

## Research Article

# Establishment and validation of a rodent model of endometriosis to evaluate the effect of new therapeutic strategies

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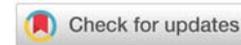
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## Abstract

**Objectives:** Endometriosis is a common disease that affects about 10% - 15% of women in their reproductive years worldwide with no curative treatment. The most common symptom of endometriosis is debilitating pelvic/abdominal pain. Current therapeutic options have limited insight into the disease mechanism and include drugs and/or surgery, which may be ineffective over the long term with unwanted side effects. We aimed at establishing a translational rodent endometriosis model that can be used to identify novel therapies. The validity of the model was confirmed by investigating the effect of the clinically-used GnRH agonist, leuprolide.

**Methods:** Endometriosis was induced by a surgical procedure in adult non-pregnant female Sprague Dawley rats in the diestrus or estrus stage (cycle determination by vaginal smear). One group of rats received a subcutaneous injection of leuprolide at 1mg/kg, every 4 weeks. Following the treatment period, we performed a direct assessment of the endometriosis-induced abdominal pain using the Von-Frey method and spontaneous pain using the abdominal licking test. Then, the lesions were excised and measured.

**Results:** Abdominal pain threshold was decreased by more than 2 fold in rats with surgically-induced endometriosis compared to sham rats. Leuprolide treatment significantly increased the threshold force required to elicit a behavioral withdrawal response in rats suffering from endometriosis. The observed pelvic floor mechanical hyperalgesia has not been correlated to the growth of endometriosis lesions. The hormonal cycle at the surgery induction influenced the endometriosis lesions growth. Leuprolide significantly inhibited the growth of endometriosis-like lesions.

**Conclusions:** we have established, based on previously reported rodent models, a model of endometriosis-associated pain that responds to clinically active drugs and can, therefore, be used to identify novel therapies and investigate some of the pathophysiological mechanisms involved in endometriosis.

## Introduction

Endometriosis is the presence of ectopic endometrial tissue outside the uterine cavity and is a common gynecological disease reported in 10% - 15% of women during their reproductive years in the world [1]. The most common symptom of endometriosis is debilitating pelvic/abdominal pain. In fact, up to 70% of women suffering from pelvic pain are affected by endometriosis. Other symptoms include

dyspareunia, severe dysmenorrhea, and dysuria. Women with the condition also suffer from co-occurring painful conditions, including interstitial cystitis/painful bladder symptoms and irritable bowel syndrome and 50% of these women suffer from infertility [2].

The disease has a huge negative impact on women's quality of life, work productivity, sexual relationship and self-esteem [3]. The most common theory proposed to explain the pathophysiology of the endometriosis is retrograde



menstruation hypothesis but also other hypotheses have been discussed like inflammatory factors, dysregulated immunity, hormones and genetic and epigenetic factors [4]. None of these mechanisms could explain alone the different types and symptoms of endometriosis. Current therapeutic options have limited insight into the disease mechanisms and include drugs and/or surgery to reduce symptoms and manage complications. These available treatments provide short-term solutions but tend to be ineffective over the long term with a high incidence of unwanted side effects such as premature bone loss and vaginal dryness. The most common medical treatment for endometriosis is gonadotropin-releasing hormone (GnRH) agonists and estrogen/progestin combinations [5,6]. In recent years, the search for more effective and less invasive therapeutic curative strategies to reduce years of suffering in women with endometriosis is receiving increased research attention. The development and validation of *In vivo* models are necessary to allow the preclinical testing of the efficacy of potential new treatment options.

Non-human primates are considered the most physiologically relevant model of endometriosis since these animals develop spontaneous endometriosis [7]. However, their use is limited by cost and ethical concerns. Thus, rodent models of endometriosis [8] have been developed to investigate the effect of the presence of endometriosis lesions on functional outcomes and the extent of its reversal by new compounds. However, it is difficult to develop a unique model that replicates all symptoms and aspects of a complex disease such as endometriosis. Indeed, endometriosis is a heterogeneous disease with poorly known etiology and several phenotypes such as ovarian endometrioma, superficial peritoneal disease and deep infiltrating endometriosis [4]. In addition, endometriosis is multifactorial and has been associated with environmental, genetic, immunological and hormonal factors [9]. Different models have been developed in mice and rats and recently reviewed by Bruner-Tran, et al. [8], each model emphasizing one or several features to explore disease-related mechanisms, to identify therapeutic targets and/or to provide insight into the development of co-morbidities. Pain is considered a significant contributor to endometriosis morbidity and pelvic pain is one of the most described symptoms [10], even though asymptomatic cases are described [11], this study aimed at establishing a rodent model of endometriosis to be a useful tool in evaluating endometriosis-related pelvic pain. To that end, we developed a rodent model of endometriosis in immunocompetent rats based on surgically-induced endometriosis originally described by Vernon and Wilson [12] to evaluate endometriosis-induced pain by assessing specifically pelvic pain while others extensively evaluated generalized pain behavior. In addition, endometriosis diagnosis is based on the analysis of the lesions collected during laparoscopic surgery [13] and the American Society of Reproductive Medicine classified the disease into 4 stages according to the evaluation of the endometriotic lesions [14]. Thus, this study also described the proliferation of endometriosis lesions being a major feature of endometriosis. Even though the model described in this study is based on previously published studies reviewed by others [8,15], very few authors reported the impact of the estrous stage on

endometriosis-induced models in rodents. Hence, this study was designed to investigate if any influence of the estrous stage at induction of endometriosis on both primary endpoints i.e., pelvic pain and endometriosis lesions size. To validate the translational value of this model, we tested the effect of one of the most clinically used medical options, a GnRH agonist on pelvic pain and lesions size.

## Materials and methods

### Endometriosis-induced surgery and treatment administration

Adult non-pregnant female Sprague Dawley rats (Elevage Janvier, Le Genest-St-Isle, France, 6–8 weeks old) were housed at least 10 days prior to the beginning of the experiments with free access to standard chow (Chow M20, 841201, SDS, UK) and water and maintained on an inverted 12h dark/light cycle (10:00/22:00). All procedures were approved by the local ethical committee (CEE47) and performed in accordance with the legislation on the use of laboratory animals (NIH publication N° 85 - 23, revised 1996) and Animal Care Regulations in force in France as of 1988 (authorization from competent French Ministry of Agriculture - Agreement No. B78-423-1, July 2017).

After the acclimation period, the reproductive status was determined by vaginal lavage using traditional nomenclature for the 4 estrous stages (proestrus, estrus, metestrus and diestrus). After the confirmation of one full cycle by daily vaginal lavage, the rats were identified with a unique identification number; then using a random number table, they were randomly allocated to different experimental groups ( $n = 12$  rats per group): sham group (SHAM), endometriosis group (ENDO) and treated group (ENDO+leuprolide).

When in the diestrus stage, all animals underwent either sham the surgical procedure or endometriosis induction by a surgical procedure based on the initial protocol described by Vernon and Wilson in rats [12] and Cummings and Metcalf [16].

In addition, in order to evaluate the influence of the estrous stage on pelvic pain and lesions size, a group of rats ( $n = 12$ ) that underwent endometriosis induction when in the proestrus/estrous stage was added to this study.

Whether surgical induction was conducted in the diestrus or proestrus/estrus stage, the rats were anesthetized with isoflurane (1% - 1.2%, Centravet, France) under aseptic conditions and using a heating pad to maintain a body temperature of the animal at 37 °C. A midline incision was made through the skin and muscle layer to expose the pelvic and abdominal organs. A segment of the mid-left uterine horn was excised, six 2x2 pieces of the excised uterus were prepared and each piece was sutured onto alternate mesenteric arteries using 4.0 nylon sutures. The same surgical procedure was applied for the sham group except for the step of suturing uterine pieces. Then, the muscle layer and the skin were closed using polyester suture (Vetsure© Bond) and the animal was closely monitored during recovery. Following surgery, animals were carefully monitored for health status and weighed twice per week. One week after surgery, the rats allocated to the

treated group received a single subcutaneous injection of the GnRH agonist, leuprolide at 1mg/kg repeated once on day 28 [17]. All endpoints were reported 6 weeks post-surgical procedure.

### Pelvic pain assessments

A vaginal cytology smear was performed to determine the estrous stage at the time of pain assessment.

A widely accepted method to evaluate mechanical hyperalgesia in conscious animals is the Von Frey filaments. This method is based on behavioral observations of animal response to mechanical stimuli of increasing forces applied to the plantar area by means of filaments with grading forces and consists of determining the hind paw withdrawal threshold as a referred pain-associated behavior. In this study, we adapted the Von Frey method to apply to the pelvic area and to determine a pelvic pain nociception threshold. Briefly, a few days before the Von Frey experiments, the rat abdomen was shaved under light isoflurane anesthesia (Centravet, France). On the day of assessment, the rat bladder was emptied by manual compression and the rat was placed in a metabolic cage for a minimum 30-minute acclimatization period before evaluation.

Monofilaments of differing forces ranging from 0.008 to 4g (Bioseb, Vitrolles, France) were applied in increasing order of force and rat behavior was recorded according to a pain behavior rating scale (score 1 to 4). The forces of the von Frey fibers used in this study were determined in a preliminary test based on the modified up-and-down method [18]. Five repeated stimulations per filament with 5 - second intervals were performed, followed by 3-minute intervals before applying the next higher force filament. Care was taken to stimulate different areas within the lower abdominal region to avoid desensitization. The pain threshold was determined by the lower force evoking a pain reaction of the rat characterized by abrupt retraction of the abdomen, jumping, and/or immediate licking of the site of application (score 3 and above).

Also, spontaneous pain was evaluated by the abdominal licking test which is used as an indicator of abdominal discomfort in various pelvic pain models [19,20]. This test was performed in an open-field home cage and consists of observing, after allowing the rat to acclimate to the cage, the number of times the rat licked the abdominal region [21]. The average number of abdominally-directed licking was recorded over two 10 min periods by an experimenter blinded to the groups.

### Lesions measurement

Following behavioral assessments, the rats were anesthetized and then euthanized by an intracardiac injection of pentobarbital (Eutasol®, Centravet, France), the abdominal cavity opened and the lesions measured by an in-house developed method adapted from a described method by Becker, et al. [22]. Briefly, the harvested lesions were aligned on graph paper, and the image was captured and analyzed using Image

J software by measuring for each lesion two perpendicular diameters (D1 and D2) and calculating a cross-sectional area (CSA) using the formula for an ellipse:  $(D1 \times D2 \times \pi / 4)$ . Then, the CSA of the lesions harvested from the same animal was averaged to calculate a mean CSA for each animal and finally to compute the average CSA per experimental group for comparison between groups.

### Statistical analysis

**All results are presented as mean  $\pm$  SEM:** Some rats were excluded if the endometriosis-like lesions were not present at harvesting or if the behavior of the rat during pain evaluation was agitated not allowing assessment. A final  $n = 10$  rats/group was included in the final analysis.

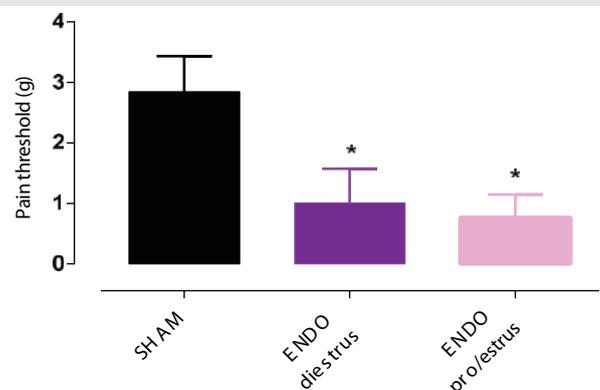
Student's t-test or one-way ANOVA followed by Dunnett's multiple comparisons post-test were used when appropriate to compare the means of two groups. Statistical analysis was performed with GraphPad Prism® 6.05 software.  $p$  values  $< 0.05$  were considered significant.

## Results

### Influence of estrous cycle on pelvic pain and lesions size

Pelvic pain threshold determined by Von Frey filaments in endometriosis rats significantly decreased by 2 fold compared to the SHAM group 6 weeks post-induction surgery independently of the estrous stage at the time of endometriosis induction (Figure 1).

However, the mean cross-sectional lesion area was significantly larger compared with the mean area of the lesions at the time of the surgical induction of endometriosis ( $3.8 \pm 0.8$  mm<sup>2</sup> vs.  $2.2 \pm 0.1$  mm<sup>2</sup>, respectively, Figure 2) when the surgical induction of endometriosis was achieved in diestrus stage but not in proestrus/estrus stage. In fact, when the surgery was conducted in the proestrus/estrus stage, the mean cross-sectional lesion area was smaller compared with the mean area of the lesions at the time of the surgery ( $1.7 \pm 0.2$  mm<sup>2</sup> vs.  $3.0 \pm 0.1$  mm<sup>2</sup>, respectively, Figure 2).



**Figure 1:** Effect of estrous cycle on pelvic pain threshold determined by Von Frey experiments. Von Frey experiments were performed 6 weeks post sham surgery (SHAM,  $n = 10$ ) or endometriosis induction surgery when rats were at diestrus stage (ENDO diestrus,  $n = 10$ ) or estrus stage (ENDO pro/estrus,  $n = 10$ ). Data are mean  $\pm$  s.e.m \* $p < 0.05$ , Dunnett's multiple post-test vs. SHAM.



Of note, the lesions prepared from the uterus when the rat was in the estrus/proestrus stage were larger than the lesions prepared when the rat was in the diestrus stage.

Consequently, the establishment and validation of the model were continued using rats in the diestrus stage at the induction of endometriosis and presented here.

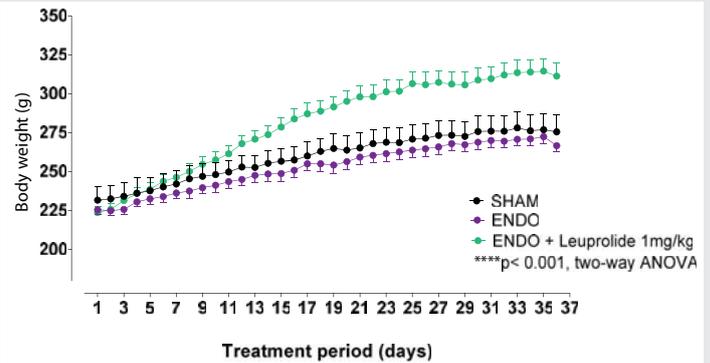
### Body weight evolution

The body weight progressively increased, starting from the first-week post-surgery until the end of the experimental period for all groups of rats. The rats in the ENDO group showed the same body weight evolution compared to sham-operated rats. However, the leuprolide-treated endometriosis rats (ENDO+leuprolide) showed significantly higher body weight evolution than ENDO rats (Figure 3). Indeed, the rats that received leuprolide showed a sharp increase in body weight gain the first 2 weeks after treatment administration compared to the day of starting the treatment ( $12 \pm 1\%$  the first week and the second week) compared to the ENDO group ( $5 \pm 1\%$  the first week and the second week)

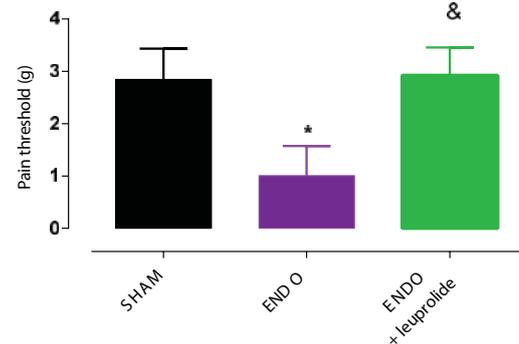
Of note, the body weights, before starting the treatment period, were comparable between all groups of rats ( $232 \pm 9$  g,  $226 \pm 3$  g, and  $224 \pm 4$  g for SHAM, ENDO and ENDO+leuprolide groups, respectively; One-Way ANOVA, ns,  $p > 0.05$ ).

### Pelvic pain assessments

As mentioned earlier, pelvic pain threshold in the ENDO group was significantly decreased by more than 2 fold compared to the SHAM group 6 weeks post-induction surgery (Figure 1). Leuprolide at 1mg/kg administered subcutaneously once every 28 days shifted pain responses towards higher forces indicating that rats in the ENDO+leuprolide group are less sensitive to pain compared to rats from ENDO group. Thus, the GnRH agonist leuprolide decreased abdominal hyperalgesia induced by endometriosis surgery by significantly increasing the pain threshold (Figure 4).



**Figure 3:** Evolution of body weight post-surgery and during the treatment period. Rats underwent either sham surgery (SHAM,  $n = 10$ ), endometriosis induction surgery (ENDO,  $n = 10$ ) or endometriosis induction surgery and treated with leuprolide (ENDO+leuprolide,  $n = 10$ ). Data are mean  $\pm$  s.e.m \*\*\*\* $p < 0.01$ , two-way ANOVA test.

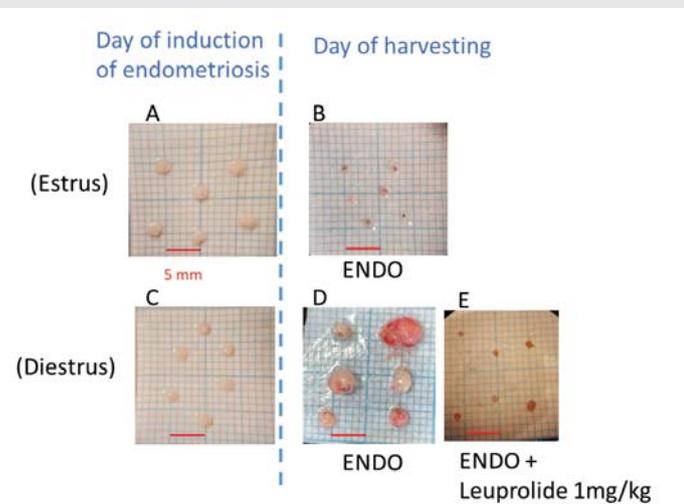


**Figure 4:** Effect of subcutaneous leuprolide at 1mg/kg on pelvic pain threshold determined by Von Frey experiments. Von Frey experiments were performed 6 weeks post sham surgery (SHAM,  $n = 10$ ) or endometriosis induction (ENDO,  $n = 10$ ) or endometriosis induction and treatment with leuprolide 1mg/kg once every 4 weeks (ENDO +leuprolide,  $n = 10$ ). Data are mean  $\pm$  s.e.m \* $p < 0.05$ , Dunnett's multiple post-test vs. SHAM, &  $p < 0.05$ , Dunnett's multiple post-test vs. ENDO.

Licking behavior remained almost unchanged in SHAM rats before and after surgery (+5%) while a slight increase in abdominal-directed licking was observed in ENDO rats after surgery (+15%), however, this increase did not reach statistical significance (Student's t-test, ns,  $p > 0.05$ ). Consequently, the abdominal-directed licking behavior assessment design should be reconsidered with some modifications to the experimental conditions to allow the evaluation of the effect of potential therapies. Indeed, some authors reported abdominal direct licking behavior in mice and rats using different conditions compared to the ones used in this study such as increasing the number of animals [21], increasing the observation window [19] and taking into account abdominal directed licking when part of the normal grooming behavior [20].

### Lesions measurement

The presence of lesions was confirmed in 90% of endometriosis rats (ENDO and ENDO+leuprolide groups). The mean cross-sectional lesion area was significantly larger compared with the mean area of the lesions at the time of the surgical induction of endometriosis ( $3.8 \pm 0.8$  mm<sup>2</sup> vs.  $2.2 \pm 0.1$  mm<sup>2</sup>, respectively). Treatment with leuprolide suppressed by 60% the growth of endometriosis-like lesions ( $p < 0.05$ , Student's t-test).



**Figure 2:** Representative pictures of the different range of lesions at induction of endometriosis and day of harvesting when rat was in estrus (A and B) or diestrus (C, D and E).

## Discussion

This study described a translational rat model of endometriosis-associated pain. The validity of the model was confirmed by investigating the effect of the clinically-used GnRH agonist, leuprolide.

First, this study investigated the potential influence of the hormonal cycle on the growth of endometriosis lesions. Some authors used uterine tissues at the proestrus/estrus phase [12,23] since the endometrium is thicker compared to other stages providing enough tissue for suturing in line with our observations. However, a correlation between the volume of endometriosis lesions and the estrous cycle has been described [24] while others reported that lesion size is independent of the estrous stages [25]. Thus, the first aim of this study was to examine the influence of the estrous stage and to set the stage to be used in subsequent experiments.

Endometriosis is an estrogen-dependent and dynamic disorder. Indeed, endometriosis lesions' appearance and progression/regression are dependent on hormonal levels. It has been reported that endometriosis lesions growth is triggered by high estrogen levels while low estrogen levels lead to lesions regression [26]. Besides, it is known that estrogen level starts to decrease at late proestrus corresponding to the follicular phase in women that is characterized by the release of luteinizing hormone and follicular-stimulating hormone [27]. The increase in follicular stimulating hormone induces ovulation matching the estrus stage of the cycle in rodents during which the estrogen levels decrease and prolactin levels peak. During the metestrus/diestrus stage, progesterone levels increase, corresponding to the luteal phase in women, followed by a peak in estrogen levels during the late luteal phase, a decline in progesterone levels, and regression of the corpus luteum, leading to menstruation in women. The late luteal phase and the regression of the corpus luteum phase correspond to the diestrus stage in rodents [28,29].

Taken together, it is plausible to suggest that the diestrus stage in rodents is comparable to the menstrual phase in women and the changes in estrogen/progesterone levels during the estrous cycle may influence the endometriosis lesions growth. Accordingly, this study is to our best knowledge among the few studies showing by direct comparison that the estrous cycle significantly affected the size and the macroscopic growth of endometriosis lesions using an autologous endometriosis rat model without ovariectomy. Indeed, significant growth of endometriosis lesions was observed when the preparation of the lesions and the induction of the surgery was done when the rats were in the diestrus stage but not when in the proestrus/estrus stage.

If the main characteristic of endometriosis is the presence of tissue lesions containing stromal, epithelial and inflammatory cells, the main clinical sign of endometriosis is pain, even though asymptomatic endometriosis has been reported [4,6]. Women suffering from endometriosis described different intensities and types of pains ranging from severe dysmenorrhea to chronic pelvic pain accompanied or not with other comorbid

pain conditions leading to a significant impact on the quality of life of patients [30]. Recent studies have illustrated that women with endometriosis develop significant sensitivity to any stimuli as a consequence of central sensitization [31]. Thus, the development of new and effective therapies is a research priority that requires the development of appropriate pre-clinical models of pain-associated endometriosis and validated tools for pain assessment which can advance our understanding of this condition of central sensitization. The first publications describing surgically induced rodent models of endometriosis focused mainly on lesion description [12,16,22,32]. More recently, investigators developing rodent models of endometriosis models have given more focus on pain endpoint but not specifically pelvic pain and rather generalized pain by performing paw withdrawal behavioral tests as an indicator of stimulus-evoked pain [21,33,34]. Indeed, only a minority of studies measured pelvic pain [20,35-37]. Thus, we aimed in this study to set up a specific pelvic-pain-associated endometriosis model to allow in the future to understand the related mechanisms. Thus, we investigated both phenotypes of pain, the stimulus evoked pelvic pain response and the spontaneous pain as indicators of chronic intermittent pain reported by patients suffering from endometriosis.

Indeed, this study showed a significant increase in pain perception in rats suffering from endometriosis. Measurement of pain was performed by evaluation of behavioural responses stimulus-dependent using Von Frey filaments. Furthermore, rats suffering from endometriosis showed exacerbated spontaneous pain behavior compared to sham rats, even though the design of this study did not allow to reach a statistical significance in the number of times the rat licked the abdominal region, and thus it needs to be fine-tuned in the future experiments. In fact, very few authors reported both phenotypes of pain in a rodent model. Indeed, pelvic and spontaneous pain was recently reported in a non-surgical mice model [36] but not yet in a rat model of endometriosis.

It is now well established that the association between the severity of pelvic pain and the endometriosis lesions growth is inconsistent [38]. Stratton and Berkley [39] have shown that a major contributing factor to endometriosis-associated pain is not the ectopic lesions growth but rather the development of nerve supply creating a direct interaction between lesions and the central nervous system. Another feature that has been associated with the severity of endometriosis pain is the vascularity of the lesions and the development of new blood vessels. This coordinated growth of blood vessels and nerves has been termed "neuroangiogenesis" [40]. In line with these suggestions, the model described in this study mimics pain-associated endometriosis and the non-correlation between the severity of pelvic pain and the growth of the endometriosis lesions.

The validation of the translational value of this model was confirmed by testing one of the most clinically-used compounds, leuprolide, a GnRH agonist. Subcutaneous leuprolide administered in a dosage regimen similar to its clinical use significantly decreased pain sensation in rats suffering from endometriosis as well as the endometriosis



lesions growth. Indeed, in clinical practice, leuprolide and more broadly the GnRH agonist are prescribed as second-line therapy in endometriosis-related pain with a satisfactory result on pain and on decreasing lesions volume when the first-line such as combined oral contraceptives and progestin fail or due to intolerance or contraindications which represent 1/4 to 1/3 of patients [41]. However, the GnRH agonists induce an artificial menopause state and thus add-on therapy is needed to limit some related risks such as premature bone mass density loss. Moreover, this type of treatment can only be prescribed to patients who do not wish to become pregnant [4].

★In conclusion, there is a need to develop more effective therapeutic and curative strategies with fewer unwanted effects. To this end, it is necessary to establish a reliable and standardized animal model of endometriosis. In this study, we have established a model of endometriosis-associated pain that responds to clinically active drugs and can, therefore, be used to identify novel therapies. It should be noted, however; no model is expected to mimic all aspects of the women's disorder and it is probable that the new therapies have to be tested in several models, each replicating some features of the disease to improve the transitional value and to increase the success rate in clinical trials.

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