Development of a subcutaneous endometriosis rat model[1]

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ABSTRACT

PURPOSE: To present a rat model of subcutaneous endometriosis for the study of pathophysiology and the effects of drugs.

METHODS: Fifty three-month-old female Wistar rats (*Rattus norvergicus*) were distributed into one control group and four treatment groups: estradiol (2.5; 5; 10mg/kg sc), medroxyprogesterone acetate (0.5; 2; 5mg/kg sc), triptorelin pamoate (0.18; 0.56mg/kg sc) and acetylsalicylic acid (3mg/kg per os). The animals were autoimplanted subcutaneously with 4x4-mm uterine fragments to induce endometriosis. The endometriomas were measured on days 1, 7, 14 and 21. The relative dry and wet weights of the endometrioma were used to evaluate response to the drug. Endometrial –like tissue was confirmed by histology. The greatest weight gain was observed on day 14 (relative wet weight: 29.1 ± 6.7mg%, relative dry weight: 5.3 ± 0.9mg %). Treatments were administered between day 5 and day 14.

RESULTS: The relative wet weight of the hemiuterus in the 10mg/kg estradiol group differed significantly from control and the other two estradiol groups ($p=0.0001$). In the medroxyprogesterone acetate group the weight decreased significantly but this decrease was not dose-dependent. Weight reduction was also significant in the triptorelin pamoate and the acetylsalicylic acid groups.

CONCLUSION: The model of subcutaneous endometriosis is reproducible, low-cost and easy to perform, and suitable for the study of pathophysiology and the effects of drugs.

Key words: Endometriosis. Disease Models, Animal. Rats.

Introduction

Endometriosis is defined as the presence of endometrial tissue (glands and stroma) outside the uterus[1]. Several theories have been proposed to explain the etiology of endometriosis[2]. Regardless of the theory, additional factors must be responsible for the expression and maintenance of the disease. The development and progression of endometriosis are associated with abnormal immune function[3].

Endometriosis may develop in any part of the organism, and one of the extra pelvic locations is the subcutaneous cell tissue, especially in incisions of pelvic surgeries. Endometrioma of the abdominal wall occurs most often in scars of Caesarean section (57%) and scars of hysterectomy (11%)[4,5]. The rate of recurrence is 4.3%[6].

The incidence of Caesarean section has increased worldwide, but nowhere as much as in Brazil[7]. Thus, in 2004, according to Knupp *et al*.[8], the incidence of Caesarean section in the state of Rio de Janeiro was 49.4% at public health facilities and 83.2% at private clinics[8]. In 2009, the overall Brazilian incidence of Caesarean section was 47%[9].

There are models of endometriosis in rabbits, mice, rats etc. In these models, various types of implants were used: in the peritoneum, under the renal capsule, in the mesentery, in the utero-ovarian ligament and in the eye ball[10]. In addition, chicken chorioallantoic membrane has been used to investigate the invasive potential of the endometrium and angiogenesis[11].

Garry[12] implanted human endometrium in the anterior chamber of the eye of sheep to study the implant and growth in its early stages through the cornea. After eight days of implantation, the development of new blood supply and new epithelial lining was observed. Some transplants developed new glands and invasion both in the iris and the cornea. The advantage of this model is to study the early stage of development in continuous fashion. Its disadvantage is that implants left for more than ten days are rejected by immune processes[12].

Nisolle *et al*.[13] implanted endometrial grafts in the peritoneal cavity of nude mice and removed them again after one, three and five days. On day 1, the stromal cells were attaching to the mesothelium; on day 3, epithelial and stromal cells had reorganized around the endometrial glands; on day 5, endometriotic cysts were seen, with greater proliferative activity in gland cells and a greater expression of vascular endothelial growth factor (VEGF) in stromal cells. These findings suggest that stromal cells are involved in the attachment process while gland cells are involved in lesion growth[13].

Banu *et al*.[14] implanted human endometrial cells in the abdominal cavity of nude mice. The cells had been collected from women in the proliferative stage of the menstrual cycle. The human origin of the cells observed in the endometrial lesions was confirmed by immunohistochemistry.

According to the most accepted theory, the presence of endometrial tissue in the peritoneal cavity, seen in humans and in some non-human primates, is required for the development of endometriosis[11]. Thus, primates have been used for the study of endometriosis, but the cost is very high. For this reason, there is a need for developing models using smaller animals, especially rodents which are less costly[11]. Monkeys develop endometriosis spontaneously, with lesions and sites identical to those which occur in human disease[15]. There is no doubt that the primate model is the best for studying endometriosis. However, In view of ethical considerations and high costs, research using this model is limited[11]. Unlike humans and other primates, rodents do not menstruate and, consequently, no endometrial blood is present in the peritoneal cavity. Nevertheless, endometriosis can be induced by implanting endometrial tissue[11].

Vernon and Wilson[16] were the first researchers to develop a model of endometriosis induced surgically in rats using autotransplantation. They removed fragments from a uterine horn and implanted them in several locations of the abdominal cavity. The model was proven by histological examination of the implants in which glands and stroma of a type similar to endometrium were verified[16].

To date, no standardized rat model of surgically induced subcutaneous endometriosis has been described in the literature. Thus, the objective of the present study was to develop and test a standardized rat model of subcutaneous endometriosis adequate for the study of pathophysiology and the effects of drugs.

Methods

The study protocol was previously approved by the Animal Research Ethics, Federal University of Ceara (UFC) School of Medicine and filed under entry 21/2011(August 29, 2011).

Fifty three-month-old female Wistar rats (*Rattus norvergicus*) weighing 150g were obtained from the UFC experimental animal facility and kept in cages in a controlled environment (circadian cycle, 24°C, food and water *ad libitum*).

The animals were anesthetized with 10% chloral hydrate i.p. and submitted to a 2.5cm longitudinal suprapubic incision starting 0.5cm above the pubis. A tunnel was made between the abdominal wall and the subcutaneous tissue, leading from the incision to the

right inguinal region. The abdominal wall was opened, the uterus was located, the uterine vessels of the left hemiuterus were ligated, and the latter was excised. The excised hemiuterus was then opened and a 4x4mm fragment was retrieved and grafted onto the muscle in the right inguinal region using mononylon thread 4-0. Finally, the abdominal wall was closed with two layers of suture using the same thread.

The experiment was performed with one control group and four treatment groups (n=10). Graft growth was quantified on post-surgical day 1, 7, 14 and 21. All animals were euthanized by an anesthetic overdose for tissue harvesting to determine the relative wet and dry weight (mg%) of the endometrioma and the right hemiuterus.

Following implantation of uterine tissue in the right inguinal region, as described above, animals in the treatment groups received estradiol (2.5 or 5 or 10mg/Kg sc), medroxyprogesterone acetate (0.5 or 2 or 5mg/Kg sc), triptorelin pamoate (0.18 or 0.56 mg/Kg sc) and acetylsalicylic acid (3mg/kg per os). Control group rats received saline 0.9% 1ml by gavage. All treatments were started on the fifth day after implantation and lasted for 9 days. The duration of the treatment coincided with the tumor growth curve observed, which peaked on the 14th day. The animals were euthanized and the relative wet and dry weight (mg%) of the endometrioma and the right hemiuterus was determined using a precision scale (Shimadzu, model AUW220D). We designed a control group only for histological analysis, because the endometriomas of the other groups were dried in an oven for measurement of dry weights.

Statistical analysis

Using the software GraphPad Prism for Windows 5.0, the normality of the data was verified with the D'Agostino-Pearson test, while the groups were compared with ANOVA, the Tukey multiple comparisons test and Student's *t* test. The level of statistical significance was set at 5% ($p < 0.05$).

Results

The relative wet and dry weight of the endometrioma and hemiuterus in each control group were expressed as mean values ± standard error of the mean (Table 1 and Figure 1).

TABLE 1 - Development of the relative wet and dry weight of the endometrioma compared to the relative wet and dry weight of the right hemiuterus in a subcutaneous endometriosis rat model.

Relative weight	Day 1 (mean ± SEM)	Day 7 (mean ± SEM)	Day 14 (mean ± SEM)	Day 21 (mean ± SEM)
Endometrioma wet weight (mg%)	8.3 ± 0.4 [a]	15.2 ± 4.4	29.1 ± 6.7 [b]	11.3 ± 4.6
Endometrioma dry weight (mg%)	1.4 ± 0.1 [a]	1.1 ± 0.3 [a]	5.3 ± 0.9 [b]	1.6 ± 0.6 [a]
Right hemiuterus wet weight (mg%)	72.6 ± 2.4	76.6 ±10.7	117.0 ±12.4	108.8 ±12.6
Right hemiuterus dry weight (mg%)	13.9 ± 0.5	14.0 ± 2.0	19.3 ± 1.7	20.8 ± 1.6

Day 14 vs. Day 1 with regard to the relative wet weight of the endometrioma: p=0.0198. Day 14 vs. all other days with regard to the relative dry weight of the endometrioma: p=0.0018.
Difference between the relative wet and dry weight of the right hemiuterus: non-significant.
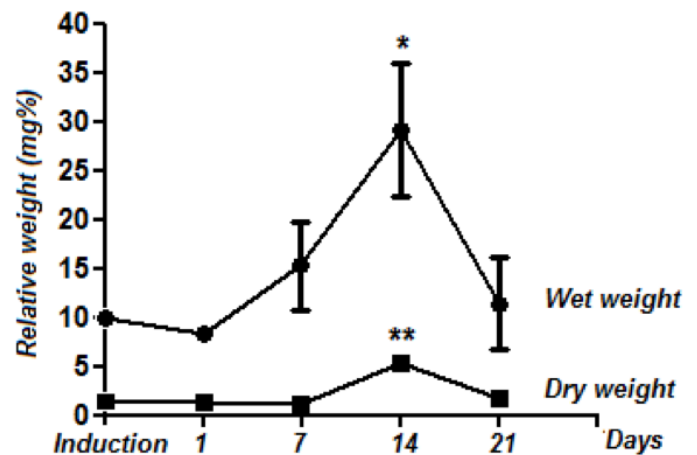One-way ANOVA and Tukey's multiple comparisons test. SEM=standard error of the mean.

FIGURE 1 - Growth curve of endometriomas in a subcutaneous endometriosis rat model.
*Day 14 compared to Day 1 with regard to the relative wet weight of the endometrioma: p=0.0198.
**Day 14 compared to all other days with regard to the relative dry weight of the endometrioma: p=0.0018. One-way ANOVA and Tukey's multiple comparisons test.

Relative wet and dry weight of the endometrioma and hemiuterus in each treatment group (estradiol, medroxyprogesterone acetate, triptorelin pamoate and acetylsalicylic acid) was expressed as mean values ± standard error of the mean (Tables 2, 3 and 4).

TABLE 2 - Effect of sexual steroids (estradiol and medroxyprogesterone acetate) on the development of the endometrioma and the right hemiuterus in a subcutaneous endometriosis rat model.

Relative weight	Estradiol (sc)			Medroxyprogesterone acetate (sc)			Control 0.9% saline per os
	2.5mg/kg	5mg/kg	10mg/kg	0.5mg/kg	2mg/kg	5mg/kg	2ml/day
Endometrioma wet weight (mg%)	36.6 ± 4.9	56.3 ± 20.1	173.9 ± 69.5	13.5 ± 2.5	14.3 ± 20.0	15.3 ± 7.1	29.1 ± 6.8
Endometrioma dry weight (mg%)	3.9 ± 1	9.1 ± 3.8	27.6 ± 11.0	2.6 ± 0.5	3.7 ± 1.0	2.68 ± 1.0	5.3 ± 1.0
Right hemiuterus wet weight (mg%)	166.5 ± 16.0	185.9 ± 16.0	223.1 ± 32.0	74.5 ± 5.0	94.4 ± 8.0	73.9 ± 12.0	117.0 ± 12.0
Right hemiuterus dry weight (mg%)	27.7 ± 2.6	31.1 ± 2.4	42.3 ± 4.1	13.4 ± 2.5	14.0 ± 0.6	15.3 ± 3.4	19.3 ± 1.7

Wet weight of the endometrioma in the 10mg estradiol group vs. all other groups: p=0.0064. Dry weight of the endometrioma in the 10mg estradiol group vs. all other groups: p=0.0032. Wet weight of the endometrioma in the medroxyprogesterone acetate group vs. control: p=0.0069. Dry weight of the endometrioma in the medroxyprogesterone acetate group vs. control: p=0.0013. Wet weight of the right hemiuterus in the estradiol group vs. all other groups: p=0.0001. Dry weight of the right hemiuterus in the estradiol group vs. all other groups: p=0.0001.Wet weight of the right hemiuterus in medroxyprogesterone acetate group vs. control: p=0.0398.
Dry weight of the right hemiuterus in the medroxyprogesterone acetate group vs. control: p=0.1462.
One-way ANOVA and Tukey's multiple comparisons test; s.c. =subcutaneous

TABLE 3 - Effect of triptorelin pamoate on the development of the endometrioma and the right hemiuterus in a subcutaneous endometriosis rat model.

Relative weight	Triptorelin pamoate sc		Control 0.9% saline per os
	0.18 mg/kg	0.56 mg/kg	2 ml/day
Endometrioma wet weight (mg%)	20.0 ± 4.0	10.8 ± 1.8[a]	29.1 ± 6.7[b]
Endometrioma dry weight (mg%)	5.2 ± 1.5	1.8 ± 0.2[a]	5.3 ± 0.9[b]
Right hemiuterus wet weight (mg%)	41.1 ± 5.3[a]	66.1 ± 6.7[a]	117.0 ± 12.4[b]
Right hemiuterus dry weight (mg%)	7.7 ± 0.9[a]	1.9 ± 0.9[a]	19.3 ± 1.7[b]

Wet weight of the endometrioma in the 0.56mg/Kg triptorelin pamoate group vs. control: p=0.0095.
Dry weight of the endometrioma in the 0.56mg/Kg triptorelin pamoate group vs. control: p=0.0038.
Wet weight of the right hemiuterus in both triptorelin pamoate groups vs. control: p=0.0119
Dry weight of right hemiuterus in both triptorelin pamoate groups vs. control: p=0.0054.

TABLE 4 - Effect of acetylsalicylic acid (ASA) on the development of the endometrioma and the right hemiuterus in a subcutaneous endometriosis rat model.

Relative weight	Acetylsalicylic acid (ASA) per os	Control 0.9% saline per os
	3 mg/kg	2 ml/day
Endometrioma wet weight (mg %)	12.8 ± 2.0[a]	29.1 ± 6.7[b]
Endometrioma dry weight (mg %)	2.0 ± 0.4[a]	5.3 ± 0.9[b]
Right hemiuterus wet weight (mg %)	82.1 ± 7.7	117.0 ± 12.4
Right hemiuterus dry weight (mg %)	15.7 ± 2.2	19.3 ± 1.7

Wet weight of the endometrioma in the ASA group vs. control: p=0.0218 (Student's t test)
Dry weight of the endometrioma in the ASA group vs. control: p=0.0092 (Student's t test)
Wet weight of the right hemiuterus in the ASA group vs. control: p=0.0655 (Student's t test)
Dry weight of the right hemiuterus in the ASA group vs. control: p=0.3231 (Student's t test)

Macroscopic aspects of the endometriomas differed according to the day of observation. Thus, on day 1, control endometriomas were whitish, loose or lightly attached and unvascularized. On day 7, they were pinkish-white, unvascularized, firmly attached, sometimes with areas of hemorrhage. On day 14, they were pinkish, vascularized (around and on the surface) and sometimes cystic in appearance, measuring 5.7 mm on the average. The aspects on day 21 were like those on day 14, but the size and weight were reduced (Figure 2).
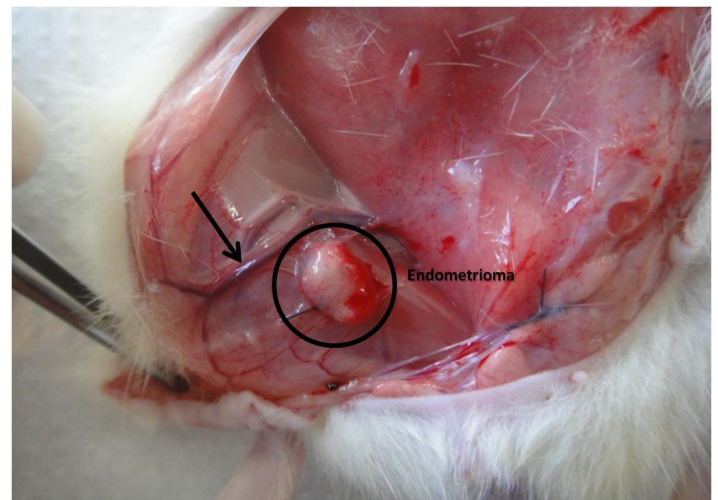
FIGURE 2 - Macroscopic aspects of endometrioma in a subcutaneous endometriosis rat model. Endometrioma from control group on day 14: pinkish and vascularized, even on the surface. The thick vessel (arrow) is part the animal's anatomy.

Endometriosis was confirmed by the following microscopic findings: i) numerous mature endometrial glands similar to those observed in the topical endometrium, surrounded by a small number of endometrial stroma, ii) exuberant granulation tissue around endometriotic foci, iii) areas of fibrosis and sporadic hemorrhagic foci, and iv) marked presence of adipose tissue (Figure 3).
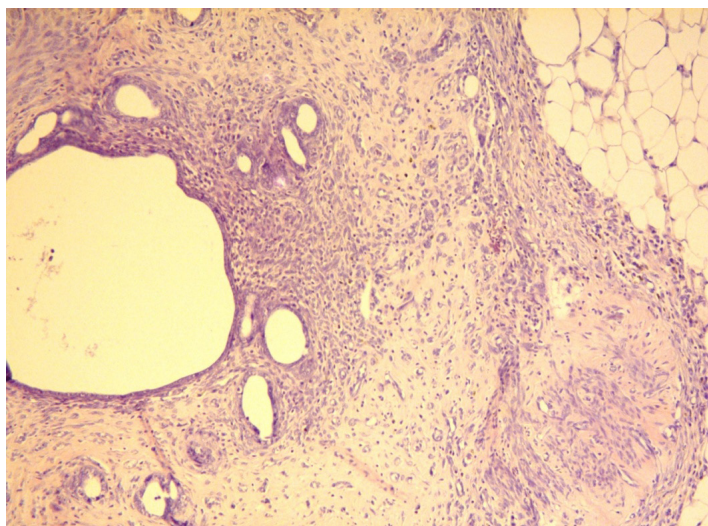
FIGURE 3 - Histological aspects of endometrioma in a subcutaneous endometriosis rat model. Stained with hematoxylin and eosin (x100). Mature endometrial glands similar to those observed in the topical endometrium, surrounded by endometrial stroma (left), adipose tissue (upper right corner), and granulation tissue between the endometrium like tissue and the adipose tissue.

Discussion

Xu *et al*.[17] used mice with deficient function of T and B lymphocytes using heterologous endometrium (human) and implanting it into the subcutaneous pouch , but did not describe how the bag. In this study, were used animals with modified immune system and heterologous endometrium, making the method more complex than this experiment. Liu *et al*.[18] used heterologous endometrium incubated with adenovirus and injected subcutaneously. In this study, one micro-organism was used and this could modify the pathophysiology of endometriosis[18]. Wang *et al*.[19] used nude mice implanted autologous endometrium in the subcutaneous pouch, but did not describe how the bag. In this work, it was also used animal with modified immune system may therefore alter the pathophysiology of endometriosis[19]. Fu *et al*.[20] implanted autologous fragments of uterus in a subcutaneous pocket, but ommited details of the bag's construction[20].

In the control group, the growth curve showed a gradual increase of the relative wet weight of the endometrioma, with a statistically significant difference between day 14 and day 1 (p=0.0198). The relative dry weight of the endometrioma also increased gradually, with a significant peak on day 14 in relation to all other days (p=0.0018) (Figure 1).

No significant increase was observed in the relative wet and dry weight of the hemiuterus in any group. This finding was expected since the controls were not treated with exogenous hormones (estrogen) to stimulate uterine growth. The estrogen

produced by the endometrioma was probably limited to local action and likely barred from entering the blood stream by the fibrosis surrounding the endometrioma[21].

The relative wet weight of the endometrioma in the 10mg/kg estradiol group differed significantly from that of the control group and the other estradiol groups (p=0.0064). However, the difference between the groups receiving estradiol at 2.5mg/kg and 5mg/kg, and between these two groups and the control, was not significant. In other words, doses up to 5mg/kg were insufficient to develop the endometriomas. The relative dry and wet weight of the endometrioma in the 10mg/kg estradiol group displayed similar patterns (p=0.0032).

The relative wet weight of the hemiuterus in the 10mg/kg estradiol group differed significantly from that of the control group and the other two estradiol groups (p=0.0001). The groups receiving estradiol at 2.5mg/kg and 5mg/kg were statistically similar, but differed significantly from the control and the 10mg/kg estradiol group (illustrated by the ascending growth curve in relation to the control), suggesting a response to estradiol stronger than that of the endometrioma. The ascending curve observed in this experiment matches the findings of Cason *et al*.[22]. This difference in response could probably be explained by the presence of fibrosis in the endometriomas which does not respond to the estrogens. Bergqvist *et al*.[23] also found fibrosis in their histological evaluation of endometriosis. The relative wet and dry weight of the hemiuterus in animals treated with estradiol displayed similar ascending curves (p=0.0001).

The relative wet weight of the endometrioma in the three medroxyprogesterone acetate groups (0.5mg/kg, 2mg/kg and 5mg/kg) decreased significantly in relation to the control (*p*=0.0069), but no difference was observed comparing subgoups treated with increasing doses of medroxyprogesterone acetate. The relative dry weight of the endometrioma in the three medroxyprogesterone acetate groups (0.5mg/kg, 2mg/kg and 5mg/kg) decreased significantly in relation to the control (p=0.0013). This is a surprising finding considering the resistance to progesterone in endometriosis and the great difference between the smallest dose (0.5mg/kg) and the highest dose (5mg/kg). Perhaps 0.5mg/kg is a high dose for this type of animal (rat) or, due to resistance to progesterone, maximum response is achieved with only 0.5mg/Kg. Even when the dose was increased, the response was the same.

The relative dry weight of the hemiuterus in the three medroxyprogesterone acetate groups (0.5mg/kg, 2mg/kg and 5mg/kg) did not differ significantly in relation to each other, nor to the control. This is probably because the hemiuterus was not affected with endometriosis. Without endometriosis, there

is no inflammatory reaction. Thus, without inflammation, there would be no accumulation of inflammatory liquid and cells with their secretory products, because progesterone, which has anti-inflammatory properties[24], would not be able to act to reduce the weight. Another explanation might be that normal endometrial and myometrial tissues do not express aromatase. In contrast, endometriotic foci produce aromatase thereby increasing the lesion. Thus, in the absence of injury there is no local estrogen in the uterus to be antagonized and no proliferative action to be inhibited by progesterone[25,26].

The relative wet weight of the endometrioma in the two triptorelin pamoate groups (0.18mg/kg and 0.56mg/kg) decreased, but the difference was only significant in relation to the control at the highest dose (p=0.0095 and p=0.0038, respectively). The reduction in the weight of the endometriomas with triptorelin pamoate was an expected result, in the case of development of the implant, because triptorelin pamoate provokes a blockage of the release of gonadotropins, leading to a reduction in the production of estrogen[27]. Thus, it could not stimulate the endometrioma, which is estrogen-dependent. Surprisingly, triptorelin pamoate was associated with the greatest reduction of weight of the endometrioma and hemiuterus among the drugs used to block endometriotic development. The relative wet and dry weight of the hemiuterus in the groups treated with triptorelin pamoate at 0.18mg/kg and 0.56mg/kg was significantly reduced in relation to the control (p=0.0119 and p=0.0054, respectively). With regard to the hemiuterus, this may be explained by the efficient blockage of gonadotropin release, leading to a lack of estrogen to which the uterus is responsive. The fact that triptorelin pamoate reduced weight more than any other substance (even in healthy hemiuterus) reflects the strength of the hormonal blockage produced[27].

The relative dry and wet weight of the endometrioma in the ASA group (3mg/kg) differed significantly from the control (p=0.0218 and p=0.0092, respectively). The same did not occur with the relative dry and wet weight of the hemiuterus. The reduction of the weight of endometriomas treated with ASA is important to validate the model of subcutaneous endometriosis since ASA has no effect on estrogen production. ASA acts by blocking the inflammatory process, demonstrating that endometriosis starts with and is maintained by inflammation. ASA prevents inflammation by inhibiting one of the most important transcription factors: NF-kB[28]. When activated, NF-kB releases many pro-inflammatory cytokines[29]. ASA also inhibits prostaglandin production. It irreversibly inhibits COX-1 and COX-2, which transform aracdonic acid into PGH2, a precursor of PGE2. Prostaglandin E2 is a potent aromatase stimulator, which in turn

increases estrogen production [21]. Since endometriomas develop in response to hormonal factors (estrogen) and inflammatory factors (proinflammatory cytokines), it is conceivable that the blockage of the inflammatory factor by ASA leads to a significant reduction in the weight of the endometrioma. The observed lack of reduction in the weight of the hemiuterus highlights the importance of the inflammatory process in the composition of endometriotic lesions. The weight of the hemiuterus remained unchanged because the organ was not affected with endometriosis and could not respond to an anti-inflammatory drug.

Conclusion

The model of subcutaneous endometriosis is reproducible, low-cost, easy to perform, and suitable for the study of pathophysiology and the effects of drugs.

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