VISCERAL HYPERALGESIA IN A RAT MODEL OF ENDOMETRIOSIS PLUS URETERAL CALCULOSIS: ROLE OF MAST CELLS

--Manuscript Draft--

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Abstract:	The effects of ultramicronized palmitoylethanolamide (PEA-um) were evaluated on pain behaviours and markers of mast cell (MC) activity in a rat model of endometriosis plus ureteral calculosis (ENDO+STONE)-induced viscero-visceral hyperalgesia (VVH). Female Sprague-Dawley rats underwent surgical induction of endometriosis, randomly assigned to receive active (PEA-um 10/mg/kg/day, 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). PEA-um-treated vs placebo-treated rats showed: significantly lower number, duration and complexity of ureteral crises, duration of uterine pain and 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 Flk1 ($p<0.01$) 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, NGF in cysts and DRG ($0.02<p<0.0002$). PEA-um 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 that PEA-um could represent a valuable treatment for VVH in patients.

RESPONSE LETTER TO THE REVIEWERS' COMMENTS

We thank both reviewers for their comments. Reviewer 1 has raised a number of important issues that we have addressed in detail in this second revision, performing a thorough re-analysis of the data, with a consequent modified approach to their interpretation. We are grateful for the opportunity this has given us to notably improve the paper, and we do hope that our revised version will be regarded as satisfactory by both the reviewer and the editors.

A point-by-point response to the reviewer's questions is provided below. Changes in the manuscript are highlighted in yellow.

Reviewer #1:

-Q. *The introduction and rational for the experiment is clearer - thank you*
I'm pleased the pilot, dose finding data, has been included. This is important in understanding what is happening in your study and it helps set the power calculation needed to determine the group size in your study.

A. Thank you

Q. *The dose finding study did not show a clear dose related effect - indeed there is a large jump in efficacy at the top dose tested. This might suggest a non-specific mechanism of action for PEA - could the authors comment on this effect - particularly in the context of clinically used doses of PEA, effective doses from other preclinical work and concentrations known to modulate specific mechanisms of action ie PPAR alpha, GPR55 etc. I think some attempt to identify the mechanism of action of PEA in the current study is still required particular as so much is already known about the contribution of several mechanisms to PEA activity in general and the inhibition of mast cell functions specifically*

A.Specific comments on these aspects – and particularly on the possible mechanism of action of PEA in this study - have been added to the text in the Materials and Method Section and in the Discussion.
In line with previous studies in other animal models (e.g., Mazzari et al., 1996, Luongo et al., 2013), we found that 10 mg/kg PEA significantly counteracted pain behaviour (here ureteral and uterine). The PEA dosage for our main experiment was

selected on the basis of these previous studies from the literature and also on the basis of our preliminary experiment. Under the experimental conditions adopted by us, a clear dose-elated effect was not observed, although the PEA dose-response effect has been reported previously in different models of inflammation and of acute and chronic/neuropathic pain (see for example Mazzari et al., 1996; Capasso et al., 2001; Farquhar-Smith 2001; Haller et al., 2006; Wise et al.,2008; Luongo et al., 2013). It is 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 with precision the range in which PEA is pharmacologically active is complicated by its lipophilic nature; further, comparisons between studies is rendered difficult by the use of different vehicles for suspension or solubilization. Our study is the first to evaluate PEAum effects in viscero-visceral hyperalgesia, in a model probably involving altered function of converging sensory projections and central sensitization sustained by dysregulation of immune cells activity such as mast cells and microglia. In this complex setting, multiple mechanisms are likely be involved both in the pathogenesis of pain and in PEA pharmacological actions. In the present study, we directed our attention to the involvement of mast cells. A more in-depth investigation will of course be needed to evaluate other possible mechanisms.

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Q. The data from your pilot study suggests a group size of 8-14 is required depending on the pain behaviour examined. Presenting the data as you have - a single study using 53 animals creates an impression that you have not followed the IASP guidelines on minimising animal use and have a disregard for the suffering the model causes in the experimental animals.

Their needs to be transparency on the animal use for your behavioural data the current study size are unnecessary and unacceptable from an ethical standpoint if not explained clearly. I don't believe the reduction reported in the complexity of ureteral crisis is meaningful, it has occurred because your sample size is too large. The reduction in the number and duration of events by contrast is meaningful. A table of animal use in the study would help. I would suggest presenting data from individual studies ie study 1 pain behaviours + mast cell histology n=15, study 2 pain behaviours + western blots n=15, separately. Data could also be combined n=30 but it needs to be clear were the n values have come from. Plus it underlines the reproducibility of the observation. I don't think you should include data from other studies unless you include the transcriptional data from these studies also.

A.We acknowledge the reviewer's concerns regarding the number of employed animals and have therefore decided to only report, for the behaviour, the number of animals for which also the morphological/biochemical analyses were performed, i.e., n. 30 animals for placebo and 30 for PEA. We chose to present behavioural data,

number/diameter of cysts and percentage of stone expulsion on the whole samples of 30 animals per group (to avoid excessive fractioning of the data, given the fact that a further subdivision of the samples was also subsequently requested by the reviewer, based on the stone expulsion/stone retention issue, see below), but have made clear, as requested, that half of this sample (15 placebo, 15 PEA) was used for the histological analysis and the other half (15 placebo, 15 PEA) for the western blot analysis [in the text, legends of figures and an additional Table (see points below)]. The difference in complexity remained significant, due to the extremely small SD/SEM. We acknowledge that the complexity graph of the previous version of the manuscript gave the impression of a small difference between placebo and PEA: this was also due to the fact that, erroneously, we had the Y axis start at 0, while it should have started at 1 since our scale of arbitrary units for complexity, validated in our previous studies, only goes from 1 to 4 (by definition complexity 0 does not exist). This has now been corrected in the present version of the manuscript.

Q.The western studies have been clarified and extended which is important as these studies contain a key finding from the work - namely changes in VEGF expression - that's great.

A.Thanks.

Q. *I still have a problem with the interpretation of the behavioural findings. The data in general is not adequately presented. For example the reference to percentage of stones expelled is misleading and creates the impression that there are multiple ureteral stones, some of which are expelled and some of which are retained. I believe the authors are instead referring to the percentage of animals with no stone at all on post-mortem. This should be made clearer and the graph should show this as a percentage not as the number of animals - therefore the bar chart would be similar between endo-stone and stone alone groups.*

A. The behavioural data are now presented more clearly, in particular the stone expulsion graph has been modified according to the reviewer's suggestion.

Q.I'm sorry but the impact of this degree of stone expulsion on the study can't be overlooked. It is much greater than previous studies by the group. As viscero-viscero hypersensitivity is a bi-directional event it is possible that all the reduction in uterine pain behaviour is due to expulsion of the stone. Indeed uterine pain behaviours are

only observed when ureteral stimulus is applied highlighting the bidirectional nature of this cross sensitisation. Furthermore, although the comparison of endo-stone and stone alone studies very nicely demonstrate the VVH due to endo. I don't believe you can definitely conclude that the greater reduction in ureteral pain behaviours is due to an effect on the cyst as you have no idea whether the impact of the cross sensitising effect of the cyst on ureteral stone is linear ie the proportional increase in ureteral pain may not be as great or not occur for a smaller ureteral stimuli. However I do accept that the data is suggestive of an effect on the cyst which is support by the cyst size/histology/protein expression data.

A. We understand the reviewer's point and have therefore performed a profound revision of data presentation and interpretation with respect to the crucial issue of stone expulsion. In particular: all behavioural data were re-analysed also separately for rats with stone expelled vs rats with stone retained, a day-by-day postoperative analysis of pain behaviour distribution was also performed in the two groups (stone expelled vs stone retained). Our new analyses showed a slight difference between stone-expelled and stone-retained (stone expelled-rats showing a lesser degree of ureteral pain behaviour for a lesser period of time) but the difference was not significant. The extent of this difference being limited, it seems unlikely for whole – large - effect of PEA on the reduction of pain behaviour, to be mediated entirely by the promotion of stone expulsion, though this mechanism certainly contributes. Uterine pain behaviour was in contrast slightly higher in stone-expelled rats, though here again, the difference was not significant. Regarding the correlation between cysts and stone, we did demonstrate in our previous paper (Giamberardino et al, PAIN, 2002), that a significant direct linear correlation exists between the mean diameter of the cysts and the global duration of the ureteral crises. We have now repeated the correlation analysis in the samples of rats in the present study, and this result was confirmed, for both the placebo and PEA treated animals. In addition, we have also performed here a correlation analysis between morphological and biochemical cyst parameters on one hand and pain behaviour on the other, as suggested in the last point of the reviewer's comments, and found a significant direct linear correlation between number of MCs, chymase and NGF levels in cysts with the ureteral pain behaviour (as well as NGF in dorsal root ganglia). Although we certainly agree that we are not allowed to draw definite conclusions regarding the stone expulsion influence on the outcome of our study, we believe that all these data together suggest that the contribution by the cysts and their algogenic potential to the

triggering of the ureteral events is substantial. Our conclusions have, however, been toned down with this respect, as requested.

Q.I'm not sure if the uterine pain behaviour should be called that, I'm not sure there is an indication that it definitely arises from the uterus that I am aware off - the location of the surviving cysts is not given for each animal and for the most part the cysts will form in the abdominal cavity - although I'm happy to be corrected

A. The reviewer is of course right in regard to the origin of the behaviour, since the cysts have locations different from the uterus. However, the name "uterine" was chosen, at the time the model was set up (Giamberardino et al, 2002), to indicate that this behaviour resembles that occurring in a model of frank "uterine pain" (a model of uterine inflammation from injection of mustard oil into one uterine horn)(Wesselmann et al 1998). Thus the endo+stone model was standardized using this terminology, which was maintained in the papers that were subsequently published on the model itself (e.g., Lopopolo et al 2014), this is the reason why we would prefer to maintain it, as a change could suggest we are using parameters different from those standardized in previous papers, which is instead not the case. We have, however, now better specified this in the text, to make the reason of this choice clearer.

Q. *This issue of the stone expulsion should be dealt with. To do this the data needs to be reanalysed. Animals in which the stone was expelled should be separated from those that retained the stone. There should be sufficient n value across all the studies to do this. Secondly the time course for effects should be plotted. If animals which lost their stones show a dramatic effect on a given day it would indicate that the loss of stone caused a change in behaviour as it was expelled. - the studies were videotaped it should be clear if there is a point after which no pain behaviours are seen for the animals with no stone post mortem.*
Additionally the duration of individual ureteral events should be presented - is the reduction in time due to a decrease in the number of events or do the events get shorter in duration - this would be interesting to know.

A. As explained above, we have re-analyzed the behaviour of all rats in regard to stone expulsion and animals in which the stone was expelled were separated from those that retained the stone, as requested. We have also performed an analysis of the time course for effects. Our analysis showed no dramatic effect on a given day for

rats which had expelled the stone with respect to those that had retained it, suggesting that stone expulsion was perhaps not the main (or not the sole) determinant of the observed behavioural effects. The duration of individual ureteral crises was calculated and it also proved to be significantly reduced by PEA, indicating that the effect of PEA was not solely due to a reduction of the number of crises. New graphs and paragraphs in the results section have now been added to address this issue.

Q. *Given the difficulty with stone expulsion using PEA - why did subsequent studies not just look at the cyst alone - this the end point that matters in terms of the mechanistic work into mast cell numbers and mediators - this would have avoided further pain studies, as I doubt the ureteral stone has an influence on the cyst pathology? Given that pain data was recorded to ureteral stone would there be value in correlating changes in cyst mast cells, mediators etc with pain behaviour to firm up a causal link? I think it would have been better to have addressed these issues prior to submitting the original manuscript to pain.*

We acknowledge the reviewer's criticism, however our aim was primarily to evaluate the effects of PEA on pain/pain behaviour, not just to assess cyst parameters and their variation with PEA; and the pain behaviour only appears in the endo model when this is combined with the calculosis, being a model of viscero-visceral hyperalgesia, which is indeed the specific pain condition we wanted to address. We do agree that the stone has no influence on the cyst behaviour, but the point we wanted to address here is that of the influence of cyst parameters on ureteral pain behaviour. With this respect, we fully agree on the importance of the correlation between behavioural parameters and cyst parameters, which had not been examined in the first version of our paper. We have now performed this analysis, as suggested, with the results already described in our response to the above-raised points. We believe that this observation is in support of an important contribution by endometrial cyst inputs to the pain behaviour expressed in our model.

Reviewer #2: THE AUTHOS HAVE CORRECTLY ANSWERED THE RECOMMENDATIONS.
Thank you.

ABSTRACT

The effects of ultramicronized palmitoylethanolamide (PEA-um) were evaluated on pain behaviours and markers of mast cell (MC) activity in a rat model of endometriosis plus ureteral calculosis (ENDO+STONE)-induced viscero-visceral hyperalgesia (VVH). Female Sprague-Dawley rats underwent surgical induction of endometriosis, randomly assigned to receive active (PEA-um 10/mg/kg/day, 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). PEA-um-treated vs placebo-treated rats showed: significantly lower number, duration and complexity of ureteral crises, duration of uterine pain and 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 Flk1 ($p<0.01$) 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, NGF in cysts and DRG ($0.02<p<0.0002$).

PEA-um 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 that PEA-um could represent a valuable treatment for VVH in patients.

ULTRAMICRONIZED PALMITOYLETHANOLAMIDE REDUCES VISCERO-VISCERAL HYPERALGESIA IN A RAT MODEL OF ENDOMETRIOSIS PLUS URETERAL CALCULOSIS: ROLE OF MAST CELLS

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Key words: ultramicronized palmitoylethanolamide, endometriosis, ureteral calculosis, viscero-visceral 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 viscero-visceral 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]. Preventative ketoprofen treatment of ENDO lesions (secretory cysts) in this model reduces cyst diameter and prevents the post-stone VVH, which confirms that reducing nociceptive inputs from the cysts is key to preventing/decreasing pain in this condition [26]. ENDO treatment with Non-Steroidal-Antiinflammatory 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 down-modulates mast cell (MC) activation and controls glial cell behaviours and angiogenetic processes alongside inflammatory reactions, thus having potential effectiveness in VVH from endometriosis [2,10,16,17,19,21,46,49,53,54,56,57,62]. Recent experimental ~~studies~~ studies,~~have,~~ 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 chemo-attractant 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]. MCs contribute to neo-angiogenesis, another ENDO feature, which guarantees oxygen supply to lesions. The expression of Vascular Endothelial Growth Factor (VEGF), the most potent pro-angiogenic factor ~~is~~, 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].
PEA, 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 ultramicronized form (PEA-um)[36]. Analgesic effects of PEA-um for pelvic pain are also suggested by clinical pilot studies [11,31,37]. No research has~~, instead,~~ tested PEA-um in VVH from endometriosis. Controlled studies in VVH would be problematic, due to high variability of the clinical parameters. Our aim was therefore to assess PEA-um effects 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

Sprague-Dawley female 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 PEA-um on ENDO+STONE

The main experiment was carried out in rats with ENDO+STONE to assess the effects of PEA-um vs placebo on pain behaviour and on morphological and biochemical parameters of MC activation.

A total of sixty rats wasere used for this experiment (30 treated with placebo and 30 with PEA-um); 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 PEA-um) and biochemical parameters (western blot analysis: chymase, VEGF, NGF, Flk1 in cysts) were assessed in the remaining half (15 placebo and 15 PEA-um). NGF was also was assessed in the Dorsal Root Ganglia (DRG) in 18 of the rats (9 placebo and 9 PEA-um) 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 one of two groups of 30 rats each, to undergo one of the following treatments:

- PEA-um, in the dose of 10 mg/kg/day, resuspended in 1.5% carboxycellulose w/v in saline;
- placebo, i.e., 1.5% carboxycellulose w/v in saline in an equivalent volume.

The PEA-um dose to employed 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 PEA-um in small groups of ENDO+STONE rats (Table 1). These preliminary experiments, although not showing a dose-response effect for PEA-um, clearly evidenced a marked efficacy of the 10mg/kg dose, which was 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, i.e., 2:00 p.m.), the last on the 25th day post-endometriosis. The timing of the daily administration (except the first day) was always 9:00 a.m.

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 post-operative days (until the 25th day post-endometriosis, i.e., 4th day post-stone), all rats (30 placebo and 30 PEA-um) were video-recorded 24-hours ~~non-stop~~ a day to evaluate their spontaneous behaviour, indicative of both ureteral and uterine pain. In the evening of the 25th day post-endometriosis (4th day post-stone formation), all animals were euthanized via 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 spinal cord (T11-L2 segments) were subsequently removed. For both the placebo and PEA-um groups, soon after the explant, the samples were immediately either stored in 4% formaldehyde or frozen in liquid nitrogen and subsequently stored at -80C°. Samples of endometrial cysts were used to perform morphological analyses (15 rats of the placebo group and 15 of the PEA-um group) and biochemical analyses (the remaining 15 rats of the placebo group and 15 of the PEA-um group). In particular, MC number and percentage of degranulation, and vessel number in the cysts were evaluated as below described in Staining Techniques. The expression in the tissues of the pro-angiogenic 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 PEAum on STONE-only

The secondary experiment was carried out in rats undergoing formation of artificial ureteral calculosis only, to assess any direct effect of PEA-um on ureteral pain

behaviour and stone expulsion in the absence of endometriosis [30]. Twenty Sprague-Dawley rats (220-240) were randomly assigned to one of two groups of 10 animals each to undergo one of the following treatments:

- PEA-um, in the dose of 10 mg/kg/day, resuspended in 1.5% carboxycellulose w/v in saline;
- placebo, i.e., 1.5% carboxycellulose w/v 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 9:00 a.m. From the 21st till the 25th day after the start of treatment (i.e., for 4 days post-stone implantation) they were video-recorded 24-hours ~~non-stop~~ a day to evaluate their spontaneous behaviour indicative of ureteral pain.

In the evening of the 25th day, all rats were sacrificed via 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 employed [7,50]. The animals were anesthetized with pentobarbital (50 mg/kg intraperitoneally), 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, i.e., three 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 anesthesia (50 mg/kg i. p.), the left ureter was approached via 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 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 ~~non-stop~~ 24 hour a day video-tape recording with a time-lapse system, with ultrared lighting for filming during the dark phase (20:00-8:00 hrs). The analysis of the whole period of recording (performed by observers blind 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 papers [26,30,44]. A "ureteral pain crisis" consists of a sequence of at least three pain behaviours (of six possible) within a period of minimum 2 minute duration. The six possible behaviours are: "hump-backed" 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 via a 4-point arbitrary scale: three movements are scored 1, four movements are scored 2, five movements are scored 3 and, lastly, all six 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 as they resemble the behaviours occurring in a model of experimental uterine inflammation (69). These consist of four 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 a 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 (4th day post-stone formation), the following were evaluated in each rat: number and diameter of endometrial cysts, and status of the urinary tract, i.e., 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 PEA-um group and immediately fixed in 4% neutral buffered formalin and routinely processed for histology. Serial sections (7μ) 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 two changes of xylene and hydrated through two changes of alcohol, 5 minutes in 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 10X 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 (400X), divided by the total MC number. Quantitative evaluations were performed by a researcher blind to the origin of the material.

2.9. Assessment of the number of vessels

For each cyst, ten sections were de-paraffinized 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 blind 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 PEA-um group) and from samples of homogenized DRG (9 rats of the placebo group and 9 of the PEA-um group). Briefly, frozen samples of both endometrial cysts and DRG, collected on the 25th day post-endometriosis, were de-frozen and then tissue lysed in 250 µl of ice-cold hypotonic lysis buffer and incubated on ice for an additional 45 min. The total protein extract was obtained by centrifugation at 14,000 rpm for 10 min 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 v/v; Merck Millipore, Darmstadt, Germania), mouse anti-β actin (1:1000 v/v; Santa

Cruz Biotechnology, Dallas, Texas), goat anti-mast cell chymase (1:1000 v/v; Santa Cruz Biotechnology, Dallas, Texas), goat anti-NGF (1:1000 v/v; Novus biologicals, Cambridge, UK), mouse anti-VEGF_r2(flk-1) (1:1000 v/v; Santa Cruz Biotechnology, Dallas, Texas). Samples from DRG (50 µg/mL) were subjected to SDS-polyacrylamide gel electrophoresis, and incubated with goat anti-NGF antibody (1:1000 v/v; Novus biologicals, Cambridge, UK).

Appropriate peroxidase-conjugated secondary antibodies (1:1000 v/v; PerkinElmer Massachusset) were used, and proteins were visualized using an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). 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).

2.11. Statistical analysis

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; number and mean diameter of endometrial cysts. For each group of rats (placebo and PEA-um), means, SD and SEM were calculated for all parameters. For the preliminary experiment, the comparison between the four groups of animals (placebo and 3 doses of PEA-um) was performed by 1-way 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 via 1-way ANOVA for both placebo and PEA-um groups. For the main and secondary experiments, the comparison between the two groups (placebo and PEA-um) was performed by Student's t-test for unpaired data.

The percentage of stone expulsions was calculated for each group of rats. The comparison between placebo-treated and PEA-um-treated groups was performed using the chi-square test.

For PEA-um-treated groups of both the main and secondary experiment, 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 experiment was performed by the chi-square test.

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

Morphological and biochemical data.

For each group of rats in~~of~~ the main experiment (placebo and PEA-um) means, SD and SEM were calculated for all parameters. The statistical comparison between the two groups was performed by 1-way ANOVA followed by Bonferroni's 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]. PEA-um rats but not placebo rats, showed a clear docility status: PEA-um 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 PEA-um than placebo rats.

3.1. Main experiment

3.1.1. Visceral pain behaviour

The video-tape analysis allowed for the evaluation of pain behaviour, both urinary and uterine.

Ureteral pain behaviour. The PEA-um group showed significantly lower number, global duration, mean duration and mean complexity of ureteral crises than the placebo group ($p<0.0001$) (Fig. 1,A-C).

Uterine pain behaviour. PEA-um rats showed a significantly lower duration of uterine pain behaviour than placebo rats ($p<0.0001$) (Fig. 1,D).

3.1.2. Autopsy findings

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

Urinary tract. In the PEA-um group, a higher percentage of rats in which the stone was expelled was found compared to the placebo group and the difference was statistically significant ($p<0.002$) (Fig. 2,C).

3.1.3 Visceral pain behaviour vs autopsy findings

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 PEAum].

Behaviour vs stone expulsion/retention. All parameters of visceral pain behaviour, calculated separately for rats which proved to have expelled the stone and rats which had retained the stone, are displayed in Fig. 3A-D, relative to the whole 4-day period post-stone formation, for both placebo and PEA-um 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 Fig.3E-F for placebo and PEA-um, 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 the placebo and PEA-um groups [placebo:

for ES, 71.06 ± 11.53 hours and for RS, 75.88 ± 15.47 hours; PEA-um: for ES, 57.2 ± 17.44 hours and for RS 66.8 ± 17.8 hours].

3.1.4. Morphological and biochemical parameters

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

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

Chymase, NGF, VEGF and Flk1 protein expression in cysts. PEA-um 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 pro-algogen neurotrophin)($p<0.05$) and VEGF (the main pro-angiogenic factor), together ~~-~~with its receptor Flk-1 ($p<0.01$), with respect to placebo (Fig. 5).

NGF in Dorsal Root Ganglia (DRG). PEA-um treatment of ENDO animals significantly reduced the level of NGF immunoreactive protein in DRGs compared to 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, NGF in cysts and DRG (Table 2).

3.2. Secondary experiment

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

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

The 10mg/kg dose of PEA-um 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 PEA-um vs placebo was significantly more pronounced in ENDO+STONE than in STONE-only rats ($p<0.0001$ for number and global duration; $p<0.007$ for complexity; chi-square test between percentages of reduction in the two groups).

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

4. DISCUSSION

Pain is the most frequent reason for medical consultation and the extreme pain of viscero-visceral hyperalgesia generated by endometriosis represents a most challenging problem for the clinician [27]. In the present study we evaluated a new possibility for VVH therapy with PEA-um by 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 PEA-um during cyst formation (starting 3 weeks before stone induction), when compared with ~~respect to~~ 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 employed a 10 mg/kg PEA-um dose, based on previous investigation in other animal models (36) and on our preliminary experiments with different doses of PEA-um, which showed a clear effect of the 10mg/kg regimen in counteracting the ureteral and uterine pain behaviour. 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 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 ~~with precision~~ the precise range in which PEA is pharmacologically active is complicated by its lipophilic nature; furthermore, comparisons between studies is ~~made~~ difficult because of~~by~~ the use of different vehicles for suspension or solubilization. Our study is the first to evaluate PEA-um effects 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 PEA-um 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 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]. MC granules contain, in fact, pro-algogenic mediators, primarily NGF [40,63]. Indeed a~~A~~fter PEA-um oral treatment, we ~~indeed~~ 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 ~~also~~ in the control of angiogenesis and neurogenesis, key features of ENDO [4,32,55]. For this reason, we first studied the role of PEA-um in angiogenesis during ENDO. Here, for the first time, we demonstrate a strong anti-angiogenic role of PEA-um in this condition, since histological analysis of ENDO cysts from animals receiving PEA-um revealed a significant reduction of blood vessels compared to placebo-treated animals. PEA-um anti-angiogenic effect was corroborated by Western blot analysis of VEGF, the main pro-angiogenic mediator, and its receptor Flk-1 [41] ~~-~~in the cysts. Our data demonstrated that PEAum oral treatment significantly inhibits VEGF pathways (expression levels of both VEGF and its receptor) in ENDO cysts. It is conceivable that VEGF down-regulation is due to MC modulation exerted by PEA-um, since VEGF is released mainly by MCs during chronic inflammation [25]. The reduced angiogenesis associated with PEA-um 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.

Secondly, biochemical analysis of DRG showed that oral PEA-um treatment also reduces NGF protein expression at this level. The latter data strongly support the notion that the PEA-um anti-algogenic effect 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 CNS [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.

On the other hand, other mechanisms beyond the anti-algogenic effect shown by PEA-um in our model cannot be excluded, since it has 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] as well as 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 MC 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 onf reduced pain behaviour can largely be attributed to the reduction, due to PEA-um, of the amount of algogenic mediators produced by MCs during ENDO and the consequent degree of central sensitization. The fact that oral PEA-um 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 which proved to have eliminated the stone vs those which had retained it. Although the former presented a slightly lesser ureteral behaviour than the latter, the difference between the two groups was not significant. There was also was no significant differential distribution of the ureteral pain behaviour over the different post-stone implantation days in the two 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 PEA-um, we also tested the effects of PEA-um 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 PEA-um was observed in the STONE-only model,- but the extent of this reduction was significantly less pronounced than in ENDO+STONE, in spite of 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 PEA-um plays a role in its antalgic effects, although the mechanism by which this occurs will need to be further investigated. On the other hand, however, it points out that a major contribution to VVH reduction by PEA-um 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 PEA-um, even though the compound is not yet available in all countries. While providing experimental support to the results of a recent clinical study showing that PEA (comicronized with trans-polydatin, 400mg + 40 mg x 2/day for 3 months) reduced chronic pelvic pain and dysmenorrhea from endometriosis [31], our findings suggest for the first time, that orally administered PEA-um 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.

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LEGENDS TO TABLES AND FIGURES

Tab. 1

Preliminary experiment for dose selection. Ureteral and uterine pain behaviours and cyst parameters in rats with ENDO+STONE treated with different doses of PEA-um (2.5, 5 or 10 mg/kg/day) and placebo, orally, for 25 days, from the day of endometriosis induction till the 4th day subsequent to stone induction (performed on day 21). Means ± 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 PEA-um 10mg/kg/day with placebo (post-hoc tests).

Tab. 2

Correlation between morphological and biochemical parameters and spontaneous pain behaviour.

Morph: rats in which morphological parameters were evaluated (n.15 placebo, n. 15 PEA-um)

WB: rats in which Western Blot parameters were evaluated (n. 15 placebo, n. 15 PEA-um)

Ureteral behaviour: global duration of ureteral crises on the post-stone days

Fig. 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 PEA.um (n. 30). Means ± SEM; a.u.: arbitrary units.

***= $p<0.001$: comparison between placebo and PEA-um.

Fig. 2

Number (A) and mean diameter (B) of endometrial cysts and percentage of rats which proved to have expelled their ureteral stone at autopsy (C). Legend as for Fig. 1. *=$p<0.05$; **=$p<0.01$.

Fig. 3

Spontaneous pain behaviour differentially shown for rats which proved to have expelled their stone at autopsy (n. 4 for the placebo group, n. 17 for the PEA-um group) and rats in which the stone was retained in the urinary tract (n. 26 for the placebo group and n.13 for the PEA-um group)(Means ± SEM).

A: total number of ureteral crises over the post-stone formation period [from day0 (stone formation) to day4 (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-F: number of ureteral crises relative to each day of recording in the post-stone formation period in placebo and PEA-um rats, respectively.

Fig. 4
Histological analysis of ENDO cysts in placebo-treated and PEA-um-treated rats. Panel A shows Toluidine Blue staining for MCs (left) and MC number per area (mm^2) (right). Panel B shows Hematoxylin & Eosin staining for morphology (left) and vessel number per area (mm^2) (right). Means ± SEM (n. 15 rats for placebo, n. 15 rats for PEA-um).
**= $p<0.01$. Comparison between placebo and PEA-um-treated animals

Fig. 5
Western blot analysis for chymase (A), VEGF (B), NGF (C), and Flk-1 (D) of ENDO cysts from placebo-treated and PEA-um-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. Means ± SEM (n. 15 rats for placebo, n. 15 rats for PEA-um).
*= $p < 0.05$, **= $p < 0.01$. Comparison between placebo and PEA-um-treated animals

Fig. 6
Western blot analysis for NGF in DRG of ENDO rats from placebo-treated and PEA-um-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. Means ± SEM (n. 9 rats for placebo, n. 9 rats for PEA-um).
*= $p < 0.05$. Comparison between placebo and PEA-um-treated animals

Fig. 7. Number, global and mean duration (logarithmic scale), mean complexity of ureteral crises and percentage of stone expulsion in rats with ureteral calculosis-only treated with placebo or PEAum (10 rats per group). *=$p<0.05$, comparison between placebo and PEAum.

Summary

In rats with endometriosis plus ureteral calculosis, oral ultramicronized palmitoylethanolamide vs placebo significantly reduces viscero-visceral hyperalgesia by downregulating mast cell activity in endometriotic lesions.

Figure 1

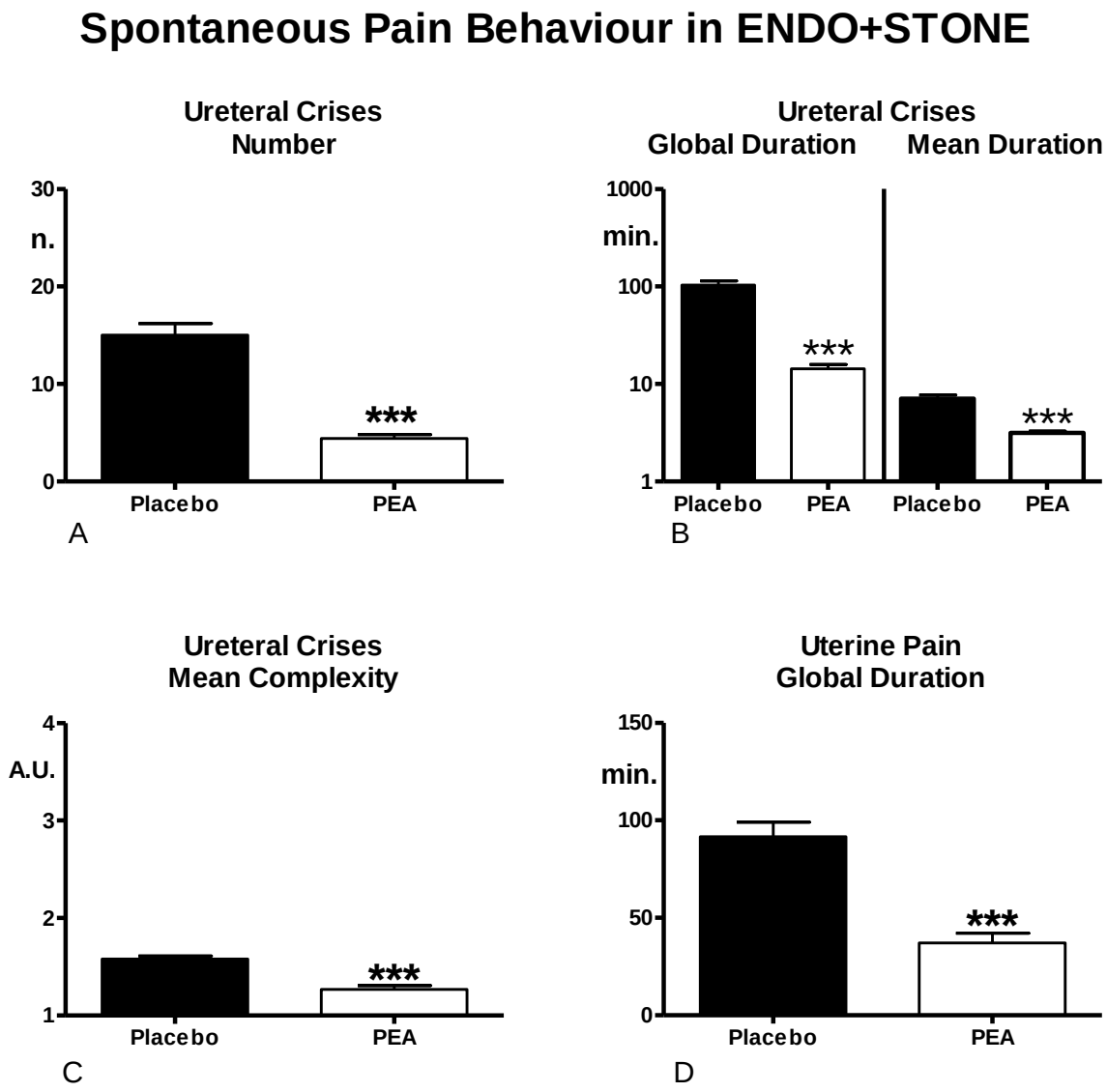

Fig. 1.

Figure 2

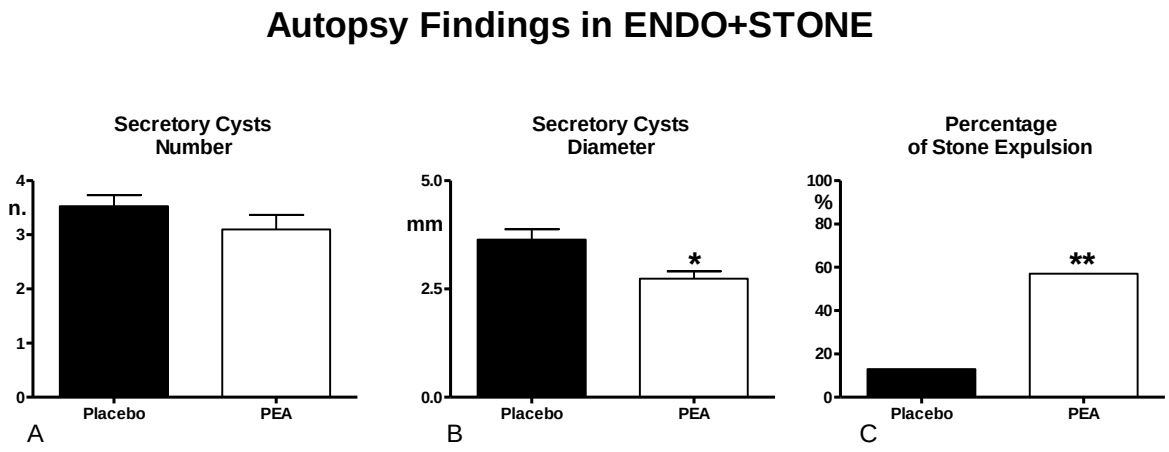

Fig. 2

Figure 3

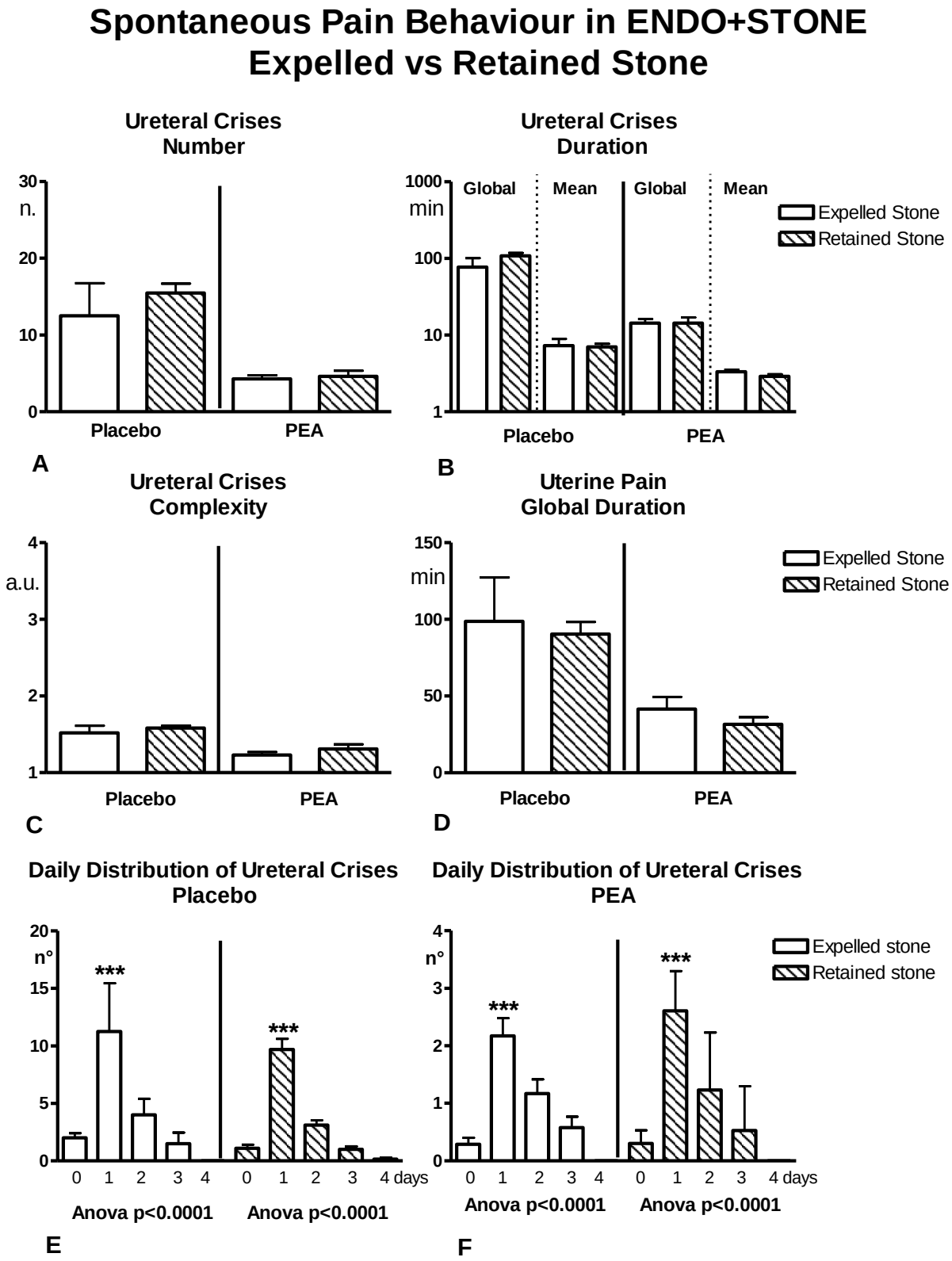

Fig. 3.

Figure 4

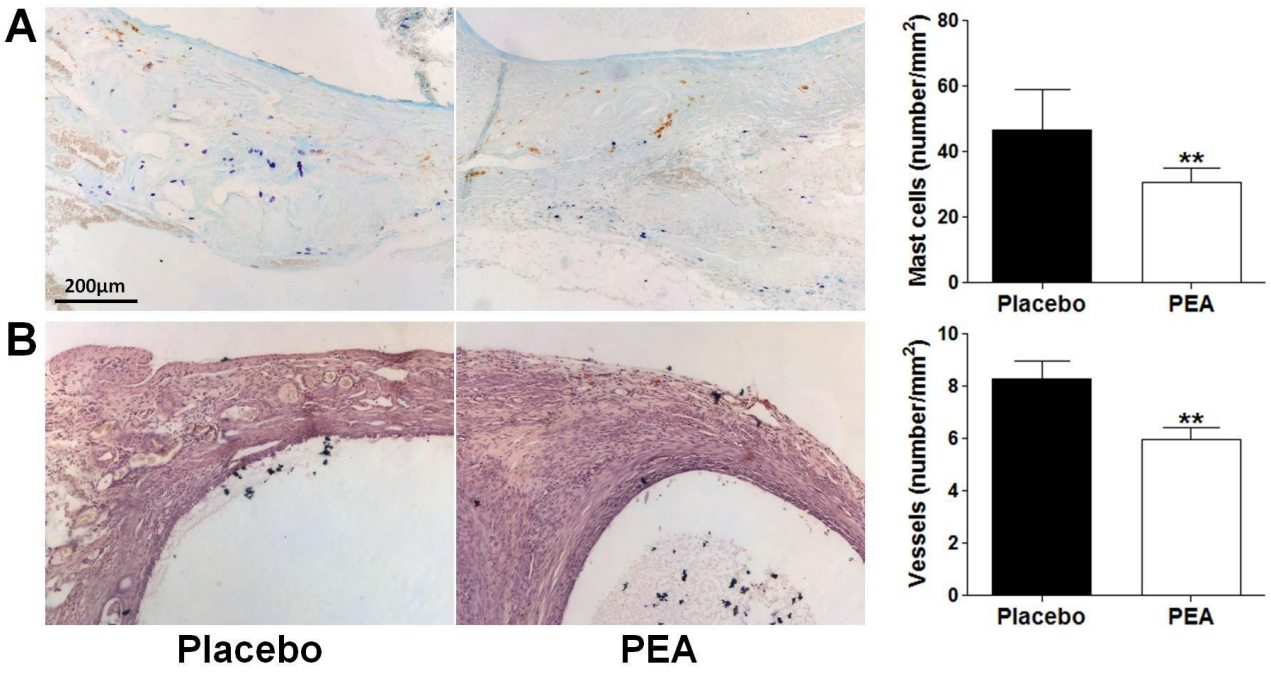

Fig. 4

Figure 5

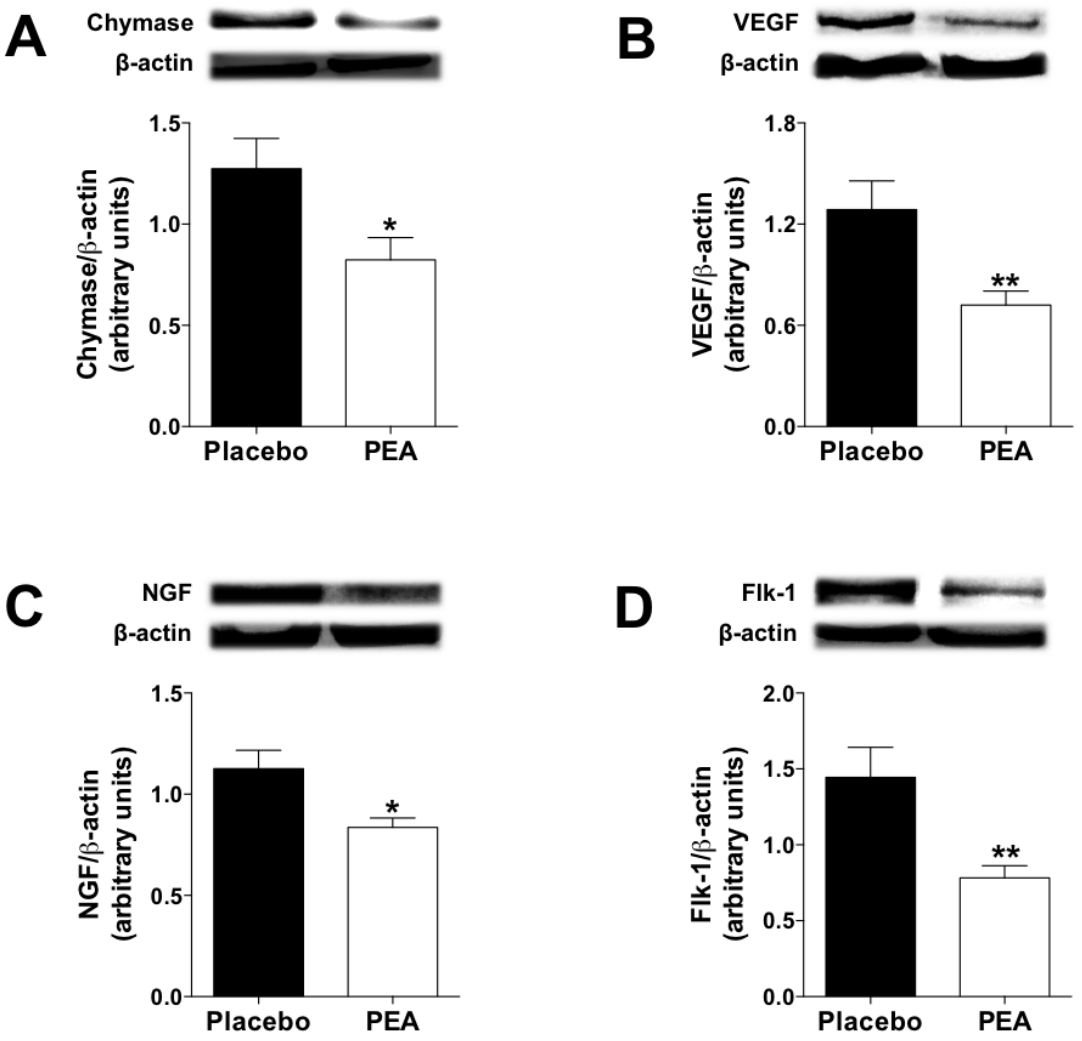

Fig. 5

Figure 6

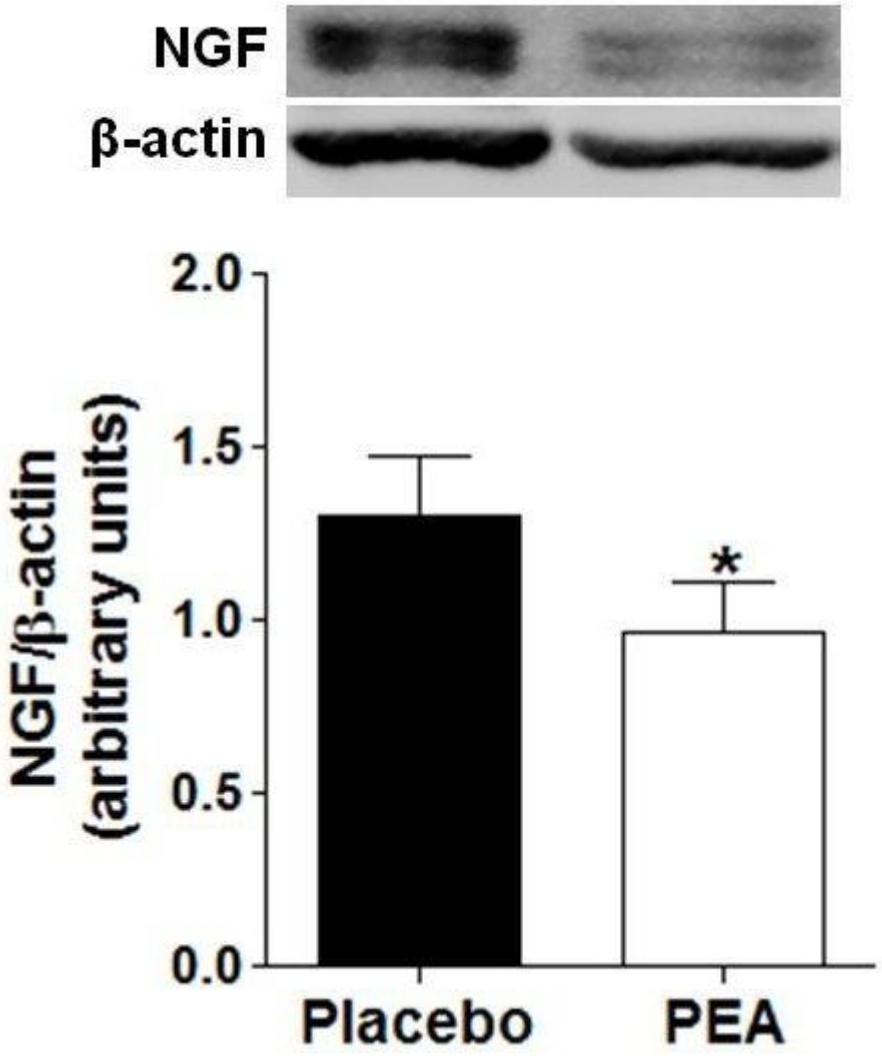

Fig. 6

Figure 7

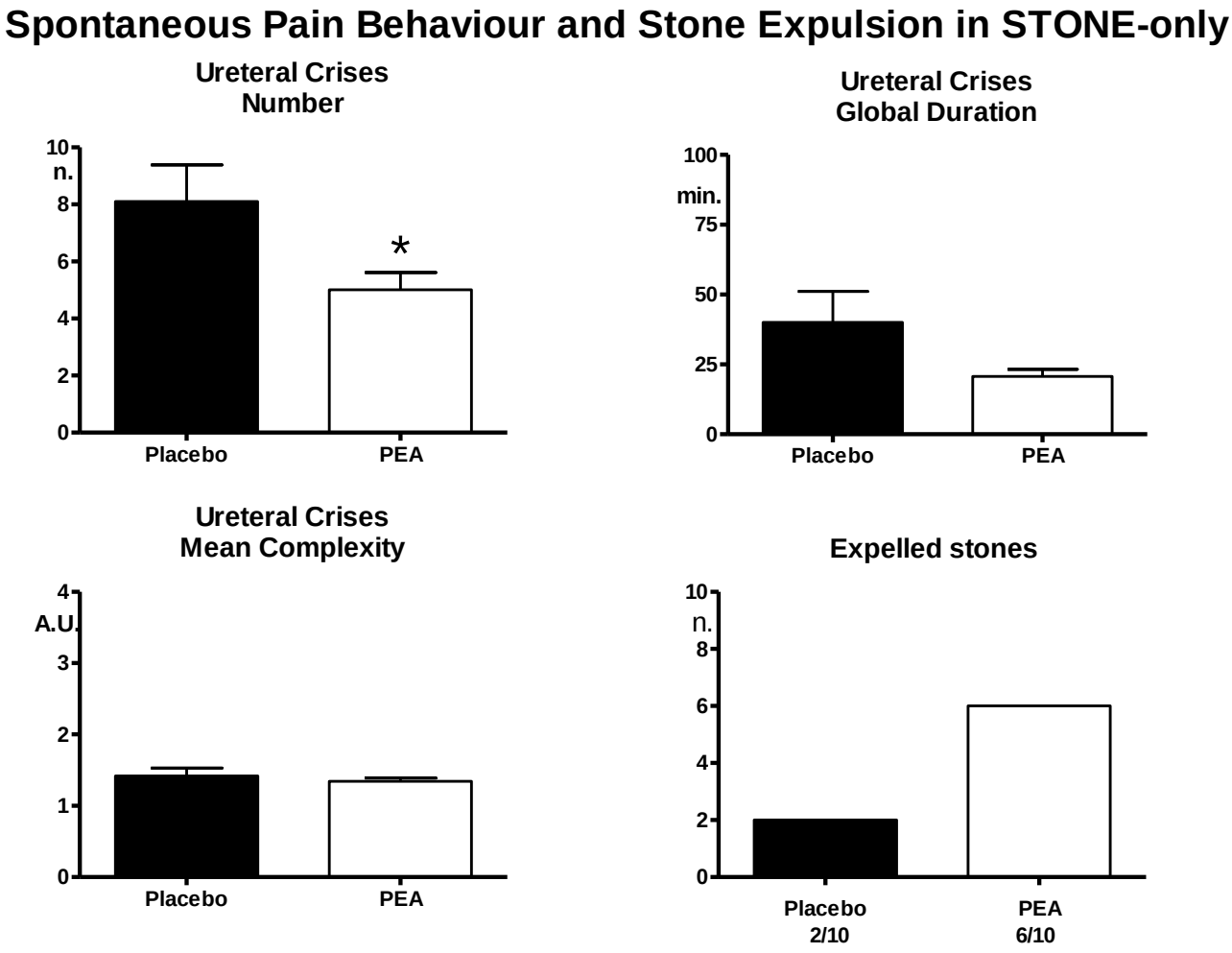

Fig. 7

Table 1

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

Tab. 1

Table 2

GROUP OF RATS	URETERAL BEHAVIOUR (N.15 per group)	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 min**	**Y= -2.003 + 2.258X (r)=0.8404 P<0.0001**	**___**	**___**	**___**
Morph PEA	**13.87±2.55 min**	**Y= -1.567+0.5033 (r)=0.8148 P<0.0003**	**___**	**___**	**___**
WB Placebo	**104.27±11 min**	**___**	**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+82.854 (r)= 0.7617 P<0.02**
WB PEA	**14.8±2.03 min**	**___**	**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**

Tab. 2.

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