Research Paper

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Ultramicronized palmitoylethanolamide reduces viscerovisceral hyperalgesia in a rat model of endometriosis plus ureteral calculosis: role of mast cells

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Abstract

The effects of ultramicronized palmitoylethanolamide were evaluated on pain behaviours and markers of mast cell (MC) activity in a rat model of endometriosis plus ureteral calculosis (ENDO+STONE)–induced viscerovisceral hyperalgesia (VVH). Female Sprague-Dawley rats that underwent surgical induction of endometriosis were randomly assigned to receive active (ultramicronized palmitoylethanolamide 10 mg·kg^{-1}·d^{-1}, orally) or placebo treatment for 25 days. At day 21, they underwent ureteral stone formation and were video-recorded till day 25 to evaluate ureteral and uterine pain behaviours. At autopsy (day 25), ureteral condition and number and diameter of endometrial cysts were evaluated. The following were then measured: number and percentage of degranulating MCs, number of vessels, chymase, nerve growth factor (NGF), vascular endothelial growth factor (VEGF), and Flk-1 (VEGF receptor) in cysts, and NGF in dorsal root ganglia (DRG). Ultramicronized palmitoylethanolamide–treated vs placebo-treated rats showed significantly lower number, duration and complexity of ureteral crises, shorter duration of uterine pain, and smaller cyst diameter ($0.0001 < P < 0.004$); a significantly higher percentage of expelled stones ($P < 0.0001$); significantly lower MC number ($P < 0.01$), vessel number ($P < 0.01$), chymase ($P < 0.05$), NGF ($P < 0.05$), VEGF ($P < 0.01$), and Flk-1 ($P < 0.01$) expression in cysts and NGF expression in DRG ($P < 0.01$). In all animals, the global duration of ureteral crises correlated linearly and directly with cyst diameter, MC number and chymase in cysts, and NGF in cysts and DRG ($0.02 < P < 0.0002$). Ultramicronized palmitoylethanolamide significantly reduces VVH from ENDO+STONE, probably by modulating MC expression/activity in cysts, thus reducing central sensitization due to noxious signals from endometriotic lesions. The results suggest potential utility of the compound for VVH in clinics.

Keywords: Ultramicronized palmitoylethanolamide, Endometriosis, Ureteral calculosis, Viscerovisceral hyperalgesia, Rat, Visceral pain, Mast cells

1. Introduction

Endometriosis (ENDO) affects over 10% of women in their reproductive years, causing sub/infertility and, frequently, pelvic pain. Deep infiltrating endometriosis is the most painful form, while ovarian ENDO cysts are generally poorly symptomatic.[6,22,29,33,34,38,60,64,67] Even when asymptomatic for pain from the reproductive organs, however, endometriosis can enhance pain from other pelvic viscera with partially overlapping sensory innervation. Women with "silent endometriosis" plus ureteral calculosis (ENDO+STONE), in fact, show enhanced urinary pain, a phenomenon known as viscerovisceral hyperalgesia (VVH), where noxious inputs from the endometriotic lesions probably sensitize neurons also receiving sensory input from the urinary tract, thus facilitating the triggering of urinary pain.[27,28] Our group set up a rat model of VVH from silent endometriosis plus artificial ureteral calculosis, where the rats show enhanced visceral pain behaviour.[26,44] Preventive ketoprofen treatment of ENDO lesions (secretory cysts) in this model reduces cyst diameter and prevents post-stone VVH, which confirms that reducing nociceptive inputs from the cysts is key to preventing/decreasing pain in this condition.[26] Endometriosis treatment with nonsteroidal anti-inflammatory drugs in humans, however, remains problematic because clinically significant results would require repeated/prolonged administration, something that is not exempt from side effects.[23,70] An imperative need therefore exists for alternative more mechanism-based treatments to control the extreme symptoms of VVH from endometriosis. Palmitoylethanolamide (PEA), an endogenous fatty acid amide, is emerging as an innovative therapeutic approach to chronic inflammation associated with pain.[47] It downmodulates mast cell (MC) activation and controls glial cell behaviours and angiogenetic processes alongside inflammatory reactions, thus

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having potential effectiveness in VVH from endometriosis.[2,10,16,17,19,21,46,49,53,54,56,57,62] Recent experimental studies, indeed, have highlighted the importance of MC activity in the development and algogenic capacity of endometriosis.[35] Stem cell factor, the major growth differentiation and chemoattractant factor for MCs, is elevated in the peritoneal fluid of ENDO patients.[52] Degranulating MCs (releasing several mediators, including nerve growth factor [NGF]) have also been found in deep infiltrating ENDO lesions, proximal to nerves, suggesting that MCs may contribute to ENDO pain by a direct effect on nerve structures.[3,5,9,39,65,68] Mast cells contribute to neoangiogenesis, another ENDO feature, which guarantees oxygen supply to lesions. The expression of vascular endothelial growth factor (VEGF), the most potent proangiogenic factor, in fact, is increased in human ENDO samples, and VEGF may be released by endometriotic and inflammatory/immune cells, including MCs.[13,20,32,42,43,48,51,55,58,66]

Palmitoylethanolamide, exogenously administered, has shown anti-inflammatory and analgesic effects in experimental models of chronic inflammation and of acute and chronic neuropathic pain, and in several human pathological pain conditions,[12,24,61] with a higher efficacy displayed by the oral ultramicronized form in a rat model of inflammatory pain.[36] Analgesic effects of micronized/ultramicronized PEA for pelvic pain are also suggested by clinical pilot studies.[11,31,37] No research has tested the compound in VVH from endometriosis. Controlled studies in patients would be problematic, due to high variability of the clinical parameters. Our aim was therefore to assess the effects of ultramicronized PEA in standardized conditions in the ENDO+STONE animal model on behavioural indicators of VVH in parallel with evaluation of ENDO morphological and biochemical parameters related to MC expression.

2. Materials and methods

2.1. Animals

Female Sprague-Dawley rats (weight: 220-240 g) were used for the study.

2.2. Experimental protocol

The experiments adhered to the guidelines of the Committee for Research and Ethical Issues of IASP, and the protocol was approved by the Ethics Committee for Animal Studies of the "G. D'Annunzio" University of Chieti (46/2012).

2.2.1. Main experiment: effects of ultramicronized palmitoylethanolamide on ENDO+STONE

The main experiment was conducted in rats with ENDO+STONE to assess the effects of ultramicronized PEA vs placebo on pain behaviour and on morphological and biochemical parameters of MC activation. The ultramicronization process reduces large drug crystals down to the submicron range (0.6–6.0 μm ultramicronized PEA vs 100–700 μm naïve PEA) and also yields a different crystalline structure increasing energy content; this enhances diffusion and distribution of the orally administered compound.[36]

A total of 60 rats were used for this experiment (30 treated with placebo and 30 with ultramicronized PEA); pain behaviour was recorded in all of them, while morphological parameters (number of MCs and their percentage of degranulation, vessel number in cysts) were assessed in half of the sample (15 placebo and 15 ultramicronized PEA) and biochemical parameters (Western blot analysis: chymase, VEGF, NGF, Flk-1 in cysts) were assessed in the remaining half (15 placebo and 15 ultramicronized PEA). Nerve growth factor also was assessed in the dorsal root ganglia (DRG) in 18 of the rats (9 placebo and 9 ultramicronized PEA) used for the biochemical parameter evaluation in cysts (see details below).

Sixty rats thus underwent induction of experimental endometriosis and were subsequently randomly assigned to 1 of 2 groups of 30 rats each, to undergo one of the following treatments:

(1) Ultramicronized palmitoylethanolamide, in the dose of 10 $mg \cdot kg^{-1} \cdot d^{-1}$, resuspended in 1.5% carboxycellulose wt/vol in saline;
(2) Placebo, ie, 1.5% carboxycellulose wt/vol in saline in an equivalent volume.

The ultramicronized PEA dose used was chosen based on previous data from the literature about PEA effects on pain behaviour in other animal models[36] and on the results of preliminary experiments with different doses of ultramicronized PEA in small groups of ENDO+STONE rats (**Table 1**). These preliminary experiments, although not showing a dose–response effect for ultramicronized PEA, clearly evidenced a marked efficacy of the 10 mg/kg dose, which therefore was selected.

The treatment was administered orally once a day for the whole experimental period; the first administration was delivered on the day of endometriosis induction (5 hours after the start of the intervention, ie, 2 PM), the last on the 25th day post-endometriosis. The timing of the daily administration (except the first day) was always 9 AM.

On the 21st day, post-endometriosis, all animals underwent stone formation in the left ureter (start of intervention soon after treatment administration). For the subsequent 4 postoperative days (until the 25th day post-endometriosis, ie, fourth day post-stone), all rats (30 placebo and 30 ultramicronized PEA) were video-recorded 24 hours a day to evaluate their spontaneous behaviour, indicative of both ureteral and uterine pain.

In the evening of the 25th day post-endometriosis (fourth day post-stone formation), all animals were killed using CO_2 and an autopsy was performed to evaluate the condition of the urinary tract and the status of the endometrial cysts (to count their number, and measure their diameter). The endometrial cysts and

Table 1

Preliminary experiment for dose selection.

Parameters	Placebo	PEA 2.5	PEA 5	PEA 10
Number of ureteral crises ($P < 0.03$), n	14.67 ± 2.75	13.83 ± 2.17	13.33 ± 2.86	4.67 ± 1.52*
Global duration of ureteral crises ($P < 0.003$), min	115.67 ± 16.70	104.17 ± 22.17	97.81 ± 20.40	14.83 ± 4.34**
Mean complexity of ureteral crises ($P < 0.04$), au	1.56 ± 0.05	1.56 ± 0.08	1.47 ± 0.12	1.22 ± 0.09
Global duration of uterine pain ($P < 0.04$), min	104.98 ± 23.28	97.40 ± 18.75	88.83 ± 14.52	32.76 ± 8.66*
Number of cysts (n.s.), n	3.83 ± 0.62	3.87 ± 0.48	3.62 ± 0.32	3.33 ± 0.67
Diameter of cysts ($P < 0.04$), mm	3.68 ± 0.46	3.57 ± 0.31	3.31 ± 0.26	2.43 ± 0.21

Ureteral and uterine pain behaviours and cyst parameters in rats with ENDO+STONE treated with different doses of ultramicronized PEA (2.5, 5, or 10 $mg \cdot kg^{-1} \cdot d^{-1}$) and placebo, orally, for 25 d, from the day of endometriosis induction till the fourth day subsequent to stone induction (performed on day 21); mean ± SEM (n = 6 rats per group). *P* values refer to 1-way ANOVA: significant trend for all parameters.
*$P < 0.05$; **$P < 0.01$, asterisks refer to comparison of ultramicronized PEA 10 $mg \cdot kg^{-1} \cdot d^{-1}$ with placebo (post hoc tests).
ANOVA, analysis of variance; ENDO+STONE, endometriosis plus ureteral calculosis; n.s., nonsignificant; PEA, ultramicronized palmitoylethanolamide.

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spinal cord (T11–L2 segments) were subsequently removed. For both the placebo and ultramicronized PEA groups, soon after the explant, the samples were immediately either stored in 4% formaldehyde or frozen in liquid nitrogen and subsequently stored at −80°C. Samples of endometrial cysts were used to perform morphological analyses (15 rats of the placebo group and 15 of the ultramicronized PEA group) and biochemical analyses (the remaining 15 rats of the placebo group and 15 of the ultramicronized PEA group). In particular, MC number and percentage of degranulation and vessel number in the cysts were evaluated as described in the Staining techniques section. The expression in the tissues of the proangiogenic mediator, VEGF, and its receptor Flk-1, was evaluated, together with the assessment of the neurotrophin NGF. Furthermore, expression of the specific MC marker chymase was evaluated.

2.2.2. Secondary experiment: effects of ultramicronized palmitoylethanolamide on STONE-only

The secondary experiment was conducted in rats undergoing formation of artificial ureteral calculosis only, to assess any direct effect of ultramicronized PEA on ureteral pain behaviour and stone expulsion in the absence of endometriosis.[30] Twenty Sprague-Dawley rats (220-240 g) were randomly assigned to 1 of 2 groups of 10 animals each, to undergo one of the following treatments:

(1) Ultramicronized palmitoylethanolamide, in the dose of 10 $mg \cdot kg^{-1} \cdot d^{-1}$, resuspended in 1.5% carboxycellulose wt/vol in saline;
(2) Placebo, ie, 1.5% carboxycellulose wt/vol in saline in an equivalent volume.

The treatment was administered orally once a day for 25 days. On the 21st day, all rats underwent stone formation in the left ureter. The timing of the daily administration was always 9 AM. From the 21st till the 25th day after the start of treatment (ie, for 4 days post-stone implantation), they were video-recorded 24 hours a day to evaluate their spontaneous behaviour indicative of ureteral pain.

In the evening of the 25th day, all rats were killed using CO_2 and an autopsy was performed to evaluate the condition of the urinary tract.

2.3. Surgical induction of endometriosis

A previously established model of endometriosis that reduces fertility and produces vaginal hyperalgesia was used.[7,50] The animals were anaesthetized with pentobarbital (50 mg/kg, intraperitoneally [i.p.]), the uterus was exposed through a midline abdominal incision, and a 1-cm segment of the right uterine horn was removed. Five pieces of endometrium were cut from this segment and were sewn around small vessels in various structures using nylon suture, ie, 3 pieces on alternate cascade mesenteric arteries that supply the caudal small intestine, one on the internal lower abdominal wall, on the right side, and one on the left ovary.[26,44]

2.4. Ureteral stone implantation

Under pentobarbital anaesthesia (50 mg/kg, i.p.), the left ureter was approached by means of laparotomy and a 0.02 mL bolus of dental cement (DuraLay, Dental Mfg. Co) was injected, while still fluid, into the upper third of the lumen, using a syringe with a 0.4 mm diameter needle according to a technique already described in detail elsewhere.[30]

2.5. Quantification of visceral pain behaviours

After surgery, each rat was placed in an individual plexiglass cage with free access to food and water. As reported above, for 4 postoperative days starting immediately after stone implantation, all rats underwent 24 hour a day videotape recording with a time-lapse system, with ultrared lighting for filming during the dark phase (8 PM to 8 AM). The analysis of the whole period of recording (performed by observers blinded to the rat's experimental group) allowed the evaluation of spontaneous pain behaviours.

Two types of pain behaviours were counted in ENDO+STONE rats: "ureteral pain crises" and "uterine pain behaviours," as characterized in previous studies.[26,30,44] A ureteral pain crisis consists of a sequence of at least 3 pain behaviours (of 6 possible) within a period of minimum 2-minute duration. The 6 possible behaviours are "humpbacked" position, licking of the lower abdomen and/or left flank, contraction of the left oblique musculature with inward moving of the ipsilateral hindlimb ("inward"), stretching of the body with raised abdomen ("stretch-stone"), squashing of the lower abdomen against the floor ("squash-stone"), and "supine" position with left hindlimb adducted and compressed against the abdomen. Complexity of each crisis is estimated using a 4-point arbitrary scale: 3 movements are scored 1, 4 movements are scored 2, 5 movements are scored 3, and, finally, all 6 movements are scored 4. For each rat, relative to the whole period of recording, the ureteral crises are characterized in terms of number, global duration (sum of duration of all crises), and complexity.

Uterine pain behaviours occur between ureteral crises; they are called uterine because they resemble the behaviours occurring in a model of experimental uterine inflammation.[69] These consist of 4 positions: "lambda" position (the rat suddenly hunches its back upwards into a sharp angle to form a triangular shape relative to the floor), "alpha" position (rat with abdomen adherent to the floor and nose curving towards the tail of the affected side), "stretch-flat" position, with stretching of the body with abdomen adherent to the floor, and "squash-pelvic" position (squashing of the lower part of the abdomen to the floor while in a standing or sitting position). For each rat, relative to the whole period of recording, the uterine pain behaviours are characterized in terms of global duration of uterine positions (sum of duration of all positions).[26,30,44]

In rats with STONE-only, which present exclusively ureteral pain behaviour, only this type of behaviour was quantified.

2.6. Evaluation of endometrial cysts and stone expulsion

At autopsy performed on the 25th day post-endometriosis (fourth day post-stone formation), the following were evaluated in each rat: number and diameter of endometrial cysts and status of the urinary tract, ie, stone absent (expelled) or present (retained).

2.7. Staining techniques

The endometrial cysts were collected on the 25th day after the surgical procedure from 15 rats of the placebo group and 15 of the ultramicronized PEA group and immediately fixed in 4% neutral buffered formalin and routinely processed for histology. Serial sections (7 μm) of paraffin-embedded tissues were cut. For each cyst, 20 sections were cut and used for the staining procedures. For histological evaluation of number and degranulation of MCs, the sections were stained according to the routine procedure with toluidine blue. For determination of vessel number, the sections were stained with haematoxylin and eosin.[15]

2.8. Assessment of density of mast cells

For each cyst, 10 sections were deparaffinized in 2 changes of xylene and hydrated through 2 changes of alcohol, 5 minutes in

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each solution. Then, the sections were kept in water for 5 minutes. The sections were then placed in a coupling jar containing toluidine blue stain for 30 minutes and then blotted carefully. They were then placed in absolute alcohol for 1 minute, cleared in xylene, and mounted on the slide using Entellan. The granules of MCs were stained purple, and the rest of the section was stained blue. The MC count was carried out on each slide using a 10× objective. Density was assessed by counting the MCs in the total section, divided by the area of tissue. The percentage of degranulation was obtained by counting the degranulated MCs at a high magnification (×400), divided by the total MC number. Quantitative evaluations were performed by a researcher blinded to the origin of the material.

2.9. Assessment of the number of vessels

For each cyst, 10 sections were deparaffinized and stained according to the routine procedure with haematoxylin and eosin. The number of vessels was determined in the total section. The results were expressed as total vessels per tissue area. Quantitative evaluations were performed by a researcher blinded to the experiment.

2.10. Western blot analysis

Western blot analysis was performed on samples of homogenized endometrial cysts (from 15 rats of the placebo group and 15 of the ultramicronized PEA group) and from samples of homogenized DRG (9 rats of the placebo group and 9 of the ultramicronized PEA group). Briefly, frozen samples of both endometrial cysts and DRG, collected on the 25th day post-endometriosis, were defrozen and then tissue lysed in 250 μL of ice-cold hypotonic lysis buffer and incubated on ice for an additional 45 minutes. The total protein extract was obtained by centrifugation at 14,000 rpm for 10 minutes at 4°C.

Endometrial cyst samples (50 μg/mL) were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred onto nitrocellulose membrane and incubated with one of the following antibodies: rabbit anti-VEGF (1:1000 vol/vol; Merck Millipore, Darmstadt, Germany), mouse anti-β-actin (1:1000 vol/vol; Santa Cruz Biotechnology, Dallas, TX), goat anti–mast cell chymase (1:1000 vol/vol; Santa Cruz Biotechnology), goat anti-NGF (1:1000 vol/vol; Novus Biologicals, Cambridge, United Kingdom), mouse anti-VEGF_r2 (Flk-1) (1:1000 vol/vol; Santa Cruz Biotechnology). Samples from DRG (50 μg/mL) were subjected to SDS-polyacrylamide gel electrophoresis and incubated with goat anti-NGF antibody (1:1000 vol/vol; Novus Biologicals).

Appropriate peroxidase-conjugated secondary antibodies (1:1000 vol/vol; PerkinElmer, Waltham, MA) were used, and proteins were visualized using an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Protein expression was quantified by densitometric analysis of the acquired images by ImageQuant 400 (GE Healthcare) and a computer program (Quantity One, Bio-Rad, Hercules, CA).

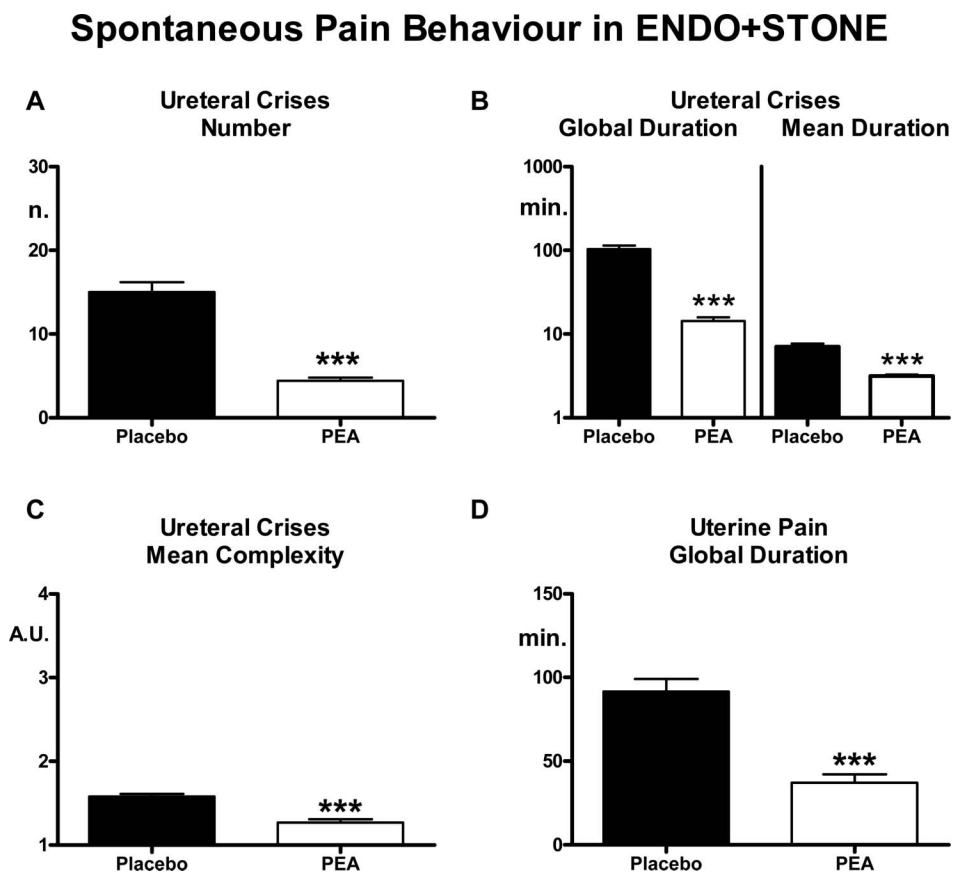

Figure 1. Number (A), global and mean duration (logarithmic scale) (B) and mean complexity (C) of ureteral crises, and global duration of uterine pain (D) in rats with endometriosis plus ureteral calculosis treated with placebo (n = 30) or ultramicronized palmitoylethanolamide PEA, (n = 30); mean ± SEM. ***$P < 0.001$: comparison between placebo and ultramicronized palmitoylethanolamide. A.U., arbitrary units.

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2.11. Statistical analysis

2.11.1. Behavioural and autopsy data

For each rat, the following were calculated: number, global duration, mean duration, and mean complexity of ureteral crises and global duration of uterine pain behaviour expressed over the whole 4-day post-stone formation period; number of ureteral crises separately for each post-stone day; and number and mean diameter of endometrial cysts. For each group of rats (placebo and ultramicronized PEA), mean, SD, and SEM were calculated for all parameters. For the preliminary experiment, the comparison between the 4 groups of animals (placebo and 3 doses of ultramicronized PEA) was performed by 1-way analysis of variance (ANOVA), followed by post hoc tests, for each parameter. For the main experiment, the trend for variation of daily ureteral crises in the post-stone period was calculated using 1-way ANOVA for both placebo and ultramicronized PEA groups. For the main and secondary experiments, the comparison between the 2 groups (placebo and ultramicronized PEA) was performed by Student *t* test for unpaired data.

The percentage of stone expulsions was calculated for each group of rats. The comparison between placebo-treated and ultramicronized PEA-treated groups was performed using the χ^2 test.

For ultramicronized PEA-treated groups of both the main and secondary experiments, the mean percentage of decrease in pain behaviour with respect to placebo was calculated (for all parameters). The comparison between these percentages in the main and secondary experiments was performed by the χ^2 test.

The correlation between cyst parameters and pain behaviour was performed by linear regression analysis.

2.11.2. Morphological and biochemical data

For each group of rats in the main experiment (placebo and ultramicronized PEA), mean, SD, and SEM were calculated for all parameters. The statistical comparison between the 2 groups was performed by 1-way ANOVA followed by Bonferroni test for multiple comparisons. The correlation of the morphological and biochemical parameters with pain behaviour was performed by linear regression analysis.

The level of significance was assessed at $P < 0.05$.

3. Results

No rat showed any sign of chronic suffering during the whole experimental period.[1] Ultramicronized palmitoylethanolamide rats but not placebo rats showed a clear docility status; ultramicronized PEA animals, in contrast to placebo animals, in fact, never showed aggressive reactions when approached, touched, or handled for the administration of therapy. Furthermore, abdominal examination during the intervention for stone formation revealed fewer visceral conglomeration/adherences in ultramicronized PEA than in placebo rats.

3.1. Main experiment

3.1.1. Visceral pain behaviour

The videotape analysis allowed for evaluation of pain behaviour, both urinary and uterine.

3.1.1.1. Ureteral pain behaviour

The ultramicronized PEA group showed significantly lower number, global duration, mean duration, and mean complexity of ureteral crises than the placebo group ($P < 0.0001$) (**Fig. 1A–C**).

3.1.1.2. Uterine pain behaviour

Ultramicronized palmitoylethanolamide rats showed a significantly lower duration of uterine pain behaviour than placebo rats ($P < 0.0001$) (**Fig. 1D**).

3.1.2. Autopsy findings

3.1.2.1. Endometrial cysts

Both placebo and ultramicronized PEA rats developed secretory cysts in variable number and dimension at the site of the implants. The 2 groups did not differ in cyst number, while cyst diameter was significantly smaller in ultramicronized PEA than in placebo animals ($P < 0.03$) (**Fig. 2A, B**).

3.1.2.2. Urinary tract

In the ultramicronized PEA group, a higher percentage of rats in which the stone was expelled was found compared with the placebo group and the difference was statistically significant ($P < 0.002$) (**Fig. 2C**).

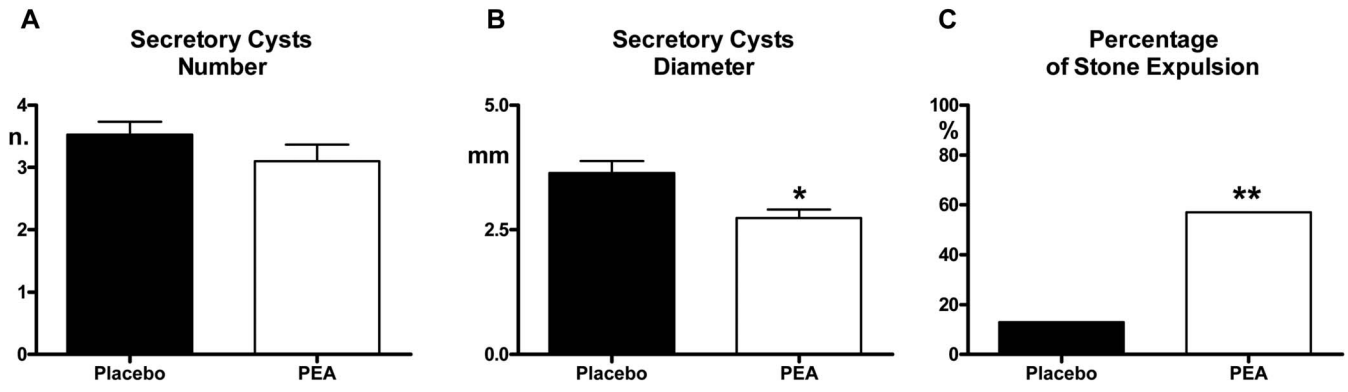

Figure 2. Number (A) and mean diameter (B) of endometrial cysts and percentage of rats that proved to have expelled their ureteral stone at autopsy (C). *$P < 0.05$; **$P < 0.01$.

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3.1.3. Visceral pain behaviour vs autopsy findings

3.1.3.1. Behaviour vs cyst parameters

In all animals, a significant direct linear correlation was found between the diameter of the cysts and the global duration of ureteral pain behaviour ($P < 0.0001$, $r = 0.8739$; $Y = -1.392 + 29.511X$ for placebo; $P < 0.0001$, $r = 0.9080$; $Y = -7.292 + 7.93X$ for ultramicronized PEA).

3.1.3.2. Behaviour vs stone expulsion/retention

All parameters of visceral pain behaviour, calculated separately for rats that proved to have expelled the stone and rats that had retained the stone, are displayed in **Figure 3A–D**, relative to the whole 4-day post-stone formation period, for both placebo and ultramicronized PEA groups. The differences between the expelled stone (ES) and retained stone (RS) animals were not significant. The daily distribution of the ureteral crises in the ES and RS animals, reported in **Figure 3E, F** for placebo and ultramicronized PEA, was similar and not significantly different. Also, the time from stone formation to the last uterine pain behaviour was not significantly different in ES and RS animals for both placebo and ultramicronized PEA groups (placebo: for ES, 71.06 ± 11.53 hours and for RS, 75.88 ± 15.47 hours; ultramicronized PEA: for ES, 57.2 ± 17.44 hours and for RS, 66.8 ± 17.8 hours).

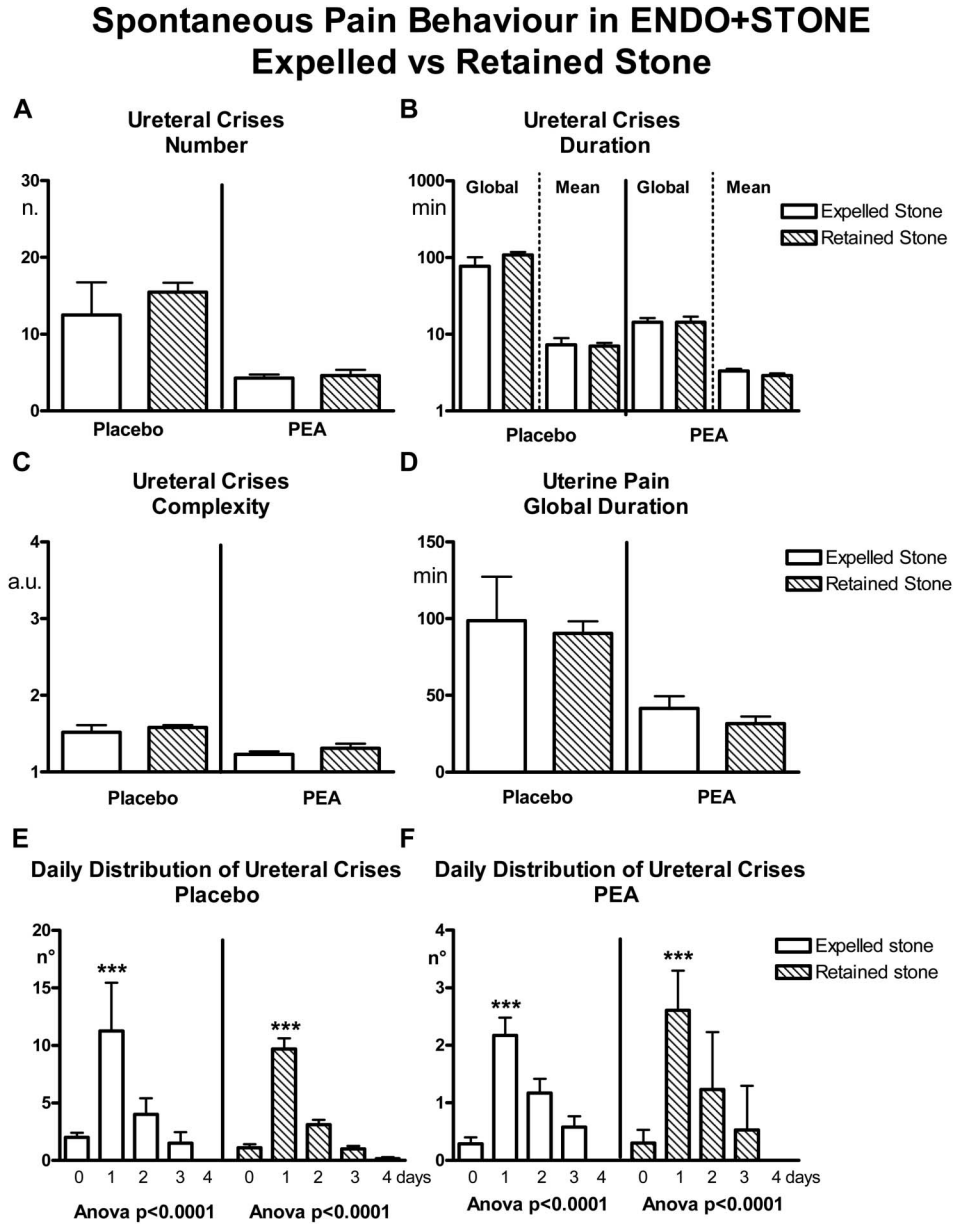

Figure 3. Spontaneous pain behaviour differentially shown for rats that proved to have expelled their stone at autopsy (n = 4 for the placebo group and n = 17 for the ultramicronized palmitoylethanolamide [PEA] group) and rats in which the stone was retained in the urinary tract (n = 26 for the placebo group and n = 13 for the ultramicronized PEA group) (mean ± SEM). (A) Total number of ureteral crises over the post-stone formation period (from day 0 [stone formation] to day 4 [suppression and autopsy]). (B) Global duration (sum of duration of all crises) and mean duration of ureteral crises over the post-stone formation period. (C) Mean complexity of ureteral crises over the post-stone formation period. (D) Global duration of uterine pain behaviour over the post-stone formation period. (E and F) Number of ureteral crises relative to each day of recording in the post-stone formation period in placebo and ultramicronized PEA rats, respectively.

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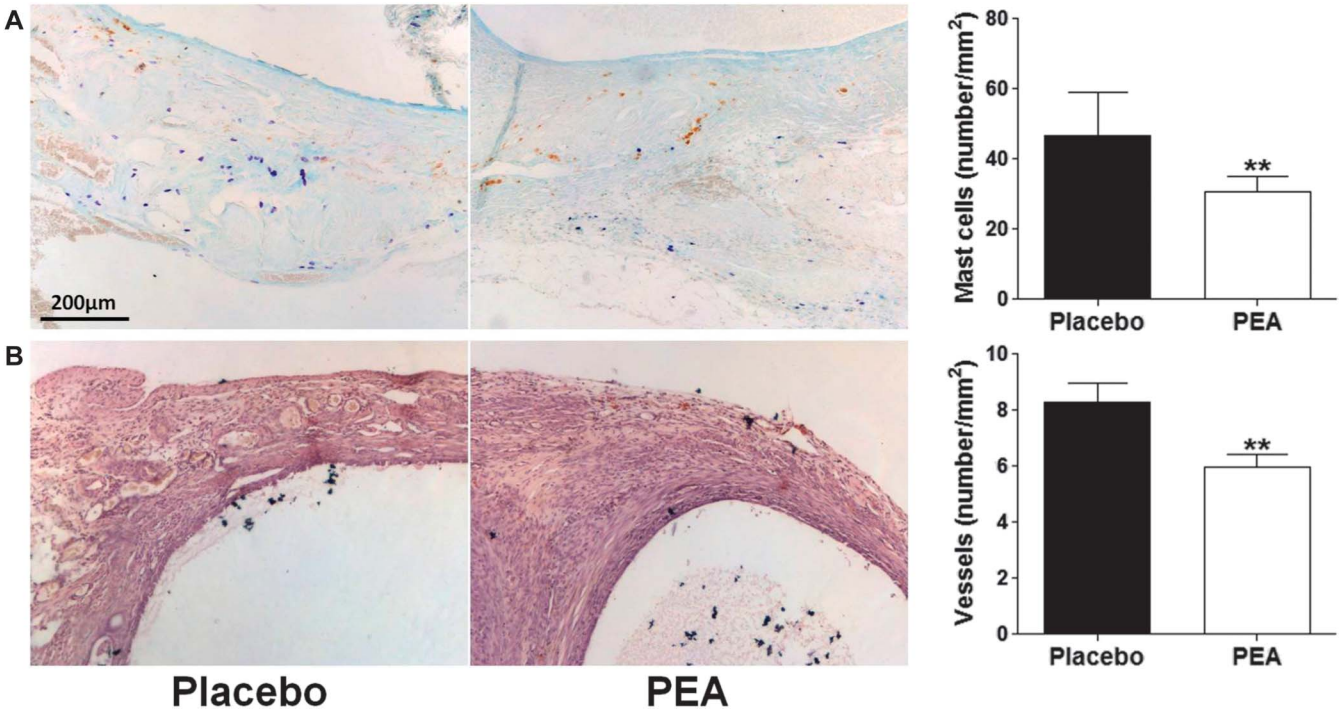

Figure 4. Histological analysis of endometriosis cysts in placebo-treated and ultramicronized palmitoylethanolamide (PEA)–treated rats. Panel A shows toluidine blue staining for mast cells (left) and mast cell number per area (in square millimeters) (right). Panel B shows haematoxylin and eosin staining for morphology (left) and vessel number per area (in square millimeters) (right); mean ± SEM (n = 15 rats for placebo and n = 15 rats for ultramicronized PEA). $^{**}P < 0.01$: comparison between placebo and ultramicronized PEA-treated animals.

3.1.4. Morphological and biochemical parameters

3.1.4.1. Mast cells and vessel density in cysts

Histological analysis of ENDO cysts from ultramicronized PEA-treated animals (**Fig. 4A**, left panel) showed lower MC density compared with the placebo group. In parallel, when MC counting was performed, a significant reduction in their number was observed in the ultramicronized PEA group compared with placebo ($P < 0.01$) (**Fig. 4A**, right panel). In a similar experiment, no differences were visible in the percentage of MC degranulation between the groups (placebo: 57.24 ± 2.32; ultramicronized PEA: 61.87 ± 2.054, in mean ± SD) (data not shown).

Moreover, histology of ENDO cysts from ultramicronized PEA-treated animals (**Fig. 4B**, left panel) also showed a reduced presence of vessels compared with the placebo group; similarly, when vessel counting was performed, a significant reduction in the number of vessels at cyst level was found in ultramicronized PEA-treated rats compared with placebo-treated rats ($P < 0.01$) (**Fig. 4B**, right panel).

3.1.4.2. Chymase, nerve growth factor, vascular endothelial growth factor, and Flk-1 protein expression in cysts

Ultramicronized palmitoylethanolamide treatment in animals with ENDO produced a significant reduction of the following protein levels in cysts: chymase (a marker of MCs) ($P < 0.05$), NGF (the proalgogen neurotrophin) ($P < 0.05$), and VEGF (the main proangiogenic factor), together with its receptor Flk-1 ($P < 0.01$), with respect to placebo (**Fig. 5**).

3.1.4.3. Nerve growth factor in dorsal root ganglia

Ultramicronized palmitoylethanolamide treatment of ENDO animals significantly reduced the level of NGF immunoreactive protein in DRG compared with placebo treatment ($P < 0.05$) (**Fig. 6**).

3.1.5. Visceral pain behaviour vs morphological and biochemical parameters

A significant direct linear correlation was found between the global duration of ureteral crises and MC number in cysts, chymase in cysts, and NGF in cysts and DRG (**Table 2**).

3.2. Secondary experiment

The results of the behavioural experiment on STONE-only rats are reported in **Figure 7**. Ultramicronized palmitoylethanolamide vs placebo treatment reduced the spontaneous ureteral pain behaviour; the difference was significant for the number of crises ($P < 0.05$). Ultramicronized palmitoylethanolamide also increased the percentage of stone expulsions with respect to placebo, although the difference was not significant, probably because of the limited sample size.

3.3. Main experiment vs secondary experiment for ureteral pain behaviour and stone expulsion

The 10 mg/kg dose of ultramicronized PEA in ENDO+STONE produced a mean reduction of ureteral pain behaviour of 71% for number, 86% for global duration, and 20% for complexity of crises.

The same dose in STONE-only rats reduced ureteral pain behaviour by 38% for number, 48% for global duration, and 6% for complexity.

The reduction of ureteral pain behaviour with ultramicronized PEA vs placebo was significantly more pronounced in ENDO+STONE than in STONE-only rats ($P < 0.0001$ for number and

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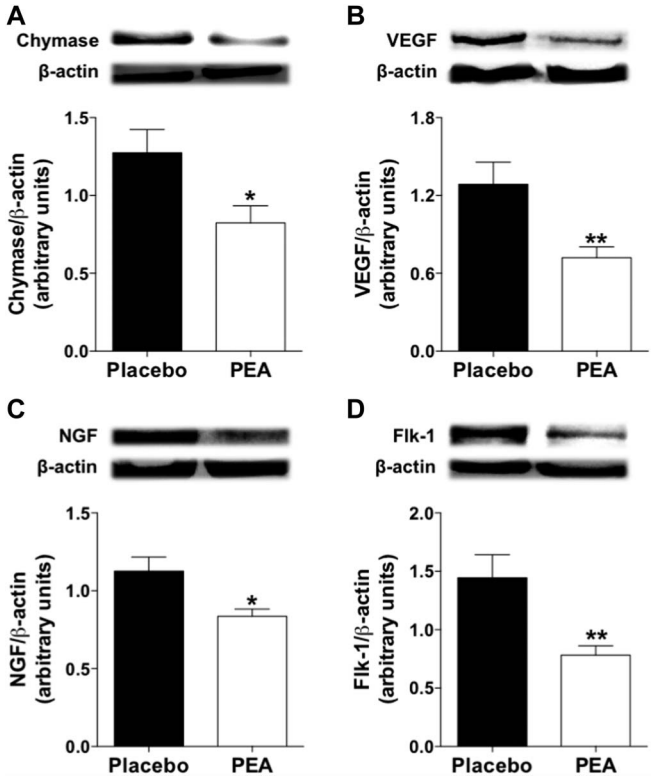

Figure 5. Western blot analysis for chymase (A), vascular endothelial growth factor (VEGF) (B), nerve growth factor (NGF) (C), and Flk-1 (D) of endometriosis cysts from placebo-treated and ultramicronized palmitoylethanolamide (PEA)–treated rats. The figure shows a representative Western blot analysis of proteins (upper) and densitometric analysis (lower) of corresponding bands. β-actin expression is shown as control; mean ± SEM (n = 15 rats for placebo and n = 15 rats for ultramicronized PEA). *$P < 0.05$, **$P < 0.01$: comparison between placebo and ultramicronized PEA-treated animals.

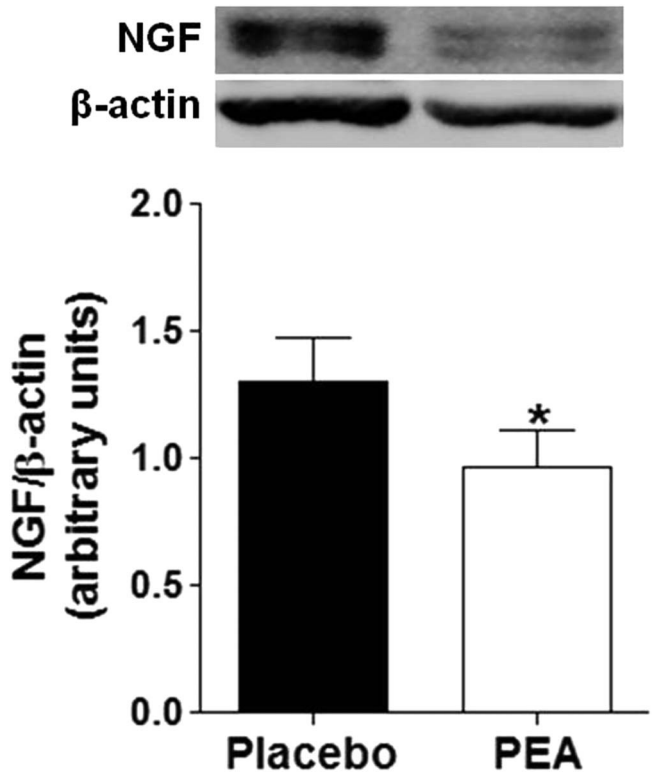

Figure 6. Western blot analysis for nerve growth factor (NGF) in dorsal root ganglia of rats with endometriosis from placebo-treated and ultramicronized palmitoylethanolamide (PEA)–treated rats. The figure shows a representative Western blot analysis of proteins (upper) and densitometric analysis (lower) of corresponding bands. β-actin expression is shown as control; mean ± SEM (n = 9 rats for placebo and n = 9 rats for ultramicronized PEA). *$P < 0.05$: comparison between placebo and ultramicronized PEA-treated animals.

global duration; $P < 0.007$ for complexity; χ^2 test between percentages of reduction in the 2 groups).

The percentage of stone expulsions was slightly less pronounced in ENDO+STONE (57%) than in STONE-only rats treated with ultramicronized PEA (60%) (17/30 vs 6/10), but the difference was not statistically significant (χ^2 test).

4. Discussion

Pain is the most frequent reason for medical consultation, and the extreme pain of VVH generated by endometriosis represents a most challenging problem for the clinician.[27] In this study, we evaluated a new possibility for VVH therapy with ultramicronized PEA using a standardized rat model of VVH from endometriosis plus ureteral calculosis, which closely mimics the clinical condition in comorbid women. Our results showed that prolonged oral treatment with ultramicronized PEA during cyst formation (starting 3 weeks before stone induction), when compared with placebo, significantly and notably reduced the behavioural indices of both uterine and ureteral pain, in parallel with a reduction of cyst diameter. This same treatment also increased the percentage of stone expulsions. The positive effects of this treatment occurred in the absence of any significant adverse event, with the animals showing no signs of chronic suffering.

In this study, we used a 10 mg/kg ultramicronized PEA dose, based on previous investigation in other animal models[36] and on our preliminary experiments with different doses of the compound, which showed a clear effect of the 10 mg/kg regimen in counteracting the ureteral and uterine pain behaviours. Under our experimental conditions, a clear dose-related effect was not observed, although a PEA dose–response effect has been reported previously in different models of inflammation and of acute and chronic/neuropathic pain (see Refs. 46,49). It is, however, not totally unexpected that the effective concentration range of PEA should vary as a function of the experimental model, the mode of administration used, and the parameters investigated. Identifying the precise range in which PEA is pharmacologically active is complicated by its lipophilic nature; furthermore, comparisons between studies are difficult because of the use of different vehicles for suspension or solubilization. Our study is the first to evaluate the effects of ultramicronized PEA in a model of VVH, which likely involves altered function of converging sensory projections and central sensitization sustained by dysregulation of immune cells activity such as MCs and microglia. In this complex setting, multiple mechanisms are likely to be implicated in both pain pathogenesis and PEA pharmacological actions. In our study, we directed our attention to the involvement of MCs, though future investigation will be needed to evaluate other possible mechanisms. The involvement of MCs in ENDO is well known, and they are a recognized target for PEA action.[3,5,39,52,65] Histological analysis showed oral ultramicronized PEA treatment to significantly reduce the number of MCs in ENDO cysts. This effect was confirmed by the biochemical analysis of chymase, a serine protease selectively stored in MC granules. This result is in line with

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Table 2

Correlation between morphological and biochemical parameters and spontaneous pain behaviour.

Group of rats	Ureteral behaviour (N = 15 per group), min	MC number in cysts vs ureteral behaviour (N = 15 per group)	Chymase in cysts vs ureteral behaviour (N = 15 per group)	NGF in cysts vs ureteral behaviour (N = 15 per group)	NGF in DRG vs ureteral behaviour (N = 9 per group)
Morph					
Placebo	103.07 ± 17.17	Y = −2.003 + 2.258X; r = 0.8404; $P < 0.0001$	___	___	___
PEA	13.87 ± 2.55	Y = −1.567 + 0.5033X; r = 0.8148; $P < 0.0003$	___	___	___
WB					
Placebo	104.27 ± 11	___	Y = 7.721 + 81.772X; r = 0.8741; $P < 0.0002$	Y = −42.955 + 137.25X; r = 0.7935; $P < 0.0005$	Y = −7.456 + 82.854X; r = 0.7617; $P < 0.02$
PEA	14.8 ± 2.03	___	Y = −4.546 + 23.402X; r = 0.8283; $P < 0.0002$	Y = −13.465 + 32.415X; r = 0.7828; $P < 0.0007$	Y = −2.497 + 17.32X; r = 0.6689; $P < 0.05$

Morph: rats in which morphological parameters were evaluated (n = 15 placebo, n = 15 ultramicronized PEA); WB: rats in which Western blot parameters were evaluated (n = 15 placebo, n = 15 ultramicronized PEA); ureteral behaviour: global duration of ureteral crises on the post-stone days.
DRG, dorsal root ganglia; MC, mast cell; NGF, nerve growth factor; PEA, ultramicronized palmitoylethanolamide.

previously reported analgesic effects of PEA, since different studies have demonstrated that PEA control of MC behaviour is reflected in a reduced pain perception.[8,18] Mast cell granules contain, in fact, proalgogenic mediators, primarily NGF.[40,63] Indeed after ultramicronized PEA oral treatment, we found, in ENDO cysts, a significant reduction of NGF levels in parallel with the reduction of MC number.

As previously reported, MCs also play a pivotal role in the control of angiogenesis and neurogenesis, key features of ENDO.[4,32,55] For this reason, we first studied the role of ultramicronized PEA in angiogenesis during ENDO. Here, for the first time, we demonstrate a strong antiangiogenic role of ultramicronized PEA in this condition, since histological analysis of ENDO cysts from animals receiving the compound revealed a significant reduction of blood vessels compared with placebo-treated animals. The antiangiogenic effect of ultramicronized palmitoylethanolamide was corroborated by Western blot analysis of VEGF, the main proangiogenic mediator, and its receptor Flk-1[41] in the cysts. Our data demonstrated that ultramicronized PEA oral treatment significantly inhibits VEGF pathways (expression levels of both VEGF and its receptor) in ENDO cysts. It is conceivable that VEGF downregulation is due to MC modulation exerted by the compound because VEGF is released mainly by MCs during chronic inflammation.[25] The reduced angiogenesis associated with ultramicronized PEA treatment may justify, at least in part, the reduction in cyst diameter reported here. In fact, new vessel formation is required for supplying oxygen and nutrients to ENDO cysts, facilitating their development and implantation.

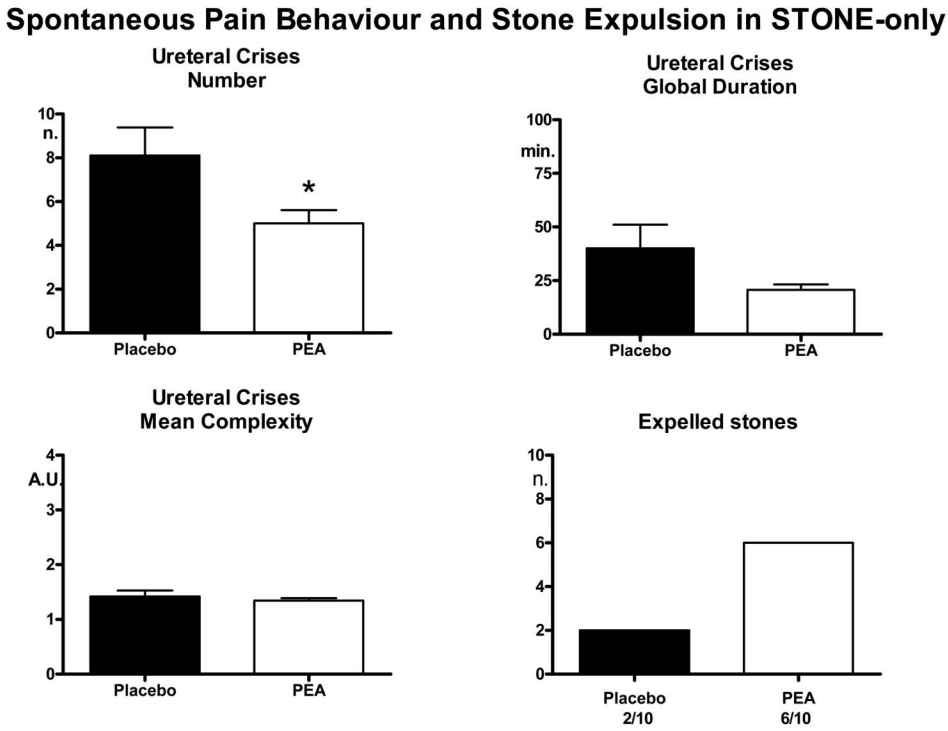

Figure 7. Number, global and mean duration (logarithmic scale) and mean complexity of ureteral crises and percentage of stone expulsion in rats with ureteral calculosis–only treated with placebo or ultramicronized palmitoylethanolamide (PEA) (10 rats per group). *$P < 0.05$: comparison between placebo and ultramicronized palmitoylethanolamide.

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Second, biochemical analysis of DRG showed that oral ultramicronized PEA treatment also reduces NGF protein expression at this level. The latter data strongly support the notion that the antialgogenic effect of ultramicronized PEA may be exerted by its control of the neurotrophin NGF, one of the main mediators activating the algogenic input from the ENDO lesions towards the central nervous system.[59] With this respect, the direct linear correlation found between NGF in DRG and ureteral pain behaviour, found in our study, is of particular relevance.

However, other mechanisms beyond the antialgogenic effect shown by ultramicronized PEA in our model cannot be excluded, since it already has been demonstrated that anti-inflammatory and analgesic effects of the compound are mediated through activation of the peroxisome proliferator–activated receptor alpha[14] and through reduction of the nuclear factor-kappa B activation in experimental models of hyperalgesia.[45]

Our results also showed a significant direct correlation between the ureteral pain behaviour and the number of MCs in cysts, chymase, and NGF expression at their level, in addition to the above-mentioned NGF expression in DRG. In view of these findings, our data on reduced pain behaviour can largely be attributed to the reduction, due to ultramicronized PEA, of the amount of algogenic mediators produced by MCs during ENDO and the consequent degree of central sensitization. The fact that oral ultramicronized PEA treatment also produced an enhanced percentage of stone expulsions with respect to placebo treatment suggests, however, that an action of the compound on ureteral activity cannot be excluded. We therefore also performed a separate analysis, in our ENDO+STONE sample, of the pain behaviour of rats that proved to have eliminated the stone vs those that had retained it. Although the former presented a slightly lesser ureteral behaviour than the latter, the difference between the 2 groups was not significant. There also was no significant differential distribution of the ureteral pain behaviour over the different post-stone implantation days in the 2 groups, suggesting that the event of stone expulsion, occurred during the recording period in some rats, had not produced dramatic effects on the subsequent evolution of the behaviour from the ureter. Regarding the uterine behaviour, this appeared instead slightly increased in the stone-expelled group as compared with the stone-retained group, although here again the difference was not significant. To further address the important point of the possible influence of stone expulsion on the reduced pain behaviour produced by ultramicronized PEA, we also tested the effects of its administration in rats with ureteral calculosis without associated endometriosis, using the same dose and duration of treatment as for rats with ENDO+STONE. A mild reduction of ureteral pain behaviour by ultramicronized PEA was observed in the STONE-only model, but the extent of this reduction was significantly less pronounced than in ENDO+STONE, despite the fact that the percentage of stone elimination was even slightly more pronounced. This outcome on one hand confirms that the promotion of stone expulsion by ultramicronized PEA plays a role in its antalgic effects, although the mechanism by which this occurs will need to be further investigated, and on the other hand, however, it points out that a major contribution to VVH reduction by ultramicronized PEA is likely to be related to an effect at the ENDO level, also considering that the compound significantly decreased uterine pain behaviour, which only appears when utereral calculosis is combined with endometriosis. Regardless of possible mechanisms, the present data are of importance in view of the clinical application of ultramicronized PEA, even though the compound is not yet available in all countries. These data provide experimental support to the results of a recent clinical study showing that PEA (comicronized with transpolydatin, 400 + 40 mg × 2/d for 3 months) reduced chronic pelvic pain and dysmenorrhoea from endometriosis.[31] While our findings suggest that orally administered ultramicronized PEA in patients could be particularly useful, alone or in combination with classic anti-inflammatory/analgesic treatments, for managing the extreme condition of VVH occurring when endometriosis is comorbid with urinary pain from calculosis, clinical studies will be needed for confirmation.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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