Effect of anti-TNF-α on peritoneal endometrial implants of rats

Efeito do anti-TNF-α em implantes endometriais no peritônio de ratas

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A B S T R A C T

Objective: To evaluate the effect of anti-TNF-α in the treatment of endometrial implants in the peritoneum of rats. **Methods**: Endometrial implants were surgically induced in 120 female Wistar-Albino rats. The animals were randomly divided into four groups. Group C (n = 36) received an intraperitoneal injection of 0.2 ml of saline. Group L (n = 41) received a subcutaneous injection of 1mg/kg of leuprolide. Group I5 (n = 20) received a subcutaneous injection of 5mg/kg of monoclonal anti-tumor necrosis factor (TNF) α (infliximab). Group I10 (n = 20) received a subcutaneous injection of 10mg/kg of infliximab. The rats were sacrificed after 21 days to assess the size of the implants and the expression of TNF. **Results**: Treatment with leuprolide (group L) promoted an absolute reduction in the surface area of the implant when compared with group C (+14 mm vs. 0mm, p = 0.013) and group I10 (+14 mm vs. +5 Mm, p = 0.018). Likewise, a percentage reduction of surface area of the implant was observed comparing group L with group C (+33.3% vs. 0%, p = 0.005) and group I10 (+33.3% vs. +18.3%, p = 0.027). Treatment with infliximab was not able to decrease the surface area of the implants when compared with group C. The expression of TNF-α in groups L, I5 and I10 was lower than in group C (505.6 mm^2 vs. 660.5 mm^2 vs. 317.2 mm^2 vs. 2519.3 mm^2, respectively; p <0.001). **Conclusion**: The anti-TNF-α therapy reduced the expression of TNF-α in endometriotic implants, but did not reduce the surface area of the lesion.

Key words: Endometriosis. Tumor necrosis factor alpha. Endometrial stromal tumors. Peritoneum. Animal experimentation.

INTRODUCTION

Endometriosis is a benign gynecological disease defined by the presence of endometrial tissue, consisting of glandular epithelium and/or stroma outside the uterine cavity[1,2]. This condition is predominantly found in women of reproductive age and affects 70-10% of all women, 71 to 87% of women with chronic pelvic pain, and 38% of infertile women[3].

Although the pathophysiology of endometriosis is poorly understood, the ability to develop endometrial implants in ectopic locations may be related to aberrant immunoreactivity resulting from the own injuries[4]. The peritoneal environment of women with endometriosis contains a rich cocktail of cytokines, angiogenic substances and growth factors[5] that are produced mainly by peritoneal immune cells and endometriotic cells[6].

The tumor necrosis factor α (TNF-α) is a key cytokine in a variety of inflammatory processes, and it is likely that it has a role in the pathogenesis of endometriosis[4]. Numerous researchers have shown that concentrations of TNF-α are elevated in the peritoneal fluid of patients with endometriosis and that its levels correlate with disease stage[7].

The anti-inflammatory effects of TNF-α blocking by monoclonal antibodies (i.e., infliximab) or TNF-α soluble receptors (i.e., etanercept) have been demonstrated in vivo, in animal models and in humans[8]. The clinical effectiveness of TNF-α blocking has been demonstrated in inflammatory conditions such as Crohn's disease and rheumatoid arthritis, but not in severe endometriosis[9]. In baboons with laparoscopically confirmed endometriosis, TNF-α blocking with p55 soluble receptors resulted in inhibition of development and growth of endometrial implants[10]. The size of peritoneal red lesions decreased compared with the control group[11], but there was no increase in pregnancy rates[12].

This association between endometriosis and increased cytokines opens the possibility for new proposals for clinical treatment, particularly using anti-cytokine therapies. The inflammation mediated by TNF-α may be a causative factor of pain associated with endometriosis and

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TNF-α blocking appears to inhibit the development of the disease in animal models.

The objective of this study was to evaluate the effect of anti-TNF-α in the treatment of surgically induced endometrial implants in the peritoneum of rats.

METHODS

One hundred and twenty Wistar-Albino rats weighing between 200 and 250g were used for the experiment. The animals were from the Pontifícia Universidade Católica do Paraná (PUC-PR) Central Vivarium. The rats were kept in proper cages with five animals, under controlled temperature, humidity and ambient light, and fed water and food *ad libitum*. After an adjustment period of one week, endometrial implants were surgically induced.

The experimental study was conducted by the Division of Biological Sciences at PUC-PR, according to Federal Law 11794 and the guidelines of the Brazilian College of Animal Experimentation. The experimental protocol used in this study was approved by the Ethics Committee on Animal Research of the Department of Biological Sciences at PUC-PR on August 19, 2008 (opinion number 243).

Surgical procedures

First operation

Surgical procedures were performed at the Laboratory of Experimental Surgery, Department of Operative Technique, Department of Health Sciences at PUC-PR.

The endometrial implants were surgically induced using the technique described by Jones[13] in 1984. The animals were anesthetized with intraperitoneal injection of 50mg/kg ketamine and xylazine 7mg/kg[14]. The animals were attached to the surgical table in the supine position, the legs in abduction.

We performed mechanical cleaning and antisepsis of the operative area using 1% iodine-active povidone-iodine. After the placement of surgical fields, a midline incision was made approximately 3 cm, starting 2 cm above the pubic bone of the animal, for the exposure of the reproductive organs. The bicornuate uterus was identified and the blood vessels of the left uterine horn were ligated using 3-0 vicryl. A 10mm segment of the middle third of the left uterine horn was resected and immersed in 0.9% saline solution at 4° C for about 2 minutes. This segment was opened on the antimesenteric border, leading to a flap, where a 5x5mm segment was removed. The implant was sutured to the abdominal wall on the right side of the rat using two simple stitches of 6-0 monofilament nylon, near a blood vessel, so that the endometrial aspect faced the abdominal cavity (Figure 1).

After checking hemostasis of the left uterine horn and the cleaning of the abdominal cavity, the abdominal wall was sutured, the muscle-aponeurotic plane with 3-0 polyglactin and skin sutures with 3-0 monofilament nylon. No hormone supplementation was administered before or after laparotomy.

After the first operation, all animals were observed for 21 days without any medication in animal facilities, except for postoperative analgesia with intraperitoneal injection of dipyrone at 1 mg per 100 grams of body weight every eight hours.

Three rats died during the period between the first and second surgery. On the morning of the second surgery, the animals were randomly divided into four groups: Group C, control (n = 36) received an intraperitoneal injection of 0.2 ml of saline 0.9% near the implant, Group L, positive control (n = 41) was treated with GnRH agonists (gonadotropin releasing hormone) with a single subcutaneous injection of leuprolide acetate in depot formulation (1 mg/kg body weight; Lucrino, Abbott, Brazil®). The dose of leuprolide acetate was based on a previous study in which 1 mg/kg was found to be optimal for rats[15], Group I5, the anti-TNF-α 5mg/kg (n = 20), received a subcutaneous injection of 5mg/kg of monoclonal anti-TNF-α (infliximab)[16]; Group I10, the anti-TNF-α 10mg/kg (n = 20), received a subcutaneous injection of 10mg/kg of anti-TNF monoclonal antibody (infliximab)[16].

Second operation

Each rat was anesthetized and a laparotomy was performed to confirm the macroscopic lesion development and viability of endometriotic implants. The surface area of the implants was measured (height x width) in millimeters and recorded.

Group C received an intraperitoneal injection of 0.2 ml of saline solution near the implant, the L group received a subcutaneous injection of leuprolide acetate (1mg/kg), the group I5 received a subcutaneous injection of anti-TNF-α (5 mg/kg) and group I10 received a subcutaneous injection of anti-TNF-α (10mg/kg).

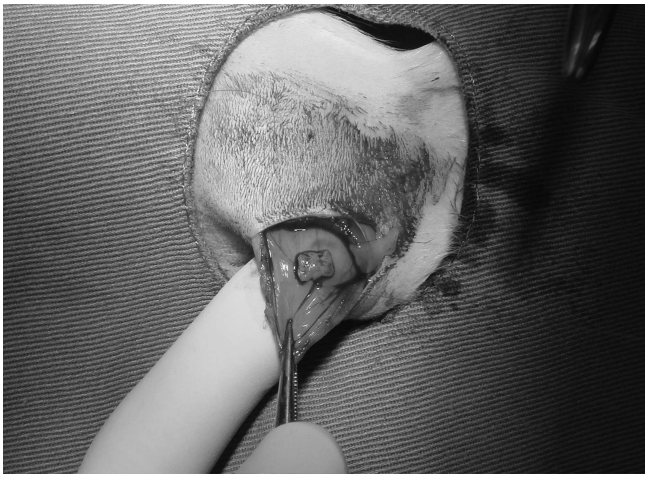

Figure 1 - *Transplantation of the endometrium on the right side of the peritoneal cavity of rats.*

The laparotomy was closed and all the rats were observed for 21 days.

Third operation

Three weeks after the second operation, the third laparotomy was performed. The effect of treatment on endometriotic implants was assessed by measuring the surface area of the implants (Figure 2). The implants were excised, fixed in 10% formalin and processed for paraffin embedding for subsequent morphological analysis at the Laboratory of Experimental Pathology, PUC-PR. After surgery, the rats were euthanized with ketamine.

Histological analysis

The pathologist assigned to assess the samples was unaware of the treatment groups. Endometrial implants were fixed in formalin prepared in paraffin blocks, sectioned at a thickness of 5mm (4 sections per sample), stained with hematoxylin and eosin and evaluated with fluorescent microscopy. The persistence of epithelial cells in endometrial implants was semiquantitatively evaluated[17] as follows: score 3 = well-preserved epithelial layer; score 2 = moderately preserved epithelium with leukocyte infiltrate; score 1 = badly preserved epithelium (occasional epithelial cells only); and score zero = no epithelium (Figure 3).

For immunohistochemical analysis of the samples we used the matrix arrangement in tissue samples or tissue microarray (TMA), described by Kononen *et al.*[18] in 1998. This is the construction of a paraffin block containing fragments of cylindrical tissue samples obtained from dozens of original paraffin blocks. The tissue cylinders are arranged in the receiver block following a pre-determined order.

To make the tissue microarray slides, the endometrial implant was identified in the histology exam and a 4 mm punch of the wall of the endometriotic cyst was performed. Each slide was electrically charged with the prepared material obtained from the punch of 15 animals. The expression of TNF-α was assessed using a commercially available immunohistochemistry kit (mouse anti-human TNF alpha, Synapse Biotecnologia Ltda, São Paulo, Brazil®) and counted using digital morphometric analysis. The images were scanned with a digital color camera (TV 0.45x Nikon lens®, Tokyo, Japan) adapted to the microscope (Nikon® Eclipse E600, Tokyo, Japan). The images were analyzed using the program Image Pro (Media Cybernetics), allowing the expression of TNF-α to be counted and integrated by area.

Statistical analysis

The four groups were compared according to the following variables: absolute reduction of the injured area, calculated by subtracting the area of injury in the third operation (after treatment) in the second operation area. A positive value indicated a reduction in the area of the lesion and a negative value indicated an increase in the area; percentage reduction of the injured area, calculated by dividing the absolute reduction in lesion area by the area in the second surgery. The result was multiplied by 100. A positive result indicated a reduction in the area of the lesion and a negative result indicated an increase in it; expression of TNF-α in immunohistochemistry.

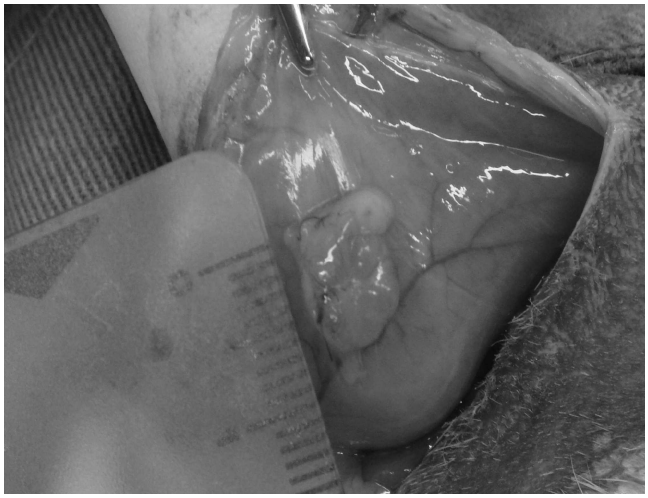

Figure 2 - *Measurement of the area of endometrial implants in the third surgery.*

The results were evaluated for normal distribution by Kolmogorov-Smirnov test. Parametric variables with normal distribution were tested by analysis of variance (ANOVA) and post hoc analysis (Bonferroni correction) to define any differences between the groups. Variables that did not follow the normal distribution were analyzed by nonparametric Kruskal-Wallis test with Bonferroni correction. P values < 0.05 were considered statistically significant. The variables with normal distribution were expressed as mean ± standard deviation. The variables that did not follow the normal distribution were expressed as median (minimum - maximum).

RESULTS

Endometrial implants produced viable lesions in all animals. They were well vascularized and adherent to the peritoneum of the abdominal wall by the time of the second laparotomy.

The area of the implants was measured on the first (area 1), second (area 2) and third (area 3) operations (Table 1). There was no difference in the transplanted segment of the endometrium in the first operation. There was an increase in the area of the implant between the first and second surgeries, but the implants in groups C and G grew more than implants of groups I5 and I10 ($p < 0.001$). Therefore, for comparison of treatment with leuprolide and anti-TNF-á, the absolute area of the lesion in the third procedure was not used, but the absolute and percentage reductions of the lesion area.

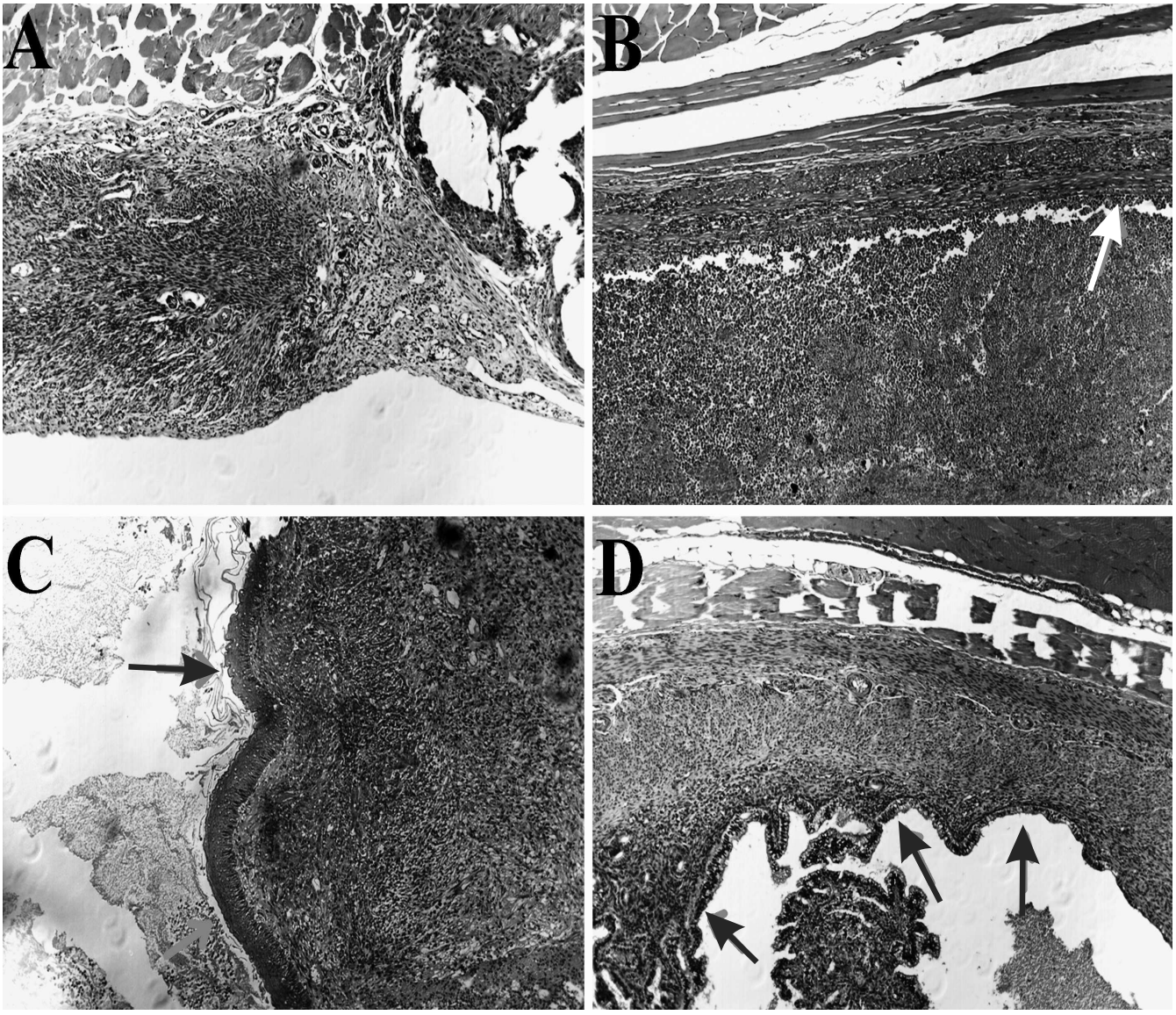

Figure 2 - *Semiquantitative evaluation of the endometrial implant (10X magnification). (A) score zero; (B) Score 1; (C) score 2; (D) Score 3. The arrows indicate areas with preserved epithelium.*

Semiquantitative analysis of the persistence of epithelial cells in endometrial implants (Table 2) showed no statistically significant difference between groups (p = 0.06).

When comparing the four groups we observed absolute and percentage reductions were statistically significant in the area of the implant from second to third surgery (Table 3). There was a statistically significant absolute reduction in the area of the implant only when the group L was compared to group C (p = 0.013) and to group I10 (p = 0.018). Similarly, there was a statistically significant reduction in the percentage of the implant when

Table 1 - Area of the endometrial implant in the three surgical procedures.

	Area 1 (mm²)	Area 2 (mm²)	Area 3 (mm²)
Group C	24 (24 a 25)	54±33.6	42 (6 a 204)
Group L	24 (24 a 25)	44.8±21.1	24 (2 a 100)
Group I5	25 (25 a 25)	27.8±11.1	22 (4 a 36)
Group I10	25 (25 a 25)	28.7±10.1	24.5 (9 a 56)
p value	1	<0.001	0.001

Table 2 - Semiquantitative Assessment of persistence of epithelial cells in endometrial implants.

	Score 0	Score 1	Score 2	Score 3	Total
Group C	4	5	7	20	36
Group L	5	4	1	31	41
Group I5	2	4	1	13	20
Group I10	2	1	7	10	20

Table 3 - Absolute and percentage reductions of the implant areas.

	Absolute reduction (mm^2)		Percentage reduction (%)	
Group C	0	(-51 a +56)	0	(-66.7 a +78.6)
Group L	+14	(-32 a +80)	+33.3	(-344.4 a +93.7)
Group I5	+6.5	(-4 a +25)	+23.3	(-20 a +73.3)
Group I10	+5	(-26 a +25)	+18.3	(-108.3 a +62.5)
p value	0.04		0.02	

group L was compared to group C (p = 0.005) and to group I10 (p = 0.027). The other comparisons between groups revealed no statistical difference in absolute or percentage reductions of implant areas.

Immunohistochemical analysis of samples using the matrix arrangement in tissue samples allowed the objective evaluation of TNF-α expression (Table 4). There was a statistically significant reduction in the expression of TNF-á in endometrial implants when comparing group L to group C (p <0.001), group I5 to group C (p <0.001) and group I10 to group C (p <0.001).

Table 4 - Expression of VEGFR and TNF-α in the endometrial implants.

	TNF-α (μm^2)	
Group C	2519.3	(109.4 a 13647.1)
Group L	505.6	(11.9 a 4811.1)
Group I5	660.5	(68 a 10678.2)
Group I10	317.2	(115.7 a 2037.2)
p value	<0.001	

DISCUSSION

Medical treatment plays a substantial role in the management of pain associated with endometriosis[19,20]. Medroxyprogesterone acetate, danazol, oral contraceptives and GnRH analogues are all effective in reducing the intensity of the painful symptoms caused by endometriosis. However, the recurrence of symptoms is common after discontinuation of medical treatment[19,20] and it has no benefit in endometriosis-associated infertility[21]. Therefore, new clinical approaches, more effective than hormonal ones, are needed to better control of this disease.

Animal models allow the study of events involving the pathophysiology of endometriosis, as well as new therapeutic approaches for this disease[15,22]. Animal models using non-primates and primates have applied for the study of endometriosis for years. Non-primates, including rodents, do not have spontaneous endometriosis, but it can be induced using autologous uterine tissue or human endometrium. Primates spontaneously develop endometriosis, and the disease can also be induced for research purposes. The advantages of using a non-primate model include its relatively low cost and ability to establish the endometriosis-like lesions[22]. Autotransplantation of uterine segments to the peritoneal cavity is a well-established method for the induction of endometrial implants in rats[23-26].

Although the term "experimental endometriosis" is used in the literature, this study involved the implantation of endometrial fragments into the peritoneal cavity of healthy rats. The actual correlation between these implants of endometrial tissue and surgically induced endometriosis that develops in women is uncertain. However, the widespread use of research protocols of "experimental endometriosis" in animal models is justified, since the complete evaluation and monitoring of endometrial lesions in humans is difficult and often not feasible due to the invasiveness of the diagnostic methods (laparoscopy or laparotomy).

The present study demonstrated that in an animal model using rats treatment with monoclonal anti-TNF-α (infliximab) decreased the expression of TNF-α in endometrial implants, but was not effective in reducing their area. Just leuprolide therapy was able to reduce the surface area of implants.

There is substantial evidence that immunological factors play an important role in the pathogenesis of endometriosis[6,27]. The increase in inflammatory cytokines

(IL-1a, IL-6, IL-8. IL-18, TNF-α) has been reported in patients with endometriosis[6,28,29]. TNF-α plays a key role in many inflammatory diseases. It is produced by macrophages, natural killer cells, neutrophils and several non-hematopoietic cells, including epithelial and stromal endometrial cells. Although TNF-α was initially identified by its cytotoxicity against certain cell lines, its primary function (associated with IL-1) is to initiate the cascade of factors associated with inflammatory response. It has been shown that TNF-α stimulates prostaglandin production by cultured endometrial epithelial cells[30] and promotes the adherence of cultured stromal cells to mesothelial cells[31]. These findings indicate that TNF-α may, as a factor of the pelvic fluid, mediate the establishment of endometrial implants. Several authors have shown that concentrations of TNF-α in peritoneal fluid of women with endometriosis are elevated[32-34]. Some researchers also suggest that levels of TNF-α in peritoneal fluid may be correlated with the intensity of dysmenorrhea[35].

In our study, we demonstrated a decrease in the expression of TNF-α in endometrial implants in groups L (505.6 μm^2, p <0.001), I5 (660.5 μm^2, p <0.001) and I10 (317.2 μm^2, p <0.001) compared with group C (2519.3 ìm^2). However, the reduced expression of TNF-α was similar using leuprolide therapy and anti-TNF-α at both doses. Despite this reduction in the expression of TNF-α, there was no significant reduction in absolute or percentage surface area of endometrial implants in the group treated with anti-TNF-α when compared to the control group. Only treatment with leuprolide reduced the surface area of the endometrial implants compared with the control group and I10 group. The group treated with a dose of 10mg/kg of anti-TNF monoclonal antibody showed the greatest decrease in expression of TNF-α in the implant, and even then, treatment with leuprolide displayed a statistically significant reduction in surface area of the endometrial implants compared with this group, showing that only a reduction of inflammation is not sufficient to reduce the area of endometrial lesions. The doses of 5mg/kg and 10mg/kg were based on a previous study using infliximab in an experimental model of colitis in rats[16].

Negative findings had already been found with the use of anti-TNF-α in endometriosis. In 2008, Koninckx *et al*.[8] evaluated 21 women with endometriosis, with important pain symptoms and rectovaginal nodule of at least 1cm. They received infliximab (5mg/kg – n = 14) or placebo (n = 7) and underwent surgery three months later. The authors observed no reduction of endometriosis-associated pain in patients treated with infliximab compared to the placebo group and also reported that the lack of efficacy of infliximab as a treatment for pain associated with endometriosis was unexpected, since they always believed that inflammation would be a major cause of pain in endometriosis. Falconer *et al*. [12] studied the role of TNF-α inhibition by using a monoclonal anti-TNF in subfertility associated with endometriosis in baboons and found that there was no significant improvement in pregnancy rates, birth rates per cycle, median time to pregnancy and cumulative pregnancy rate when compared to placebo[36]. A systematic review aimed to determine the effectiveness and safety of anti-TNF-α in the management of pelvic pain associated with endometriosis found no evidence to support the use of these drugs in the treatment of women with endometriosis in order to improve pelvic pain.

In our animal model we demonstrated the reduced expression of TNF-α in endometrial implants using anti-TNF-α therapy and leuprolide. However, the TNF-α reduction promoted by anti-TNF-α therapy did not correlate with the regression of surface area of the lesions, contrary to what was observed with the use of leuprolide. Consequently, simply reducing local inflammation seems to be insufficient to reduce the surface area of endometrial implants.

R E S U M O

Objetivo: *Avaliar o efeito da terapia anti-TNF-α no tratamento de implantes endometriais no peritônio de ratas.* ***Métodos:*** *Os implantes endometrióticos foram induzidos cirurgicamente em 120 ratas Wistar-Albino. Os animais foram aleatoriamente distribuídos em 4 grupos. O grupo C (n=36) recebeu uma injeção intraperitoneal de 0,2ml de solução salina. O grupo L (n=41) recebeu uma injeção subcutânea de 1mg/kg de leuprolide. O grupo I5 (n=20) recebeu uma injeção subcutânea de 5mg/kg de anticorpo monoclonal anti-fator de necrose tumoral (TNF) a (infliximab). O grupo I10 (n=20) recebeu uma injeção subcutânea de 10mg/kg de infliximab. As ratas foram sacrificadas após 21 dias para se avaliar o tamanho dos implantes e a expressão do TNF-α.* ***Resultados:*** *O tratamento com leuprolide promoveu uma redução absoluta na área de superfície do implante comparado com o grupo C (+14mm vs. 0mm;* p*=0,013) e com o grupo I10 (+14mm vs. +5mm;* p*=0,018). Da mesma forma, uma redução percentual da area de superfície do implante foi observada comparando o grupo L com o grupo C (+33,3% vs. 0%;* p*=0,005) e com o grupo I10 (+33,3% vs. +18,3%;* p*=0,027). O tratamento com infliximab não foi capaz de diminuir a área de superfície do implante comparado com o grupo C. A expressão de TNF-α reduziu nos grupos L, I5 e I10 comparado com o grupo C (505,6μm^2 vs. 660,5μm^2 vs. 317,2μm^2 vs. 2519,3μm^2, respectivamente;* p*<0,001).* ***Conclusão:*** *A terapia anti-TNF-α reduziu a expressão de TNF-α nos implantes endometrióticos mas não reduziu a área de superfície da lesão.*

Descritores*: Endometriose. Fator de necrose tumoral alfa. Tumores do estroma endometrial. Peritônio. Experimentação animal.*

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