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Original article

Identification of local angiogenic and inflammatory markers in the menstrual blood of women with endometriosis

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ABSTRACT

The aim of this study was to evaluate the presence of myeloperoxidase (MPO), N-acetyl-β-D-glucosaminidase (NAG), tumor necrosis factor alpha (TNF-α) and vascular endothelial growth factor (VEGF) in peripheral and menstrual blood in women with ($n = 10$) and without ($n = 7$) endometriosis. NAG and MPO activities were evaluated by enzymatic methods, whereas TNF-α and VEGF by immunoassay. No significant differences were found for these markers, neither in menstrual nor in peripheral blood between groups. Menstrual blood NAG ($P = 0.039$) and MPO ($P = 0.0117$) activities in the endometriosis group were significantly higher than in peripheral blood. NAG and MPO presented positive linear correlation in peripheral ($P = 0.07$; $r = 0.641$) and menstrual blood ($P = 0.01$; $r = 0.603$). These findings point to the existence of an increased local inflammatory activity in women with endometriosis.

1. Introduction

Endometriosis is a benign gynecological disorder characterized by the presence and growth of endometrium-like tissue in sites outside the uterine cavity, primarily on the pelvic peritoneum and ovaries [1–4]. In spite of affecting 5 to 10% of women in reproductive age, the exact etiology of the disease remains an intriguing enigma, since it is currently accepted that multiple factors are involved in the pathogenesis of endometriosis [2–8]. In addition to an invasive phenotype of endometrial cells, neoangiogenesis and sustained local inflammatory response are believed to be crucial points in the pathophysiology of endometriosis [9–15]. Neoangiogenesis is evident by the visualization of peripheral vascularization of ectopic endometriotic tissue at laparoscopy and increased angiogenic activity and vascular endothelial growth factor (VEGF) levels found in peritoneal fluid of patients with endometriosis [3,11,12,16–18].

Immune dysfunction has been identified in these patients either as a result or as a consequence of disease [7,19–21]. In recent years, alterations in both cell-mediated and humoral immunity have been observed in monkeys and in women with endometriosis [17,21–24].

Myeloperoxidase (MPO), an enzyme restricted to the azurophil granules of neutrophils, has been extensively used as a marker of polymorphonuclear leukocytes accumulation in tissue samples [25–30]. N-acetyl-β-D-glucosaminidase (NAG), present in lysosomes, has been employed to detect macrophage accumulation/activation in a variety of animal and human tissues [26–31]. Both NAG and MPO evaluated in infertile women with endometriosis undergoing in vitro fertilization, showed a distinct pattern of macrophage/neutrophil activation in the serum and follicular fluid. This suggests the possibility of an altered immunologic function in the follicular fluid of patients with endometriosis, which could contribute to infertility in these women [28].

Tumor necrosis factor-α (TNF-α) plays a pivotal role in the establishment and maintenance of endometrial deposits [32] and high concentrations in the peritoneal fluid of women with endometriosis suggest local release from activated peritoneal macrophages [32–34]. Published evidence shows that TNF-α,

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directly or indirectly promotes the proliferation and adhesion of endometrial cells and associated angiogenesis seen in endometriosis [32,35]. The search for an innate or acquired survival advantage of eutopic endometrium favoring ectopic implantation has fueled a number of studies comparing eutopic endometrium from women with and without endometriosis [10,36–40]. Menstrual blood offers a unique minimally invasive diagnostic opportunity, as it has elements from endometrium and has already been used for the diagnosis of dysfunctional uterine bleeding [41]. The study of inflammatory markers in the endometrium and menstrual blood samples may help to elucidate the steps of the pathogenesis of endometriosis and to find a biomarker for the disease.

Our study sought to determine whether the menstrual blood of women with endometriosis expresses inflammatory and angiogenic markers differently from that of women without endometriosis, comparing the activity of NAG and MPO, and the expression of TNF-α and VEGF in menstrual and peripheral blood.

2. Materials and methods

2.1. Patients

The study protocol was approved of by the local ethics committee. A written informed consent was obtained from all patients involved before the procedure.

Our transversal study involved seventeen women undergoing infertility or chronic pelvic pain treatment at Hospital das Clínicas at Universidade Federal de Minas Gerais (HC-UFMG). These patients were recruited among those referred to our department from February 2011 to December 2012 and were divided into two groups: endometriosis ($n = 10$) and normally cycling women without endometriosis ($n = 7$), which served as a control group. Inclusion criteria were regular menstrual cycles in the six months preceding sample collection, no use of hormonal nor anti-inflammatory agents in the previous three months and surgical confirmation or exclusion of endometriosis in agreement with the ESHRE guidelines [42]. The control group has undergone surgery as part of infertility evaluation or tubal sterilization. None of the women had a significant past medical history. Endometriosis was staged according to the classification proposed by the American Society for Reproductive Medicine (ASRM, 1996) [43].

Menstrual and peripheral blood samples were collected in the follicular phase (1st to 4th day of menses). The activity of inflammatory markers (NAG and MPO) was evaluated by enzymatic methods whereas TNF-α and VEGF were measured by ELISA (Kit Duoset R&D Systems). NAG, MPO, TNF-α and VEGF were evaluated in serum and menstrual blood. Menstrual blood samples were collected at the external cervical os by gentle syringe aspiration, and peripheral blood was withdrawn from antecubital vein concomitantly. Blood was centrifuged (3600 rotations per minute for 20 minutes at 4 °C). Serum was withdrawn and stored at −25 °C until assayed for markers.

2.2. Assays

2.2.1. Determination of NAG activity

Accumulation of mononuclear cells in menstrual and peripheral blood was quantified by measuring the levels of the lysosomal enzyme NAG, which is present in high levels in activated macrophages [26–28]. An aliquot of the serum/menstrual blood (50 μL) was homogenized in 150 μL NaCl solution (0.9% w/v) containing 0.1% v/v Triton X-100 (Promega, São Paulo, Brazil) and then centrifuged (3000 rpm; 10 min at 4 °C). Samples of the resulting supernatant (50 μL) were incubated with 100 μL of p-nitrophenyl-N-acetyl-beta-D-glucosaminide (2.24 mM) prepared in 50 μL citrate/phosphate buffer (39 mM pH = 4.5). The reaction was stopped by the addition of 100 μL of 0.2 M glycine buffer and the product was detected colorimetrically at 400 nm. NAG activity was expressed as change in optical density (OD).

2.2.2. Determination of MPO activity

The extent of neutrophil accumulation in menstrual and peripheral blood was measured by assaying MPO activity as previously described [44]. An aliquot of serum/menstrual blood (50 μλ) was homogenized in 150 μL sodium phosphate pH 5.4 buffer for 30 seconds. The supernatants (200 μL) were then resuspended in 400 μL (hexadecyltrimethylammonium bromide) HTAB and centrifuged at 5000 g for 10 min at 4 °C. This solution (200 μL) was added to 100 μL of H2O2 and 100 μL of tetramethylbenzidine. The reaction was stopped by the addition of 100 μλ of H_2SO_4 (4 M). MPO activity in the samples was obtained by measuring the change in absorbance (OD) at 450 nm.

2.2.3. Determination of TNF-α and VEGF

TNF-α and VEGF were evaluated using commercial specific enzyme-linked immunosorbent assays (ELISA). Kits for Human VEGF (Duoset R&D Systems DY293B range: 31,2-2000 pg/mL) and Human TNF-α (Duoset R&D Systems DY210, Minneapolis, MN – USA, range: 15,6-1000 pg/mL) were used to quantify the expression of each protein. Samples from each patient were tested in duplicate, according to the detailed protocol provided by the manufacturers. Briefly, samples and standards were added to a 96-well antibody-coated plate, which were shaken, sealed and stored overnight at 4 °C. The plate was then washed with wash buffer. After then, the biotin conjugate-labeled second antibody was added in plates and stored for a further one hour at room temperature. The plate was washed again and the estreptavidin conjugate was added, sealed, shaken and stored at room temperature for 20 minutes and after washed again. The OPD (o-phenylenediamine dihydrochloride) substrate was added in all plates and stored at room temperature out of light for 10 minutes and then the stop solution – H_2SO_4 4 M was added. The absorbance was measured by spectrophotometry.

2.3. Statistical analysis

Calculations were carried out using GraphPad Prism 5 and IBM SPSS Statistics 21(SPSS Inc; Chicago; IL; EUA), and $P < 0.05$ was considered statistically significant for all analysis. Mann-Whitney test was used to compare of unpaired groups, the Wilcoxon test was used to compare paired groups, once normality could not be assumed after application of the Kolmogorov-Smirmov test. Correlations were calculated using Spearman's rho coefficient.

Power calculations based on the expected or desired effect size showed that 10 patients in the case group and seven individuals in the control group showed a confidence level of 90%, statistical power of 80% and a minimal detectable difference of 4 and a standard deviation of 3.

3. Results

The age of the patients ranged from 31 to 48 years, and in both groups the median was 36. No significant difference was found between endometriosis and control group, neither for BMI (median 27,17 kg/cm^2 in control group versus 24,86 kg/cm^2 in endometriosis) or menstrual cycle length (median 28,5 in control group and 28 days in endometriosis) (Fig. 1). Endometriosis stage according to ASRM was stage II in the five patients (50%), IV in two (20%) and in three (30%) no surgical staging was available in spite of surgical confirmation. The majority of women in the endome-