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Original Article

Bone marrow derived mononuclear stromal cells and experimental model of deep endometriosis

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Abstract: Objective: To evaluate the effect of the treatment with mononuclear bone marrow stromal cells and the expression of metalloproteinases MMP-1, MMP-9 and the factor TGF-β1 in Wistar rats submitted to induction of deep/retrocervical endometriosis. Methods: A total of 120 female Wistar rats were induced to a deep endometriosis and after development of the disease, the animals were randomized into three different groups, referring to the treatment applied: saline (n = 38), leuprolide (n = 30) and stem cells (n = 35). Results: There was a significant difference in reduction of implant in the stem cell (P = 0.017) and leuprolide (P = 0.025) groups. However, there was no significant difference in the saline solution group (P = 0.771). In metalloproteinases expression there was no significant difference among groups. Conclusions: Treatment with bone marrow derived-mononuclear stromal cells was effective in reducing ectopic implant area. However, there were no significant differences between groups in relation to the expression of MMP-1, MMP-9 and TGF-β1.

Keywords: Endometriosis, mononuclear bone marrow stromal cells, metalloproteinases, transforming growth factor-β, experimental model

Introduction

Endometriosis is a frequent gynecological disease, determined by the presence of endometrial tissue, characterized by glands and/or stroma outside of the uterine cavity, inducing a chronic inflammatory reaction and exerting a function similar to the normally situated endometrium [1, 2]. According to current estimates, it affects 10% to 20% of women at a reproductive age [3].

Considered by some authors as a specific entity of the disease, deep endometriosis differs from the peritoneal and ovarian types [3, 4] and affects 20% to 35% of women who suffer from endometriosis [5]. Defined as a subperitoneal invasion by endometrial lesions that are over five-millimeter deep, this type of disease may involve pelvic fibromuscular structures and is usually related to worsening of symptoms that are characteristic of the illness and are not always present in other types [6]. This type of the disease affects mainly the retrocervical region, uterosacral ligaments, rectum, rectovaginal septum and bladder. The so-called retrocervical region is an extraperitoneal space located behind the cervix and above the rectovaginal septum.

Although hormone treatments are efficient to relieve pain, side effects are inconvenient and there are risks of recurrence after therapy discontinuation. Therefore, the standard treatment for deep endometriosis-an important gynecological disease that causes painful disabling symptoms-is surgical procedure [5-7].

Currently, a promising alternative for endometriosis is the use of stem cells, and perhaps they can be a treatment option. These cells have already been used as a therapeutic approach for many diseases due to their anti-inflammatory and regenerating action [8, 9]. Thus, since endometriosis is considered an inflammatory disease [10-12], the use of bone marrow derived-mononuclear stromal cells for

the treatment of endometriosis might bring good results.

Several studies have investigated the relation of the immune system with endometriosis [13, 14]. The altered expression of the MMPs (matrix metalloproteinases) is associated with the alteration of endometrial function, and may cause endometriosis, uterine cancer, and also potentially affect pregnancy [15, 16]. The elevated expression of these molecules leads to an increase in vascularization, a very significant event in progression of endometriosis, facilitating tissue degradation and the invasive formation of the disease [17]. Additionally, it is also associated with other inflammatory and degenerative diseases [18]. Therefore, studies performed in rodents investigated the possible role of MMPs in experimental endometriosis and demonstrated that the inhibition of these enzymes may prevent the formation of endometrial lesions [18].

Another molecule involved with the disease is the TGF-β (transforming growth factor-β) a multifunctional cytokine that modulates various biological processes [19, 20]. The TGF-β acts in extracellular matrix reposition, increasing its synthesis and impeding its degradation, and it is also a regulating mediator of inflammation. It is very important in regulation of tissue healing processes, and it is involved in repair of connective tissue in general as well as in bone regeneration [19]. Alterations in the expression of this factor were related to various abnormalities in the body, including endometrial cancer and endometriosis [19]. Some studies reported that the peritoneal fluid of women with endometriosis contains ten times more TGF-β than that of women without the disease [21]. A high expression of TGF-β1 by activated macrophages in the peritoneal fluid may contribute towards the formation of a microenvironment favorable for the formation of fibromuscular fiber around the endometrial lesions [22]. Additionally, TGF-β1 is also involved in the formation of adhesions, which is also a characteristic of deep disease [23].

Thus, our objective was to evaluate the expression of metalloproteinases MMP-1 and MMP-9 and the factor TGF-β1 in Wistar rats subjected to induction of deep retrocervical endometriosis and treated with mononuclear bone marrow stromal cells from bone marrow mononuclear cells.

Materials and methods

Development of the experimental disease

After approval by the Ethics Committee on the Use of Animals (CEUA) at the *Pontifícia Universida de Católica do Paraná* (PUC-PR), Brazil, registration number 479, the study was carried out with 120 mature female Wistar-albino rats (200-250 g) from the PUC-PR Animal Reproduction Center.

The animals were kept at appropriate levels of temperature and humidity, with ad libitum feeding and water. After the one-week adaptation period, the surgical procedures were initiated.

After preoperative fasting for 12 hours, the animals were taken to the Laboratory of Operative Technique and Experimental Surgery for the surgical procedures. The anesthesia protocol was an intramuscular injection of 0.2 mL for every 100 grams of body weight of a mixture of one milliliter of ketamine (50 mg/mL) with one milliliter of xylazine (20 mg/mL) for all painful procedures (laparotomies).

Endometriosis was induced in order to establish a new technique for experimental endometriosis of the retrocervical type, seeking to characterize a deep and infiltrative disease. A median incision was made along the lineaalba with approximately three centimeters, and the bicornuate uterus of the animal was exposed. From the left uterine tube a segment was removed, which was later measured, maintaining a standard of 5 × 5 mm. The endometrium was exposed from a longitudinal incision of the segment. With a 6-0 non-absorbable polypropylene thread the segment was sutured at the retrocervical portion of the uterus, with the endometrial surface facing the cavity.

After three weeks, all animals were submitted to a new laparotomy to diagnose the endometriosis. At this stage, the viability of the implants was evaluated, measuring the area (mm^2) affected with a digital pachymeter.

The implant volume was calculated with the following formula using the Degree Classification System of Growth Implants: [4π(length/2) × (width/2) × (height/2)/3. Therefore, the remaining was randomly divided into three groups: saline (n = 38) (corresponding to the control group); leuprolide (n = 30); and stem cells (n =

35). In the animals from the saline group, saline solution (sterile 0.9% salt water) was injected close to the lesions. In the animals from the second group, a 1 mg/kg dose of leuprolide acetatedepot formulation (Lupron Depot, Abbott, Brazil) was injected subcutaneously, already used in previous studies [10, 24]. In the group stem cells, the rats were treated with mononuclear bone marrow stromal cells, a 0.2 mL dose of 50×10^6 cells were injected next to the lesions, as per the same protocol of Guarita-Souza et al. [25] e Kondo et al. [24].

Mononuclear bone marrow stromal cells harvesting

This process occurs in two phases: mononuclear bone marrow stromal cells harvesting and other stage corresponds to isolation of mononuclear bone marrow stromal cells. During the first phase, the method used was puncture aspiration in bone marrow of the iliac crest, with a heparinized 5 mL syringe and a 40 × 12 needle.

Approximately 4 mL of bone marrow was removed from each rat. The material collected in the puncture aspiration was taken to the laminar flow hood for isolation of the mononuclear cells.

Isolation of bone marrow mononuclear cells was done as per the modified method described by Böyum [40]. For this process, the Ficoll-Hypaque (Ficoll-Hypaque, Sigma, St. Louis, MO) gradient and the Iscove's Modified Dulbecco's Medium (IMDM) were used, the latter for containing important nutrients to maintain the cells. After the preparatory phase, the cells were injected in an autologous transplant into the animals.

Next, 42 days after induction of the disease and 21 days following application of treatments, all animals were submitted to the same anesthesia protocol, and after laparotomy, the aspect of the endometrium implants was assessed macroscopically, along with its vascularization and size, comparing the three groups. The tissue implanted was removed, fixed in 10% formalin, and biopsied to diagnose the presence of endometrial tissue and its components (glands and/or stroma). All animals were euthanized after the experiment, with a lethal dose of 250 mg/kg of sodium thiopental given by intraperitoneal route.

Histopathological procedures

Initially the preparation of the slides was done, with 5-μm slices stained with hematoxylin and eosin for histological evaluation by optical microscope. The presence of epithelial cells in the implants was semiquantitatively evaluated as described by Keenan et al. [26]. This classification has been already used in other studies to assess onset of experimental disease [24, 27]. Presence of a well-established epithelial layer = score 3, presence of a moderately preserved epithelial layer with leukocyte infiltration = score 2, very rare presence of epithelial layer (with occasional cells) = score 1, and absence of epithelium = score 0.

Next the preparation of slides for manual tissue microarray (TMA) was carried out. This technique consists in mounting paraffin blocks, with multiple samples, to prepare multisampling histological slides.

For the immunohistochemical tests, the slides prepared by manual TMA were submitted to the immunohistochemical process, which is divided into three distinct phases: standardization of the TMA technique for primary antibodies selected for the study; application of the standardized technique on the study material; and reading of the manual TMA slides.

During the first phase, a TMA was set up with known positive controls for each of the primary antibodies chosen, which were tested as per the technique. The slides were deparaffinized with xylol at room temperature, dehydrated with successive alcohol baths and rehydrated with water. Methyl alcohol was used, as well as H_2O_2 for the first endogenous peroxidase blockage, and distilled water and H_2O_2 for the second blockage. Later the incubation was performed with the primary antibodies anti-MMP-1 (Rabbit Anti-Human MMP-1 Polyclonal Antibody-Spring Bioscience, Fremont, California, USA); anti-MMP-9 (Mouse Anti-Human MMP-9 Monoclonal Antibody-Novocastra Laboratories, 15W2, Newcastle Upon Tyne, Northumberland, UK) and anti-TGF-β1 (Mouse Anti-Human TGF-β1 Monoclonal Antibody-Santa Cruz Biotechnology, Inc., Heidelberg, Baden-Württemberg, GER) at set dilutions. For development, the complex

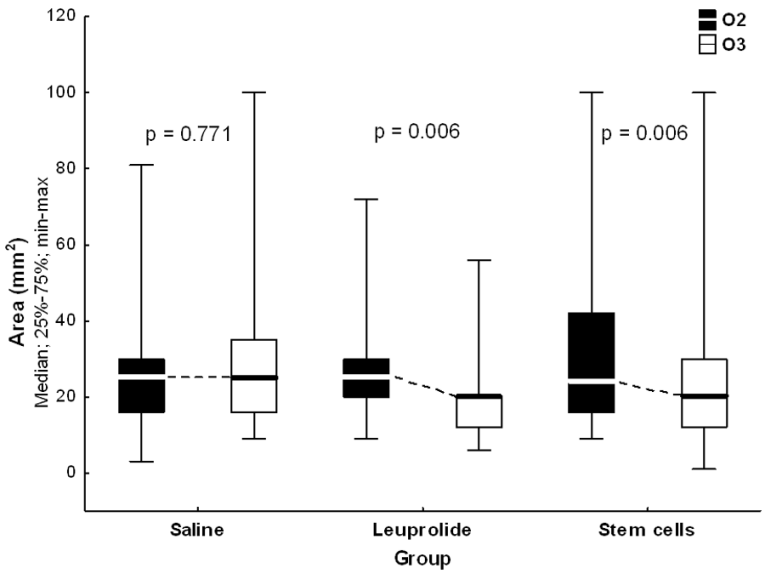

Figure 1. Comparison between the value of the area of implantation at the second operation with the area of implantation at the third operation and comparison among groups.

DAB (1:1) (Dako®) plus substrate complex was added to the slides and counter-staining was conducted with Harris hematoxylin.

During the second phase of the process, after reading of the controls and certification that the antibodies had a good level of staining reliability, the technique was applied to the study material always paired with negative and positive controls. Lastly, images were captured with a Zeiss Mirax Midi Slide Scanner (manual brightfield scan, version 1.11.45.0, Jena, Germany) and were later read with Mirax Viewer software (version 1.11.49.0, Jena, Germany).

Statistical analysis

Data analysis was performed with the software IBM SPSS Statistics v.20.0. Calculation of the sample size was based on the first part of the study (pilot) which included 20 animals in the saline group, 20 animals in the leuprolide group and 20 in the mononuclear bone marrow stromal cells group. Considering a 5% significance level and 80% power, and the minimal difference of 10.5 in the area difference between second and third operations among the groups, at least 34 animals would be necessary in each group. Observing the lack of symmetry of area data (non-parametric tests should be used) and considering that some animals could die during the experiment, 40 animals were assigned to each group. During the study two animals of saline group died and 15 animals were excluded for presenting score zero (absence of epithelium) indicating absence of disease. Thus, the saline solution group comprised 38 animals, whereas the leuprolide group had 30 animals and the stem cell group 35 animals.

To estimate the percentage of animals that presents endometriosis (effectiveness of the technique) a 95% confidence interval was set. One-way analysis of variance model (ANOVA) and the least significant difference test (LSD) for multiple comparisons were considered to compare the groups regarding expression of the markers. To compare the groups as to area of the implant, the non-parametric Kruskal-Wallis test was applied. The non-parametric Wilcoxon test was employed to compare the implant areas in the second and third surgeries. The symmetry condition of the variables was assessed by the Kolmogorov-Smirnov test. *P*-values below 0.05 indicated statistical significance.

Results

Results of the experiment indicated that 87.5% (95% CI: 81.6%-93.4%) of the animals developed the disease at any degree as per the classification by Keenan et al. [41] confirming the effectiveness of the retrocervical model.

Analyzing the progression of the disease from the second to third operation, for the saline group there was no significant difference (P = 0.771) in the area of implantation between the surgeries. On the other hand, for the leuprolide (P = 0.006) and mononuclear bone marrow stromal cells (P = 0.006) groups, the reduction in area of implantation from one operation to another was significant (**Figure 1**). Also, it was observed that the treatment was effective in reducing the size of the implant in 60% of leuprolide group and 77% of stem cells group, while

Table 1. Progression of implants from the second to the third operations, in all groups, determined in median, minimum value and maximum value

Group	N	Area O2[a] (mm^2)	Area O3[b] (mm^2)	Absolutedifference (03-02)	% difference	*p*-value* (02 vs 03)
Saline	38	25 (3; 81)	25 (9; 100)	0, 5 (-45; 39)	2 (-70; 75)	0.771
Leuprolide	30	25 (9; 72)	20 (6; 56)	-4, 5 (-40; 26)	-20 (-81.6; 55)	0.006
Stemcells	35	24 (9; 100)	20 (1; 100)	-7 (-60; 61)	-33.3 (-93.8; 75.3)	0.006
p-value (groups)				0.025	0.017	

[a]O2: Second operation; [b]O3: Third operation. *Non-parametric Wilcoxon test, P<0.05; **Non-parametric Kruskal-Wallis test, P<0.05.

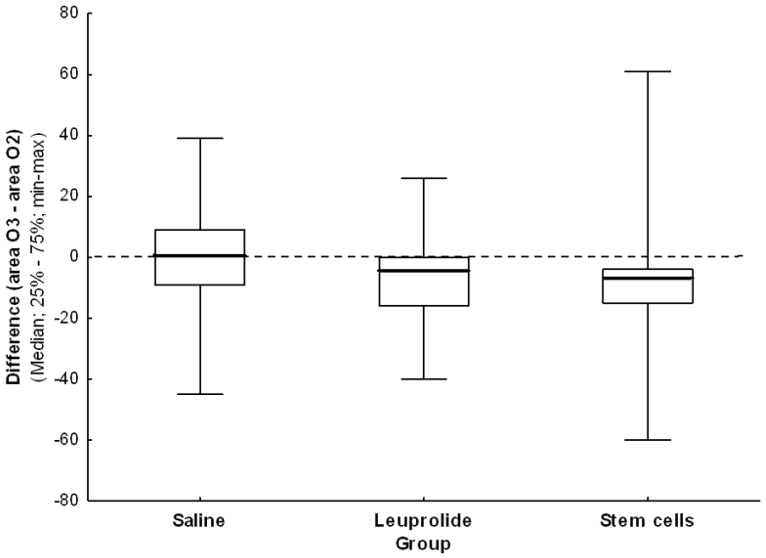

Figure 2. Comparison among groups as to the absolute difference in the area of implantation from the second operation to the third operation.

for soline group it ocurred in 39% of cases. Comparing the three groups as to the absolute difference in the size of the implant (area in mm^2) between the second and the third operation, a significant difference was noted among groups (P = 0.025). The saline group was different from the leuprolide group (P = 0.040) and from the mononuclear bone marrow stromal cells group (P = 0.011), and with the treated groups there was no difference among them (P = 0.688) (**Table 1** and **Figure 2**).

Flow cytometric analysis

Flow cytometric analysis (FACS Calibur; Becton Dickinson, USA) was performed to validate the bone marrow origin of the stem cells. Immunophenotyping of CD34 and CD45 were performed with a commercially available kit using a single-platform method according to the International Society of Hematotherapy and Graft Engineering (ISHAGE) guidelines.

All flow cytometric analysis was performed in duplicate, and the mean was calculated from the results. The cells were CD45+ and CD34- as immunophenotypical characterizes of mononuclear mononuclear bone marrow stromal cells. The mononuclears cells obtained were 5.0 × 105. (**Figure 3**).

The immunohistochemical analyses that evaluated the expression of the metalloproteinase markers 1 and 9 and TGF-β1 (**Figure 4**) showed that there was no significant difference among the groups as to any of the markers analyzed.

Discussion

There are several ongoing experimental studies, aiming at elucidation of the disease and effective therapies [29, 30]. To confirm the characterization of the disease, Keenan et al. [31] developed a classification based on epithelial persistence in ectopic implants. Quereda et al. [31] prepared another classification based on cyst development, in which stage zero means disaperance of the cyst; stage I is the presence of a cystic formation measuring less than 2-milimiter diameter; stage II, a cystic formation with a diameter of 2 to 4.5 mm; and stage III, a cystic formation with over 4.5-mm-diameter. Since cystic formation is not always observed, we adopted the first classification

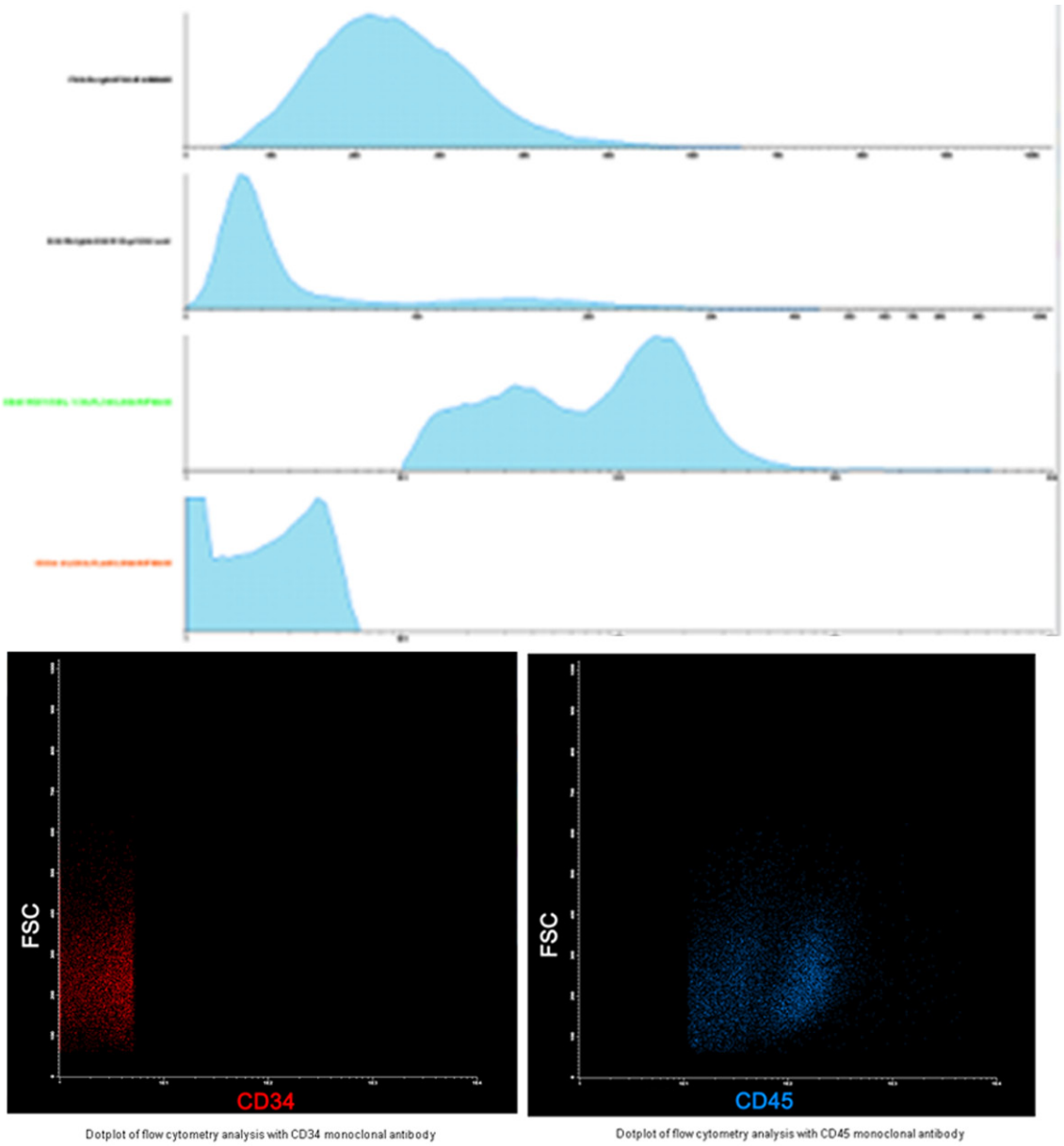

Figure 3. Mononuclear bone marrow stem cells flow cytometric analysis from rat.

system in our study. However, unlike other studies [42, 43] that reported a relation between treatment used and a significant reduction in Keenan grade, we did not observed such relation. Approximately 80% of the animals in each group presented the disease at any grade, that is, the grade in the stem cell group, which is the treatment group, was similar to that in the saline solution group. This may have occurred due to the model used, since the Keenan classification was developed in experimental endometriosis model in the mesenterium. Other investigations [27, 28] utilized this classification in peritoneal model, but other grading parameters may be established for deep endometriosis model, in which our study is pioneer.

The present study demontrated that leuprolide acetate significantly reduced the size of the implants in cases of experimental endometriosis, as already observed in a previous research [12]. Furthermore, it was observed that the group treated with mononuclear bone marrow stromal cells showed a diminished implant area in 82.9% of the cases, unlike what was demonstrated by our group's previous study [24].

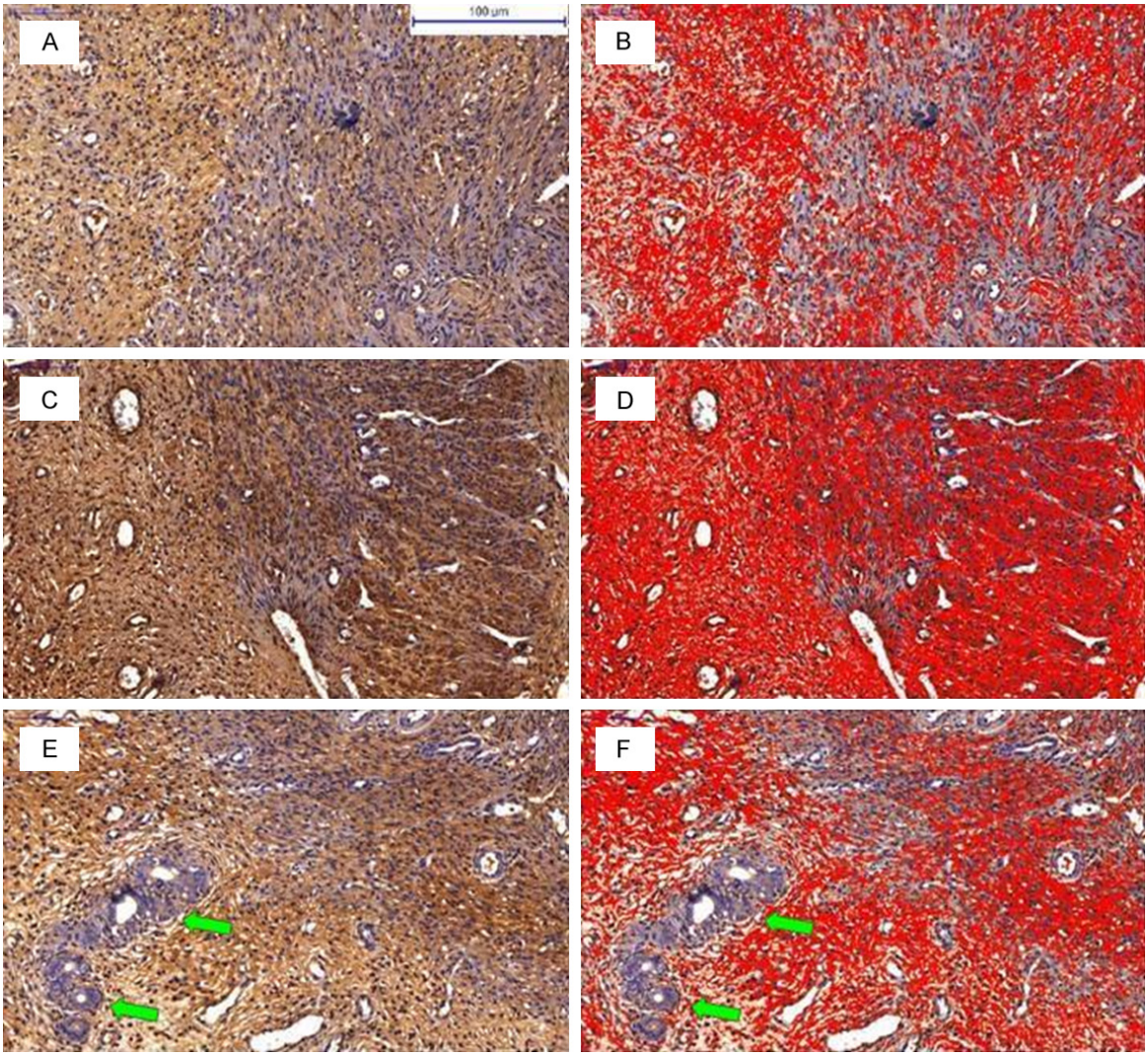

Figure 4. Immunohistochemical analysis of the markers MMP-1, MMP-9 and TGF-β1. A. Region in brown showing the expression of the MMP-1 marker in endometrial stroma (magnification 40 ×). B. Mensuring the expression of the MMP-1 marker in the endometrial stroma using digital morphometric evaluation. C. Region in brown showing the expression of the MMP-9 marker in the endometrial stroma (magnification 40 ×). D. Mensuring the expression of the MMP-9 marker in endometrial stroma using a digital morphometric evaluation. E. Region in brown showing the expression of the TGF-β1 marker in the endometrial stroma (magnification 40 ×). F. Mensuring the expression of the TGF-β1 marker in the endometrial stroma using a digital morphometric evaluation. The arrows indicate endometrial glands (Anova test).

However, despite employing the same treatment protocol, this study used a peritoneal model, which may be related to diverse results.

In this aspect, it was contrary to the hypothesis that stem cells could contribute to progression of endometriosis, as reported by Du and Taylor [1]. In this study, the authors demonstrated that stem cells-not derived from the uterus-originated endometrial cells in transplants for ectopic uterus. And suggested that stem cells could be related to a new mechanism of endometriosis and contribute to progression of the disease.

Assuming some studies [8, 32] have used mononuclear bone marrow stromal cells as therapy due to their anti-inflammatory potential, one would consider that, in our experiment, reduced implant area in this group may have occurred by inhibiting the inflammatory process generated by the disease. In such aspect, this would corroborate the results of Kondo et al. [24], who showed reduction in expression of

inflammatory cytokines in the group treated with stem cells.

Other studies demonstrated that the initial step for onset of endometriosis depends on breaking down the extracellular matrix, which directly involves the matrix metalloproteinases [33, 34]. Research conducted in humans and animals revealed changes in the expression of these enzymes in endometriosis when compared with those who did not suffer from the disease; and both MMP-1 and MMP-9 presented over expression in the cases with endometriosis [35, 36]. Nevertheless there was no statistically significant difference in our study when comparing the expression of MMP-1 and MMP-9 in the treated groups in relation to the control group. Considering that a significant reduction of these markers was expected in the treatment groups due to decreased implant size, proven in the leuprolid and stem cell groups, longer treatment period or changes in dosages, or even a larger animal sample might be needed for this process occur.

Affara et al. [37] demonstrated that MMP-1 plays a fundamental role in inflammatory diseases, and Amălinei et al. [37] referred to MMP-9 as extremely important enzymes in invasion and metastasis processes. Hence, taking into account the anti-inflammatory and regenerative properties of stem cells, MMP-1 and MMP-9 expression should diminish in this group. And it might be necessary to have a longer post-treatment interval to obtain a drop in expression of these markers, in such circumstances. Or, yet, these markers may not present the desired sensitivity regarding the disease. Bilibio (56) has already reported that researchers try hard to find serum markers of endometriosis that assist in clinical management, avoiding unnecessary surgeries and reducing time to diagnosis, which is often made late. And even with many ongoing investigations, an ideal marker has not been found yet, and some studies have evaluated a panel of markers seeking better results. Amălinei et al. [37] stated that several experimental studies conducted in rodents demonstrated to what extent the effects of the MMPs are complex and paradoxical when related to carcinogenesis. The effects of these enzymes in relation to endometriosis might follow the same trend.

Likewise, no significant difference was noted in the groups as to expression of TGF-β-1. This molecule has been much associated to the disease, but its action is the opposite of that by the MMPs, hindering degradation of the matrix and enhancing its synthesis, and works also as a modulator of MMPs and their inhibitors [20].

The innumerous functions of TGF-β include fibrosis formation [24]. This cytokine is responsible for inducing the synthesis of α-actin in fibroblasts of smooth muscle and for stimulating the production of type-1 collagen; therefore, it is considered a key element in the evolution of lesions characterized by myofibroblasts. Since deep endometriosis lesions are characterized by prevalence of fibromuscular tissue in their composition and formation of adhesions, the increased expression of TGF-β may be related to the features of a more severe type of the disease [22, 23]. Our results, nonetheless, could not effectively confirm this relation, because further data are required to characterize this new model of experimental endometriosis as a model that mimics the severe form of the disease. Many studies would be needed with this same model and with new approaches to prove it is effective in deep endometriosis.

Moreover, TGF-β1 inhibits the expression of inflammatory molecules, and promotes repair and regeneration [38, 39]. Thus, once again the contradiction is observed in the results obtained, considering the anti-inflammatory and regenerating action of stem cells. According to this trend, this growth factor should have presented over expression in this treatment group. And this makes us argue again about time to assess expression of the marker and its sensitivity related to the disease.

Kondo et al. [24] conducted the only investigation currently known to test the effect of bone marrow stem cells in experimental endometriosis, and contrary to the present study, there was a significant reduction in expression of the immunologic markers evaluated-TNF-α and VEGF-R-but with no significant changes in the implant area. However, this makes us believe that the experimental endometriosis model may have great influence in these results. There is scarce literature correlating the factors tested in this study to help understanding the obtained results. Hence, new investigations in the area are needed to better assess the stan-

dard experimental model and the correlations that may be established among stem cells, metalloproteinases and TGF-β.

Concluding, treatment with bone marrow derived-mononuclear stromal cells was effective in reducing the area of ectopic implant. However, no significant difference was obtained among the groups as to the expression of MMP-1, MMP-9 and TGF-β1.

Disclosure of conflict of interest

None.

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