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Evaluation of N-acetilglucosaminidase and myeloperoxidase activity in patients with endometriosis-related infertility undergoing intracytoplasmic sperm injection

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Abstract

Aim: Inflammation is as an important factor in ovulation with the active participation of leucocytes and their inflammatory mediators. The present study was performed to compare the activity of the inflammatory enzymes myeloperoxidase (MPO) and N-acetylglucosaminidase (NAG) in patients with endometriosis-related infertility and in normally ovulating women undergoing intracytoplasmic sperm injection (ICSI).

Material and Methods: This prospective study included infertile women undergoing ICSI treatment. These women were divided into two groups: endometriosis anovulation ($n = 18$) and normally ovulating ($n = 20$). NAG and MPO activity was evaluated colorimetrically in serum and in follicular fluids obtained at the time of oocyte retrieval.

Results: There was a significant correlation between the serum and follicular fluid activities of NAG and MPO ($\tau = 0.256$, $P = 0.025$; and $\tau = -0.234$, $P = 0.041$; respectively). Both serum and follicular fluid NAG activities were higher in patients with endometriosis compared to the control group ($P < 0.001$). MPO follicular fluid activity was lower in patients with endometriosis compared to normally ovulating women ($P = 0.016$).

Conclusion: Infertile patients with endometriosis show a distinct pattern of serum and follicular fluid macrophage/neutrophil activation compared to normally ovulating women undergoing ICSI, which may reflect the role of immune and inflammatory alterations in endometriosis-related infertility.

Key words: endometriosis, infertility, inflammation, myeloperoxidase, N-acetylglucosaminidase.

Introduction

Inflammation apparently has a significant role in gynecology and infertility, affecting the ovary and uterus, as well as the embryo and implantation. Endometriosis is an estrogen-dependent inflammatory disease that affects 5–10% of women of reproductive age.[1] It causes infertility by interfering with vital steps in the reproductive process. Its defining feature is the presence of endometrium-like tissue in sites outside the uterine cavity, primarily on the pelvic peritoneum and ovaries.[1,2] The mechanisms of infertility associated with endometriosis remain controversial and include abnormal folliculogenesis, elevated oxidative stress, altered

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immune function and hormonal milieu in the follicular and peritoneal environments, as well as reduced endometrial receptivity. Endometriosis may also distort pelvic anatomy and produce large ovarian masses.[2,3] It is not clear, however, whether all these alterations are a cause of infertility or a paraphenomenon. Despite decades of research, the pathophysiology of endometriosis remains unclear. While the implantation of viable endometrium on peritoneal surface after retrograde menstruation remains the most widely accepted theory, clearly other factors, such as genetic predisposition, aberrant immunological response as well as local peritoneal factors seem to be involved in the pathogenesis of endometriosis.[4] Endometriosis results in tissue damage and production of antibodies against the endometrium and the ovary. These antibodies, phospholipids and histones are associated with other autoimmune diseases, such as thyroiditis and systemic lupus erythematosus (SLE), all of which suggest an autoimmune cause of inappropriate immune response.[5] The development of *in-vitro* fertilization (IVF) as a therapeutic tool in patients with endometriosis has provided information about the disease, especially on aspects of the reproductive process in humans, particularly folliculogenesis, fertilization, embryo development and implantation.[6]

It is well-recognized that many cytokines are of paramount importance for reproductive processes, such as follicular development, ovulation, fertilization, implantation and embryonic development.[7,8] The role of cytokines in the female reproductive system has been investigated during controlled ovarian stimulation (COS) in IVF attempts, which is clearly different from those of natural cycles.[9–11] Some studies have tried to assess the endocrine, paracrine, and autocrine milieu in patients with endometriosis on the basis of the measurement of several cytokines in serum and follicular fluid (FF) and *in vitro* culture of granulosa luteal cells. The results demonstrate that cytokines are regulated differently in patients with endometriosis, suggesting that a fine hormonal modulation of cytokines occurs at the systemic and local (ovarian) levels, which may contribute to reduced oocyte and embryo quality in endometriosis and thus result in a poor IVF outcome.[12,13]

Much interest has been focused on the mechanisms responsible for the attraction and activation of the leukocytes into any inflammatory tissue. Among the products released by inflammatory cells, myeloperoxidase (MPO), an enzyme restricted to the azurophil granules of neutrophils, has been extensively used as a marker for measuring polymorphonuclear leukocytes accumulation in tissue samples.[14] N-acetylglucosaminidase (NAG), present in lysosomes, has been employed to detect macrophage accumulation/activation in a variety of animal and human tissues.[15]

The study of substances present in human follicular fluid, which originate from plasma or are produced by follicular structures, reflect oocyte maturation and can be used to assess oocyte quality.[16] Several ovulation-associated mediators are produced by most leukocyte subtypes, including neutrophils, macrophages and mast cells and it seems that there are cyclic changes in the leukocyte population in the ovary, which is vital for normal ovarian function.[17] The aim of this prospective study was to compare the activity of inflammatory enzymes MPO and NAG in women with endometriosis-related infertility and normally ovulating women undergoing intracytoplasmic sperm injection (ICSI) due to male factor infertility.

Material and Methods

The study protocol was approved by the national and local ethics committees. A written informed consent from all patients involved was also obtained before the procedure.

Patients were divided into two groups: endometriosis ($n = 18$) and normally ovulating women ($n = 20$), which served as control group. Both groups were prospectively studied as they underwent ICSI and embryo transfer (ET). Inclusion criteria for the study group were patients with endometriosis diagnosed during laparoscopy and confirmation by histological examination of the endometriotic lesions. All patients were in the first phase of the menstrual cycle and none had taken hormonal medications for at least 3 months prior to surgery.

Endometriosis was staged according to the classification proposed by the American Society for Reproductive Medicine (ASRM)[18] Other causes of infertility were excluded by performing complete diagnostic work-up for couple infertility: hysterosalpingography, sperm evaluation, and measurements of serum follicle-stimulating hormone (FSH), prolactin and thyroid-stimulating hormone levels on the third day of the menstrual cycle. In the control group, subjects had regular menses and levels of progesterone above 5 ng/mL in the luteal phase of the preceding menstrual cycle, demonstrating normal ovulation.[19] None of these patients were diagnosed with endometriosis, all of them were fertile and none had a significant past

medical history. The indication for ICSI in this group was association with male factor infertility. NAG and MPO activities were evaluated in serum and in follicular fluids obtained at the time of oocyte retrieval. These samples were centrifuged (200 *g*, 10 min), and supernatants of serum and follicular fluid were carefully extracted and then stored at –80°C until analysis.

Assisted reproductive technology (ART)

The patients were downregulated with gonadotropin releasing hormone agonist (3.75 mg; leuprolide acetate depot suspension [Lectrum; Novartis, Entre Rios, Argentina]) once a month administered in the mid-luteal phase of the preceding cycle or first day of the stimulation cycle. Subsequent stimulation with Human Menopausal Gonadotropin (Merional [Meizler, São Paulo, Brazil] 150–300 IU/day) was started once there was no sonographic evidence of ovarian follicular activity (endometrium thickness <5 mm and no follicles in both ovaries). Human chorionic gonadotropin (hCG 10.000 IU [Choriomon; Meizler]) was administered when at least two of the leading follicles exceeded 17 mm in diameter each. Oocyte retrieval was performed 35–36 h later using vaginal ultrasound guidance. Follicles were carefully measured before retrieval and only follicular fluid from follicles larger than 16 mm in diameter was collected and pooled from each individual patient for analysis. Follicular fluid with blood contamination and flushing media were excluded.

Metaphase II oocytes, identified by the presence of the first polar body, were injected. Single sperm injection (ICSI) was performed 3–6 h after oocyte retrieval using previously described techniques and equipment.[20] Day 2/3 embryos were transferred back to the uterine cavity using a fine transcervical catheter (Sydney; Cook Medical, Bloomington, IN, USA) under ultrasound guidance. All women received luteal phase supplementation (natural micronized progesterone 200 mg vaginally TID [Evocanil; Zodiac, St. Petersburg, FL, USA] for two weeks until the result of the pregnancy blood test was available and progesterone supplementation was continued up to the 12th week of pregnancy.

Determination of NAG activity

Accumulation of mononuclear cells in follicular fluid and blood was quantitated by measuring the levels of the lysosomal enzyme NAG present in high levels in activated macrophages[21] An aliquot of the supernatants was homogenized in NaCl solution (0.9% w/v) containing 0.1% v/v Triton X-100 (Promega, São Paulo, Brazil) and then centrifuged (3000 *g*; 10 min at 4°C). Samples of the resulting supernatant (100 µL) were incubated for 10 min with 100 µL of p-nitrophenyl-N-acetyl-beta-D-glucosaminide (2.24 mM) prepared in citrate/phosphate buffer (39 mM pH = 4.5). The reaction was stopped by the addition of 100 µL of 0.2 M glycine buffer. The reaction product was detected colorimetrically and was performed at 400 nm. NAG activity was expressed as change in optical density (OD).

Determination of MPO activity

The extent of neutrophil accumulation in follicular fluid and blood was measured by assaying MPO activity as previously described.[21] An aliquot of the supernatants was homogenized in pH 4.7 buffer (0.1 M NaCl, 0.02 M NaPO4, 0.015 M NaEDTA) and centrifuged at 12 000 *g* for 10 min. The supernatants were then re-suspended in 0.05 M NaPO4 buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) followed by three freeze-thaw cycles using liquid nitrogen. MPO activity in the samples was assayed by measuring the change in absorbance (OD) at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.3 mM). The reaction was terminated by the addition of 50 µL of H_2SO_4 (4 M). Results were expressed in OD.

Statistical analysis

Calculations were carried out using Statistical Package for Social Science, Windows version 16.0 (SPSS, Chicago, IL, USA). The Mann–Whitney test was used for group comparison of unpaired continuous data because after application of the Kolmogorov–Smirnov test, normality could not be assumed. Correlations were calculated using the Kendall's tau correlation coefficient. $P < 0.05$ was considered statistically significant for all analyses. Power calculations based on the expected or desired effect size showed that 18 patients in the case group and 20 individuals in the control group allowed a power of 90% and a type I error of 10%.

Results

The age of the patients ranged from 20 to 41 years (32.1 ± 0.8 years). The mean BMI was 21.7 ± 0.3 kg/cm^2 (ranging 17.6–26.2 kg/cm^2). There were no differences between groups regarding age, time of infertility, body mass index (BMI), basal FSH, basal luteinizing hormorne (LH) or basal number of antral follicles (Table 1). 2. The ASRM stage of endometriosis was I in six patients (33.3%), II in six (33.3%), III in two (11.1%) and four (22.2%) patients.

Table 1 Baseline characteristics of infertile patients with endometriosis (cases) and normally ovulating women (control group) undergoing ICSI

	Control group $n = 20$	Cases $n = 18$	*P*-value
Age (years)	29 ± 0.8	28.8 ± 0.9	0.859
Time of infertility (years)	3.9 ± 0.6	4.3 ± 0.9	0.742
BMI (kg/cm^2)	21.6 ± 0.6	21.9 ± 0.4	0.648
Basal FSH (mUI/mL)	5.8 ± 0.2	5.6 ± 0.3	0.541
Basal LH (mUI/mL)	7.8 ± 0.8	8.4 ± 0.7	0.345
Basal antral follicles (*n*)	30.2 ± 1.4	29.1 ± 1.6	0.742

Data are expressed as means ± SEM and *n* (%). Differences between groups were assessed by Student's *t*-test. The level of significance was set at $P < 0.05$. BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

There was a significant correlation between the serum and follicular fluid activities of NAG ($\tau = 0.256$; $P = 0.025$) and MPO ($\tau = -0.234$; $P = 0.041$), respectively (Fig. 1). As illustrated in Figure 2, serum and follicular fluid NAG activities were higher in patients with endometriosis compared to the control group ($P < 0.001$). In follicular fluid, MPO activity was lower in patients with endometriosis compared to normally ovulating women ($P = 0.016$). There was no difference between groups regarding serum MPO activity ($P = 0.114$) (Fig. 3).

NAG and MPO activities were not associated with the occurrence of ovarian hyperstimulation syndrome and presence or absence of pregnancy in patients with endometriosis-related infertility (cases) and control group (Table 2).

Discussion

Our prospective study measured the activity of the inflammatory enzymes MPO and NAG in patients with infertility caused by endometriosis and normally ovulating women undergoing ICSI. To the best of our knowledge, no previous studies have been published regarding the activity of these two enzymes in endometriosis. No significant differences regarding age, time of infertility, BMI, basal FSH, basal LH or basal number of antral follicles were found between the two groups studied.

The extent of neutrophil accumulation in follicular fluid and serum was measured by assaying MPO, which showed lower activity in patients with endometriosis compared to normally ovulating women ($P = 0.016$). Leukocytes present within the ovary may constitute potential *in situ* modulators of ovarian function that act through local secretion of regulatory soluble factors, including numerous cytokines.[22]

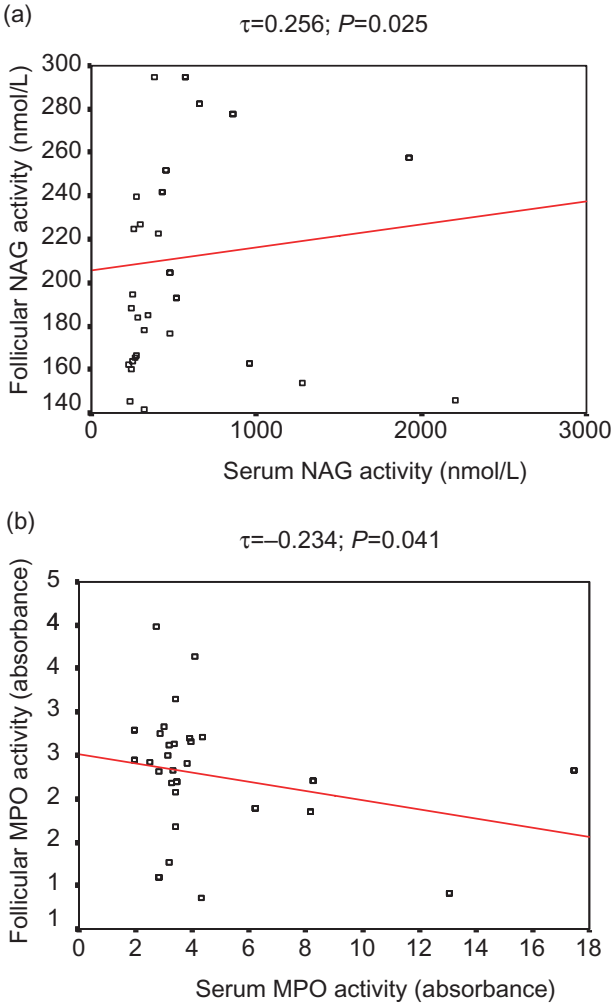

Figure 1 Correlation between serum and follicular fluid activities of (a) N-acetilglucosaminidase (NAG) and (b) myeloperoxidase (MPO) activities in infertile patients with endometriosis (cases) and normally ovulating women (control group) undergoing ICSI. Correlation between variables was performed by Kendall's tau test.

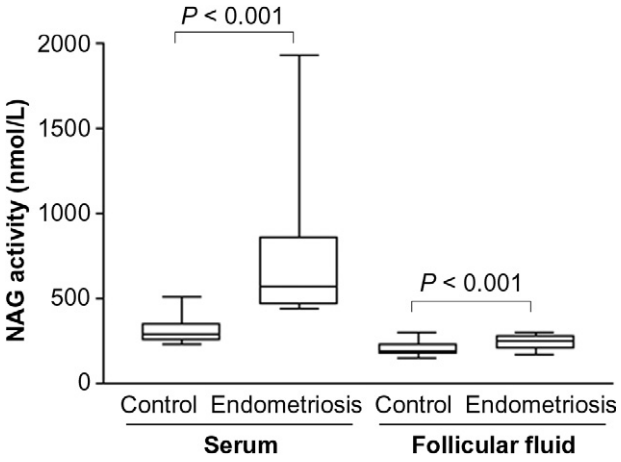

Figure 2 Evaluation of serum and follicular fluid N-acetilglucosaminidase (NAG) activity in the control group ($n = 20$) and in patients with endometriosis ($n = 18$) undergoing intracytoplasmic sperm injection (ICSI). Comparison between groups was performed by Mann–Whitney test.

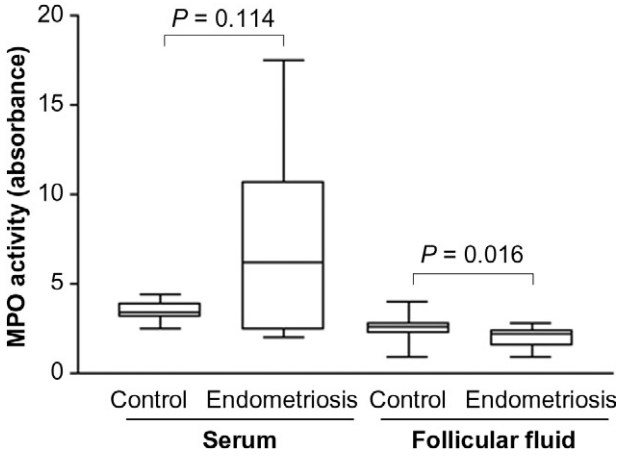

Figure 3 Evaluation of serum and follicular fluid myeloperoxidase (MPO) activity in control group ($n = 20$) and in patients with endometriosis ($n = 18$) undergoing ICSI. Comparison between groups was performed by Mann–Whitney test.

Several ovulation-associated mediators are produced by most leukocyte subtypes, including neutrophils, macrophages and mast cells and it seems that there are cyclic changes in the leukocyte population in the ovary and that these cell types actively participate in the functional and structural changes of the follicle and corpus luteum.[17] Leukocyte activity in our study was assessed by MPO levels, which were lower in endometriosis, a fact that could be related to disrupted folliculogenesis and reduced oocyte quality described in endometriosis.[2,6,23]

Serum and follicular fluid NAG activities were higher in patients with endometriosis compared to the control group ($P < 0.001$). As NAG is present in high levels in activated macrophages, its levels may reflect macrophage activation which is increased in endometriosis.[24] Macrophages are known to play diverse roles in intraovarian events, including folliculogenesis, tissue restructuring at ovulation and corpus luteum formation and regression.[25] Thus, increased macrophage activation in the follicular fluid may result in disruption of folliculogenesis and decreased oocyte quality observed in endometriosis.[2,23] Brandeli and Passos[26] found elevated NAG activity enzymes in the peritoneal fluid of infertile women with endometriosis, suggesting a possible deleterious effect on gametes, which could explain some cases of infertility in endometriosis. The increased macrophage activation in women with endometriosis has a stimulatory effect on endometriotic tissue, although in normal populations macrophages might locally act to suppress endometrial proliferation.[27] Our study demonstrated an association between the presence of endometriosis and elevated number of macrophages and it appears therefore that impaired function, and not population size of macrophages is important for endometriotic tissue proliferation.[28]

There was a significant correlation between the serum and follicular fluid activities of NAG and MPO. Studies have shown that cytokines are regulated differently in patients with endometriosis, suggesting that a fine hormonal modulation of cytokines occurs at the systemic and local (ovarian) levels, which may contribute to reduced oocyte and embryo quality in endometriosis and result in a poor IVF outcome.[12,13] We found a lower MPO activity in the follicular fluid of women with endometriosis which reflects reduced neutrophil accumulation and may be related to altered folliculogenesis and ovulation seen in these patients. However, NAG activity was significantly higher in the endometriosis group, which reflects increased macrophage activation in this group. As these cell types actively participate in the functional and structural changes of the follicle and corpus luteum,[17] alterations in their expression could be related to compromised gamete quality and infertility. Impaired immune response that results in inadequate removal of refluxed menstrual debris has been proposed as a possible causative factor in the development of endometriosis. Alterations in cellular immunity

Table 2 Association between the occurrence of ovarian hyperstimulation syndrome and pregnancy with follicular fluid and serum N-acetilglucosaminidase (NAG) and myeloperoxidase (MPO) concentration levels in patients with endometriotic-related infertility (cases) and control group

	Ovarian hyperstimulation syndrome		*P*-value	Pregnancy		*P*-value
	No ($n = 35$)	Yes ($n = 3$)		No ($n = 21$)	Yes ($n = 17$)	
NAG (nmol/L)						
Follicular fluid	168.2 (162.5–194.4)	224.9 (165.1–224.9)	0.25	184.4 (167.5–192.9)	176.5 (153.8–232.3)	0.805
Serum	276.1 (240.1–320.6)	267.9 (256.2–267.9)	0.117	267.1 (242.1–315.4)	276.1 (244.6–393.8)	0.461
MPO (absorbance)						
Follicular fluid	2.6 (2.1–2.7)	2.4 (2.2–2.4)	1.0	2.6 (2.4–2.8)	2.3 (1.9–3.2)	0.775
Serum	3.4 (3.2–3.9)	3.2 (2.8–3.2)	0.122	3.2 (3.0–3.4)	3.4 (3.3–4.2)	0.422

Data are expressed as medians and interquartile ranges. Differences between groups were assessed by Mann–Whitney test. The level of significance was established at $P < 0.05$.

include increased number and activation of peritoneal macrophages, decreased T cell and natural killer (NK) cell cytotoxicity result in inadequate removal of ectopic endometrial cells from the peritoneal cavity. Moreover, increased levels of several cytokines and growth factors which are secreted by either immune or endometrial cells seem to promote implantation and growth of ectopic endometrium by inducing proliferation and angiogenesis. In fact, various studies have demonstrated that women with endometriosis show altered immune response and cytokine secretion at the local and systemic level, which, in turn, could affect ovarian and endometrial function and impair fertility.[12,13,24,27,28]

Endometriosis is associated with inflammatory changes in the follicular fluid. An increased percentage of B lymphocytes, natural killer cells, and monocyte-macrophages in the follicular fluid have been noted in a case-controlled study of patients with endometriosis compared with those with other causes of infertility.[29]

Our results show that infertile patients with endometriosis show a distinct pattern of serum and follicular fluid macrophage/neutrophil activation compared to normally ovulating women undergoing ICSI. These findings suggest the possibility of an altered immunologic function in the follicular fluid of patients with endometriosis, which could contribute to infertility in these women. Our findings thus strengthen the role of inflammatory and immunological alterations in endometriosis-associated infertility. Further clinical studies with a larger number of patients and other inflammatory biomarkers are indicated to establish the role of inflammation in endometriosis-related infertility.

Disclosure

The authors have no relationships with any companies that may have a financial interest in the information contained in the manuscript.

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