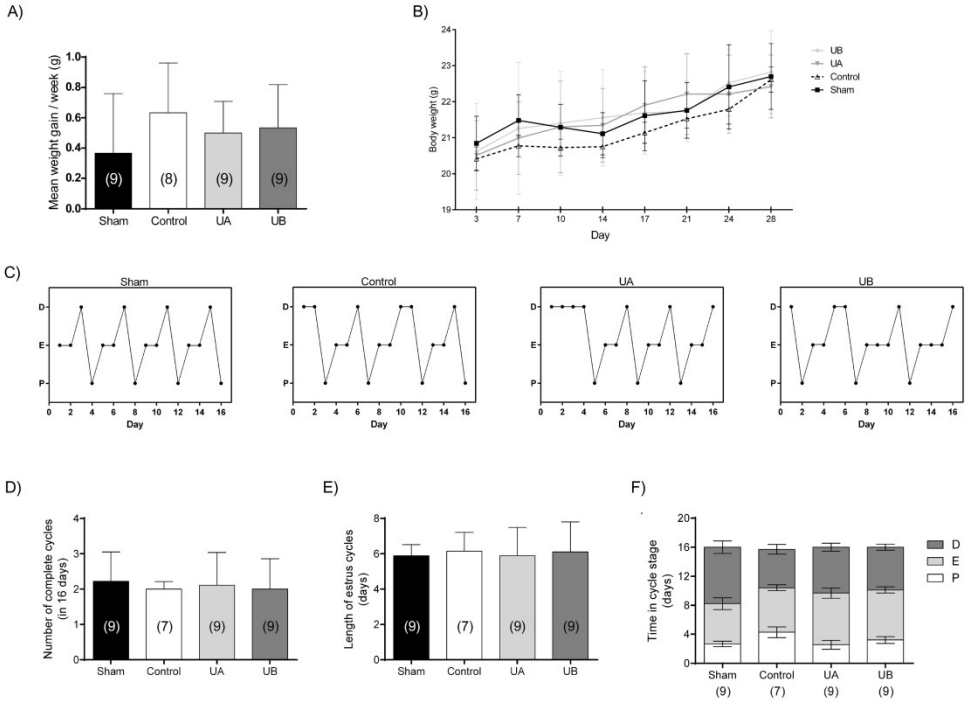

Figure 1: Weight variations and estrous cyclicity of mice. All mice were weighed twice a week and the mean per week was calculated. (A) Mean weight gain per week per group. (B) Progression of mice body weight throughout the treatment. Estrous cycle (P: proestrus, E: estrus and D: diestrus) of all the animals were 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) estrous cycle total duration; (F) time spent in each stage of the estrous cycle.
Results are expressed as a mean ± SEM. N expressed in parenthesis in the graphs.

158x115mm (600 x 600 DPI)

View Article Online
DOI: 10.1039/D1FO01702K

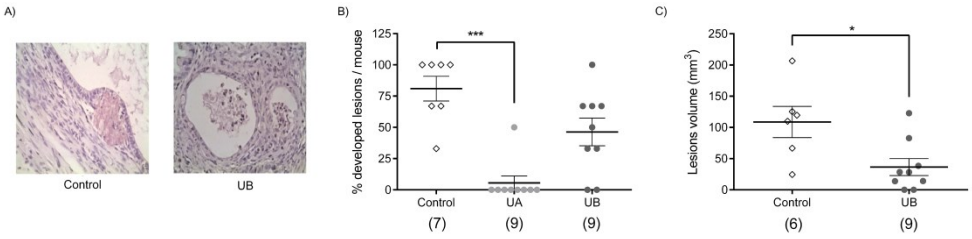

Figure 2: Endometriotic-like lesions development. After 28 days of treatment the animals were sacrificed and the peritoneal cavity was examined. Representative images of endometriotic-like lesions: Control (A) and UB (B) groups (UA image is not shown since only one lesion was found). Magnification 400x. (C) Percentage of lesions developed per mice and (D) 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 expressed in parenthesis in the graphs.

1530x389mm (120 x 120 DPI)

View Article Online
DOI: 10.1039/D1FO01702K

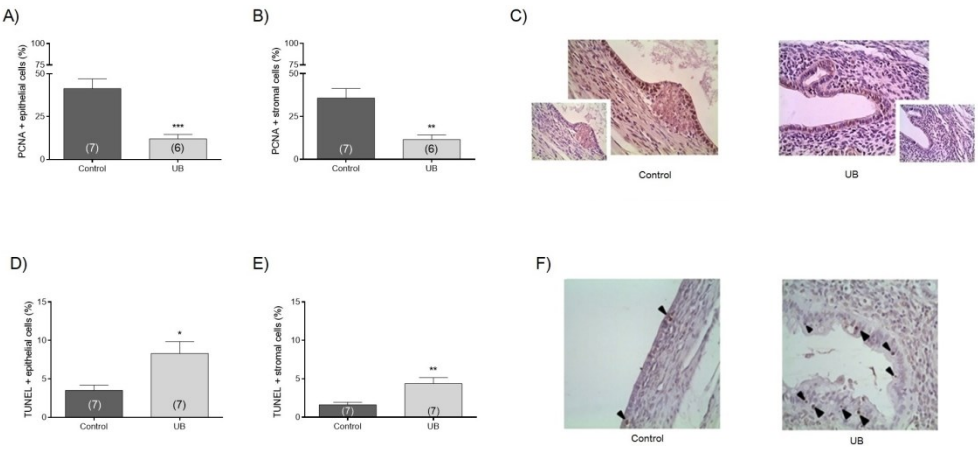

Figure 3: Immuno-histochemical assessment of proliferation and apoptosis on endometriotic-like lesions. After 28 days of treatment the developed lesions were removed and fixed. Cell proliferation within the implants was evaluated by immunohistochemistry of PCNA. The percentage of PCNA+ (A) epithelial 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 400x. Apoptosis within the implants was evaluated by 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 400x.
Results are expressed as mean ± SEM. **p<0.01 and ***p<0.001 with respect to the Control group. N expressed in parenthesis in each bar.

416x195mm (120 x 120 DPI)