

Quantitative and qualitative peritoneal immune profiles, T-cell apoptosis and oxidative stress-associated characteristics in women with minimal and mild endometriosis

Jennifer Mier-Cabrera,^{a,b} Luis Jiménez-Zamudio,^b Ethel García-Latorre,^b Oliver Cruz-Orozco,^c César Hernández-Guerrero^d

^a Departamento de Investigación en Nutrición, Subdirección de Investigación en Salud Pública, Instituto Nacional de Perinatología 'Isidro Espinosa de los Reyes', Mexico City, Mexico ^b Departamento de Inmunología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico City, Mexico ^c Coordinación de Infertilidad, Instituto Nacional de Perinatología 'Isidro Espinosa de los Reyes', Mexico City, Mexico and ^d Departamento de Salud, Universidad Iberoamericana Ciudad de México, Mexico City, Mexico

Correspondence: Dr C Hernández-Guerrero, Departamento de Salud, Universidad Iberoamericana Ciudad de México, Prol. Paseo de la Reforma 880, Col. Lomas de Santa Fe, C.P. 01219, México, D.F. Email hehecatlzin@gmail.com

Accepted 30 September 2010. Published Online 18 November 2010.

Objectives To assess immunological variables, T-cell apoptosis and oxidative stress markers in the peripheral blood and peritoneal fluid of women with (WEN) and without (WWE) endometriosis.

Design Observational and transverse case-control study.

Setting National Institute of Perinatology, Mexico City, Mexico.

Population and sample Peripheral blood and peritoneal fluid obtained from 30 WWE and 32 WEN.

Methods Blood was drawn before surgery and peritoneal fluid was collected during surgery but before any surgical procedure had been carried out. Flow cytometry, spectrophotometry, high-performance liquid chromatography and multiplex immunoassay analyses were performed.

Main outcome measures Peripheral and peritoneal lymphocyte subpopulations (CD3⁺, CD4⁺ CD3⁺, CD8⁺ CD3⁺, CD16⁺ CD56⁺, human leucocyte antigen-DR⁺ CD3⁺ and CD19⁺), intracellular CD4⁺ CD3⁺ and CD8⁺ CD3⁺ cytokine synthesis (interleukin-2 [IL-2] and interferon- γ [IFN- γ]), CD3⁺ apoptosis, malondialdehyde and ascorbate concentrations and peritoneal cytokine concentrations.

Results No differences were found in peripheral and peritoneal lymphocyte subsets between the groups. Peritoneal T lymphocytes from WEN produced less IL-2 and IFN- γ than those from WWE. Peritoneal malondialdehyde concentrations were higher and ascorbate concentrations were lower in WEN than in WWE. Higher peritoneal concentrations of pro-inflammatory cytokines (IL-1 β , tumour necrosis factor- α and IL-6) and chemokines (IL-10, IL-8, eotaxin, vascular endothelial growth factor, monocyte chemoattractant protein-1 and regulated upon activation, normal T-cell expressed, and secreted) and lower concentrations of IFN- γ , IL-1 receptor antagonist and IL-15 were found in WEN. No statistical differences were found in IL-2, IL-4, IL-12 and IL-13 concentrations.

Conclusion The alterations observed in WEN were associated with a diminished peritoneal T helper type 1 immune response. Pro-inflammatory, chemotactic, angiogenic and oxidative stress markers were altered in the peritoneal milieu of WEN. These changes appeared to contribute to the peritoneal immune alterations found.

Keywords Apoptosis, endometriosis, intracellular cytokines, lymphocyte subpopulations, multiplex immunoassay.

Please cite this paper as: Mier-Cabrera J, Jiménez-Zamudio L, García-Latorre E, Cruz-Orozco O, Hernández-Guerrero C. Quantitative and qualitative peritoneal immune profiles, T-cell apoptosis and oxidative stress-associated characteristics in women with minimal and mild endometriosis. BJOG 2011;118:6–16.

Introduction

Endometriosis is a common gynaecological disorder characterised by implantation and growth of endometrial tissue

outside the uterine cavity. This pathology is identified in 35–50% of infertile women as the sole cause of primary and secondary infertility. The precise mechanism involved in its aetiology is unknown; however, an impaired T helper

(Th) immune response has been identified as a main factor in the development and progression of endometriosis.¹ Decreased cytotoxic T (Tc) activity has been observed in peripheral Th lymphocytes (CD3⁺ CD4⁺), Tc lymphocytes (CD3⁺ CD8⁺) and natural killer (NK) cells (CD16⁺ CD56⁺) in women with endometriosis.^{2,3} This diminished cytotoxic activity seems to be caused by a lack, or a decreased amount, of the cytokines required for the development of the cellular or Th1 immune response.⁴ Studies have demonstrated decreased expression of Th1 cytokines such as interferon- γ (IFN- γ), interleukin-2 (IL-2), IL-12 and IL-18 by peritoneal and peripheral T lymphocytes in women with endometriosis.^{5,6} Oxidative stress, an imbalance between the production of free radicals and antioxidant concentrations, could also be involved in the modulation of T-lymphocyte activation, given that several mechanisms are abrogated by free radicals.^{7,8} The aim of the present work was to compare lymphocyte subsets, apoptosis and intracellular IFN- γ and IL-2 cytokine synthesis in the peripheral and peritoneal compartments of women with and without endometriosis as quantitative and functional markers of the immune response. The roles in endometriosis of general markers of oxidative stress, such as malondialdehyde and ascorbic acid, and a panel of cytokines related to the Th1/Th2 immune response, pro-inflammation, angiogenesis and chemotactic activity have not been fully elucidated, but these may be involved in mechanisms commonly implicated in the aetiology of this phenomenon.

Materials and methods

Study population

Thirty-two women with endometriosis (WEN) and 30 women without endometriosis (WWE) from the Instituto Nacional de Perinatología 'Isidro Espinosa de los Reyes' (INPerIER) participated in the study. The eligibility criteria for the WWE group were as follows: fertile women presenting to the INPerIER for tubal ligation as a permanent contraceptive method and having the absence of endometriosis confirmed during surgery. The WEN group consisted of infertile women who underwent diagnostic laparoscopy for infertility and presented with stage I or II endometriosis according to the revised criteria of the American Society for Reproductive Medicine (r-ASRM).⁹ Women in the WEN group had never received any hormonal treatment and those in the WWE group had not taken contraceptive hormones in the last 3 months.

The exclusion criteria for both groups were as follows: the presence of pelvic inflammatory disease, autoimmune and/or endocrine metabolic diseases, use of antioxidant medication in the last year, mononuclear peritoneal cell viability <80% and a final reconstituted peritoneal cell number <2 \times 10⁶ cells/ml.

All the participants were provided with information about the study and gave written consent. The Medical Ethics Committee of the INPerIER approved the study protocol.

Peritoneal fluid and blood samples

In both groups, peritoneal fluid collection during laparoscopic surgery was performed in the peri-ovulatory phase. The peri-ovulatory phase was defined as days 12–16 of the menstrual cycle. To prevent contamination with blood, all peritoneal fluid was aspirated from the pouch of Douglas immediately after the insertion of the trocar and before any surgical procedure was performed. After all the peritoneal fluid available had been collected, the peritoneum was washed with 100 ml sterile saline solution, which was recovered to obtain the greatest possible number of peritoneal immune cells. The peritoneal fluid sample and the saline solution recovered from the peritoneum were transported to the laboratory and centrifuged. Peritoneal fluid was stored in 1.5-ml aliquots at -70°C until analysis. Cell pellets from the saline solution washed from the peritoneum and from the peritoneal fluid were pooled, washed and re-suspended in RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 2% fetal bovine serum. Cells were counted and examined using the trypan blue exclusion test and adjusted to a final concentration of 2×10^6 cells/ml.

Peripheral blood samples were drawn into 7-ml heparin vacutainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) before surgery in both groups and were taken to the laboratory immediately for processing. To evaluate lymphocyte subsets and Th and Tc intracellular cytokine production, a sample of whole blood was taken and stained within 1 hour of drawing for optimal results; the rest of the sample was centrifuged, and the plasma was separated and stored at -70°C .

To assess the percentage of apoptosis of T lymphocytes in peritoneal and peripheral blood in WEN and WWE, lymphocytes were isolated using LymphoprepTM (Axis-Shield PoC AS, Oslo, Norway) gradient centrifugation method following the manufacturer's instructions. Recovered lymphocytes were washed three times and re-suspended in RPMI-1640 medium supplemented with 2% bovine serum albumin (Invitrogen Life Technologies).

Flow cytometric analysis

For lymphocyte subpopulations, whole blood and total peritoneal cells were stained with the following monoclonal antibodies: Th and Tc lymphocytes, fluorescein isothiocyanate-conjugated (-FITC) CD4/phycoerythrin-conjugated (-PE) CD8/PE-Cychrome 5-conjugated (-PECy5) CD3; B and T lymphocytes, CD3-FITC/CD19-PE; activated T lymphocytes, CD3-FITC/HLA-DR-PE; and NK cells, CD3-FITC/CD16⁺ CD56-PE; these were processed according to

the manufacturer's instructions (Beckton Dickinson, San Jose, CA, USA).

For activation, whole blood and peritoneal cells were stimulated for 4 hours with ionomycin, phorbol 12-myristate 13-acetate and Brefeldin A (Sigma-Aldrich, St Louis, MO, USA). A culture without ionomycin and phorbol 12-myristate 13-acetate was obtained to determine basal intracellular cytokine production. Plates were incubated at 37°C with 5% CO₂. Intracellular cell staining was performed according to the manufacturer's instructions (Beckton Dickinson) with the following antibodies: CD4-FITC/CD3-PECy5, CD8-PE/CD3-PCy5, anti-human IFN- γ -PE and anti-human IL-2-PE.

To evaluate the degree of apoptosis in T lymphocytes from peripheral and peritoneal samples, cells were washed with cold phosphate-buffered saline and processed for cell cycle analysis. Briefly, the cells were fixed in absolute ethanol. The fixed cells were then centrifuged at 196 \times g and washed twice with cold phosphate-buffered saline. RNAase A (final concentration 20 μ g/ml) and propidium iodide staining solution (final concentration 50 μ g/ml) were added to the cells, which were incubated for 30 minutes at 37°C in the dark. The cells were analysed using a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA) equipped with CELLQUEST 3.3 software (Becton Dickinson Immunocytometry Systems, Mansfield, MA, USA). Cells with a DNA content less than that of G0/G1-phase cells were considered to be apoptotic (sub-G0/G1). The ModFit LT 3.1 trial cell cycle analysis software was used to determine the percentage of cells in the different phases of the cell cycle.

Acquisition was performed on a FACSCalibur flow cytometer (Becton Dickinson). A minimum of 10 000 lymphocyte-gated events were acquired using CELLQUEST 3.3 software and analysed with WINMDI 2.8 software (<http://fac.scripps.edu/software.html>). Lymphocytes were appropriately gated on forward¹⁰ and side scatter.

Malondialdehyde and ascorbic acid determinations

The concentrations of thiobarbituric acid reactive substances were determined according to the method developed by Ohkawa *et al.*¹¹ Briefly, plasma and peritoneal fluid were mixed with 12.5 mmol/l butylated hydroxytoluene, 0.11 mol/l orthophosphoric acid and 0.1 mol/l thiobarbituric acid. The mixture was homogenised and boiled at 95°C for 45 minutes. After cooling, the reaction mixture was mixed with *n*-butanol and a saturated solution of NaCl and centrifuged at 1761 \times g for 5 minutes. Malondialdehyde concentrations were estimated by spectrophotometric measurement (UV/visible Lambda 45; Perkin Elmer Instruments, Foster, CA, USA) at 530 nm to evaluate the production of a pink chromogen. A standard curve was prepared with tetramethoxypropane and thiobarbituric acid, and then the malondialdehyde values were calculated.

Plasma and peritoneal ascorbate concentrations were analysed by paired-ion, reverse-phase high-performance liquid chromatography coupled with electrochemical detection (Perkin Elmer Instruments) as reported by Lee *et al.*¹² A C18 reverse-phase column with a mobile phase of sodium acetate buffer (pH 4) containing *n*-octylamine (1 mmol/l) as the paired-ion reagent, at a flow rate of 1 ml per minute, was used for maximum separation with minimal elution time. An electrochemical detector pre-set at 0.7 V and a current of 100 mA/V were used for the determination of ascorbic acid concentration. Briefly, 10% (volume/volume) metaphosphoric acid was added to the plasma and peritoneal fluid. After 10 minutes, the samples were centrifuged at 2397 \times g for 10 minutes and the supernatants were stored at -80°C. For determinations, samples were thawed and diluted 1:10, and the ascorbic acid standard was added to the samples. After mixing, the samples were ready for high-performance liquid chromatography determination.

All reagents were purchased from Sigma (Sigma-Aldrich). The coefficients of variation for intra- and inter-assays were 3.5 and 7.5%, respectively, for malondialdehyde measurements and 5 and 8%, respectively, for ascorbic acid measurements.

Multiplex immunoassay for cytokine determinations

A Bio-Plex human cytokine assay (Bio-Plex, Hercules, CA, USA) for simultaneous quantification of the concentrations of various cytokines was run according to the recommended procedure. Briefly, the pre-mixed standards were reconstituted, generating stock concentrations of 50 000 pg/ml for each cytokine, which were serially diluted to generate eight points for the standard curve. The assay was performed in a 96-well filtration plate supplied with the assay kit. Pre-mixed beads coated with target capture antibodies were transferred to each well and washed twice with Bio-Plex wash buffer. Pre-mixed standards (50 μ l) or samples (50 μ l) (without dilution) were added to each well. The plate was shaken and incubated at room temperature for 30 minutes with low-speed shaking. After incubation and washing, pre-mixed detection antibodies were added to each well. The incubation was terminated after shaking for 10 minutes at room temperature. After three washes, the beads were re-suspended in Bio-Plex assay buffer. Beads were read on the Luminex system (Bio-Plex) and the data were analysed using BIO-PLEX MANAGER software (v 3.0; Bio-Rad, Hercules, CA, USA) with 5PL curve fitting.

Standard curves for each cytokine were generated using the reference cytokine concentrations supplied with the kit, and resulted in correlation coefficients ranging from 0.85 to 0.99. Intra- and inter-assay variation coefficients were 2–7% and 3.5–12%, respectively.

Statistical analysis

Statistical analyses were performed using SIGMASTAT 3.1 software (Systat Software Inc., San Jose, CA, USA). Epidemiological and obstetric data, data from flow cytometric analyses, and malondialdehyde, ascorbic acid and cytokine concentrations were analysed using Student's *t* test or the Mann–Whitney rank sum test, for parametric and nonparametric data, respectively. Comparisons between compartments (peripheral blood versus peritoneal fluid) in WWE and WEN were carried out using the appropriate parametric or nonparametric paired test. A value of $P < 0.05$ was considered to indicate a significant difference.

Results

General characteristics of women with and without endometriosis

All participants in the study lived in Mexico City or the metropolitan area and were of middle/low socio-economic status. The mean (\pm standard deviation) age of women in the WWE group was 33.8 (\pm 5.4) years, which was not statistically different ($P > 0.05$) from the mean age of women in the WEN group, which was 32.7 (\pm 2.5) years. Obstetric characteristics of women in the WWE group were as follows (given as median [minimum–maximum]): gravidity, 3 (1–7); vaginal delivery, 1 (0–4); caesarean delivery, 1 (1–2); and miscarriage, 0 (0–2). None of the women in the WEN group had ever been pregnant.

Flow cytometric analyses

Lymphocyte subsets.

In both groups, peripheral lymphocyte subpopulation values were within the reference range for lymphocyte subsets in healthy adults.¹³ Comparison of the percentage of positive cells for total lymphocytes, B cells, Th cells, Tc cells, T-activated lymphocytes and NK cells from peripheral blood and peritoneal fluid between WWE and WEN showed no statistically significant differences ($P > 0.05$) (Figure 1A, B).

The comparison between peripheral blood and peritoneal fluid lymphocyte subsets in the WWE and WEN groups revealed statistically significant differences in the percentages of all subsets of cells (total lymphocytes, Th, Tc and NK cells) analysed in both groups ($P < 0.001$) (Figure 2A, B), with the exception of activated T-lymphocyte subsets, for which the percentage was significantly higher ($P < 0.05$) only in the peritoneum of WWE than in peripheral blood ($21.85 \pm 15.22\%$ versus $12.12 \pm 5.71\%$ for peritoneal fluid versus peripheral blood). The percentage of activated T lymphocytes in WEN showed no significant difference.

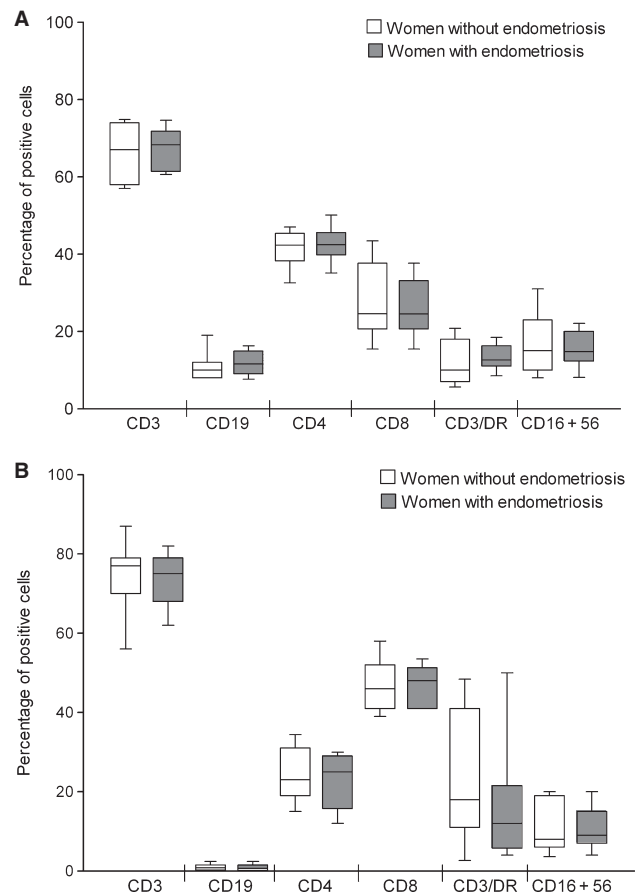


Figure 1. Intergroup lymphocyte subpopulations comparison.

Peripheral (A) and peritoneal (B) lymphocyte subpopulations were compared between women without endometriosis (open box; $n = 30$) and those with endometriosis (grey box; $n = 32$). Data are expressed as percentages of positive cells. Boxes represent median (black line) \pm 5th/95th percentiles.

Intracellular cytokines

The percentage of peripheral Th lymphocytes producing IFN- γ in WEN was lower than that in WWE ($P < 0.001$). No differences were found in the percentage of peripheral Th lymphocytes producing IL-2, or in the percentage of peripheral Tc lymphocytes producing both cytokines (Figure 3A–E). Similarly, the percentages of peritoneal Th and Tc lymphocytes producing both cytokines (IL-2 and IFN- γ) were lower in WEN (Figure 4A–E; $P < 0.001$). Moreover, we found a higher percentage of peritoneal Th and Tc lymphocytes producing IL-2 and IFN- γ in WWE than in WEN when the two compartments were compared ($P < 0.05$; Table 1).

Apoptosis

There was no statistically significant difference in the percentage of peripheral apoptotic lymphocytes between the

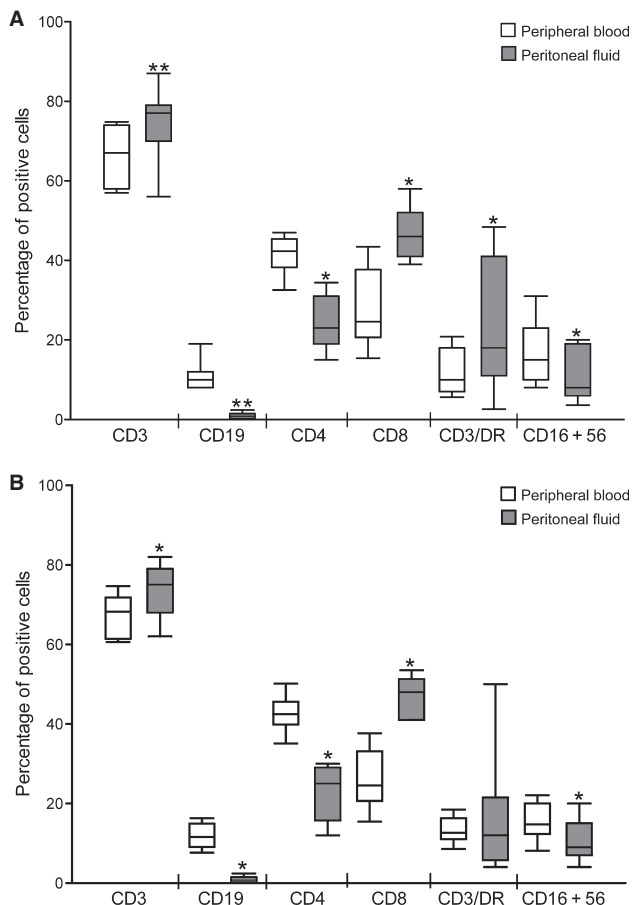


Figure 2. Intragroup lymphocyte subpopulations comparison. Lymphocyte subsets were compared between peripheral and peritoneal compartments in women without endometriosis (**A**; $n = 30$) and those with endometriosis (**B**; $n = 32$). Data are expressed as percentages of positive cells. Boxes represent median (black line) \pm 5th/95th percentiles. * $P < 0.001$, paired t test; ** $P < 0.001$, Wilcoxon rank sum test.

two groups (Figure 3F). Peritoneal lymphocytes from WEN showed a higher percentage of apoptosis than those from WWE (Figure 4F) ($P < 0.001$). A higher percentage of apoptosis was observed in the peritoneal compartment for WEN when the two compartments were compared ($P < 0.05$). No statistical difference was observed in the comparison between compartments in WWE (Table 2). All the cell cycle values obtained for peripheral and peritoneal T lymphocytes were within the normal ranges and showed no statistically significant differences between the groups.

Oxidant and antioxidant markers

No statistically significant difference was found in the comparison of peripheral malondialdehyde concentrations between WWE and WEN. However, the peritoneal fluid from WEN showed a higher concentration of malondialdehyde than that obtained from WWE ($P < 0.05$). Moreover,

in WEN, the malondialdehyde concentration was higher in the peritoneal compartment than in peripheral blood ($P < 0.05$). No significant difference was found in the comparison of compartments in WWE (Table 3).

The peritoneal fluid from WEN showed a lower ascorbic acid concentration than that obtained from WWE ($P < 0.05$). No significant difference was found in peripheral ascorbate concentrations. However, when the peritoneal and peripheral compartments were compared, the peritoneal ascorbate concentration was lower in both the WWE and the WEN groups ($P < 0.05$) (Table 3).

Peritoneal cytokine concentrations

A total of 16 cytokines were evaluated using a multiplex immunoassay. Cytokines related to pro-inflammation (IL-6, tumour necrosis factor- α [TNF- α] and IL-1 β), angiogenesis (IL-8 and vascular endothelial growth factor [VEGF]), chemotaxis (monocyte chemoattractant protein 1 [MCP-1], regulated upon activation, normal T-cell expressed, and secreted [RANTES] and eotaxin) and anti-inflammation (IL-10) showed a higher concentration in the peritoneal milieu of WEN than in that of WWE ($P < 0.05$). The concentrations of Th1 cytokines (IFN- γ and IL-15) and IL-1 receptor antagonist were reduced in WEN ($P < 0.05$). We found no significant differences between the groups for the concentrations of IL-2, IL-12, IL-4 and IL-13 (Table 4).

Discussion

Endometriosis is a multifactorial disease in which an altered peritoneal immune response has been identified as a factor involved in the development and spread of endometrial tissue outside the uterus. It has not yet been conclusively determined why there is a diminished cytotoxic response in women with endometriosis; however, the evidence suggests that a lack of, and/or alterations in, T-cell functions rather than quantitative immune cell differences between women with and without endometriosis may explain this reduced cytotoxic response in women with endometriosis.^{3,5,6} In the present work, we evaluated the possible causes of this reduced cytotoxic response by determining whether there were numerical alterations in lymphocyte subpopulations or qualitative changes in the effector characteristics of T lymphocytes obtained from the peritoneal milieu and peripheral blood of women with endometriosis.

Studies on the frequencies of peripheral and peritoneal lymphocytes characterised by flow cytometry have also produced inconsistent results in women with endometriosis. In the present study, we found no differences between WEN and WWE in any of the lymphocyte subsets analysed from either the peritoneal or the peripheral compartment, similar to the findings of Opsahl *et al.*¹⁴ and Zhang *et al.*¹⁵

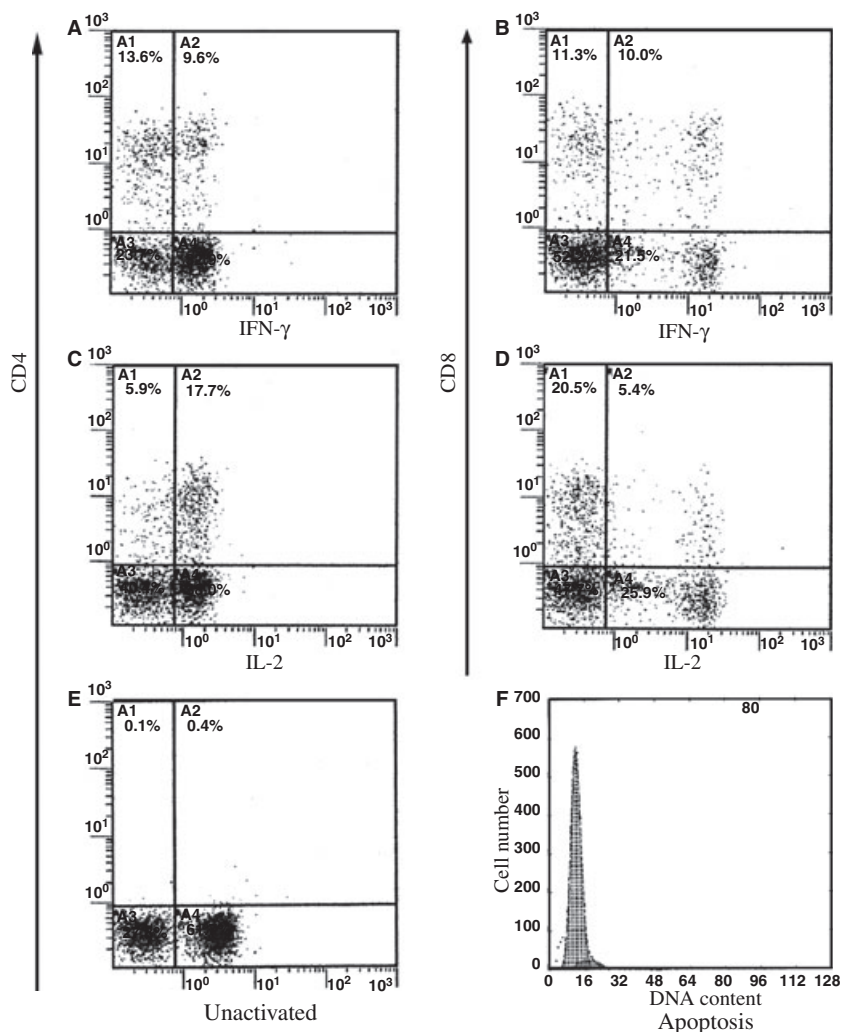


Figure 3. Representative dot plots showing peripheral activated CD4⁺ and CD8⁺ T-lymphocyte synthesis of IFN- γ (**A** and **B**, respectively), IL-2 (**C** and **D**, respectively) and unactivated CD4⁺ lymphocytes (**E**) and a representative DNA histogram (cell cycle and apoptosis) for peripheral CD3⁺ lymphocytes (**F**) from women without endometriosis. Data in dot plots are expressed as percentages of positive cells.

Szylo *et al.*¹⁶ found that the percentages of peripheral CD3⁺ and CD4⁺ cells did not differ between women with and without endometriosis, but found a decreased percentage of peripheral CD8⁺, CD19⁺ and NK cells in women with endometriosis. Hill *et al.*¹⁷ found that peripheral blood leucocyte profiles in women without endometriosis ($n = 8$) and those with I–II stage endometriosis ($n = 33$) were indistinguishable from reported normal values, although peritoneal fluid profiles were increased in the numbers of total leucocytes, macrophages, Th lymphocytes and NK cells and in the CD4⁺:CD8⁺ ratio. Significantly higher numbers of T cells and NK cells and fewer B lymphocytes were observed by Dmowski *et al.*¹⁸ in the peritoneal fluid of women with endometriosis compared with those in women without the disease. Tariverdian *et al.*¹⁹ like us, found the percentages of peritoneal CD4⁺ and

CD8⁺ cells to be slightly increased in early endometriosis when compared with controls, but differences were not significant. In addition, Ho *et al.*²⁰ found a significant decrease in NK cytotoxicity in the peritoneal fluid of women with endometriosis compared with those without endometriosis, but no differences in the proportion of NK cells in either the peripheral blood or the peritoneal fluid between women without and with stage III–IV endometriosis. It is interesting to note, however, that in endometriosis, despite decreased NK cell activity, the percentages of peripheral NK cells have been found to be unchanged^{14,20,21} or increased.¹⁷ These inconsistencies may be attributable to methodological differences, different sample sizes, samples taken at different phases of the menstrual cycle, different diagnostic criteria or different stages of endometriosis, or to comparison of women with various stages

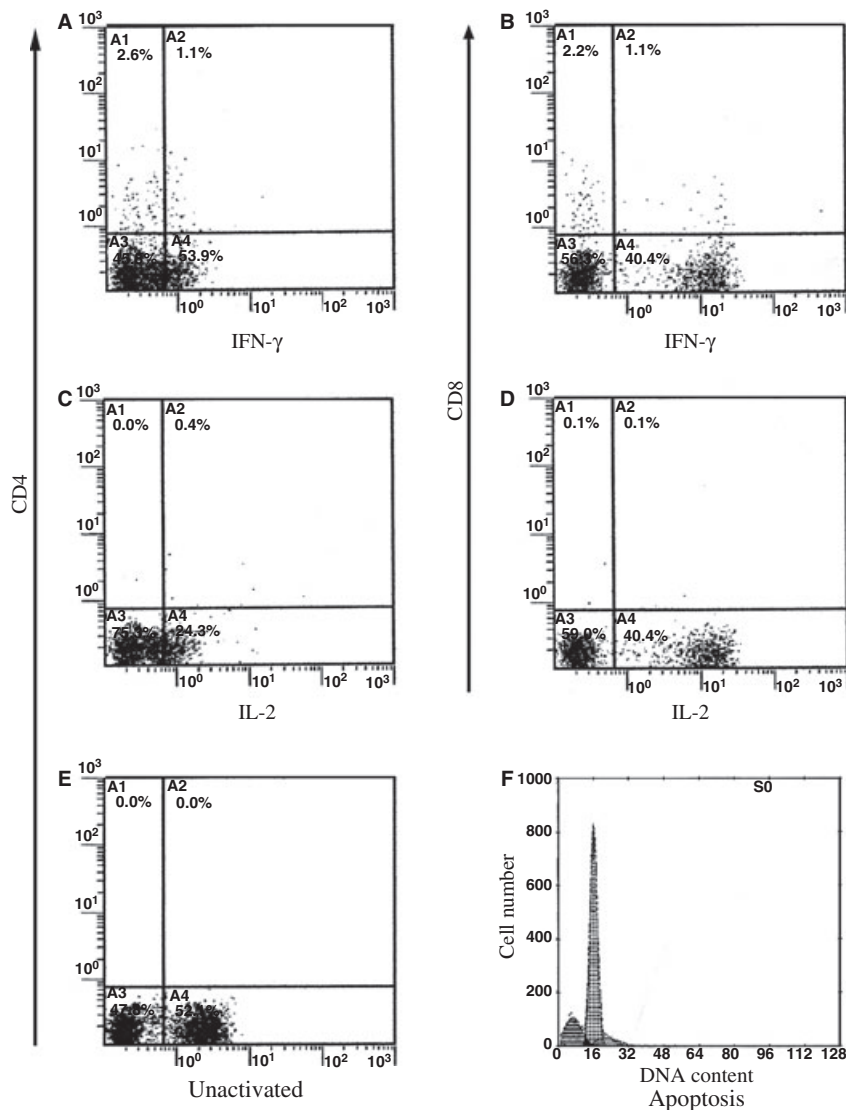


Figure 4. Representative dot plots showing peritoneal activated CD4⁺ and CD8⁺ T-lymphocyte synthesis of IFN- γ (A and B, respectively), IL-2 (C and D, respectively) and unactivated CD4⁺ lymphocytes (E) and a representative DNA histogram (cell cycle and apoptosis) for peritoneal CD3⁺ lymphocytes (F) from women with endometriosis. Data in dot plots are expressed as percentages of positive cells.

of endometriosis versus different groups considered as controls (e.g. infertile women without endometriosis, women with uterine leiomyoma, or fertile women without endometriosis).

However, comparisons revealed that the peritoneal cavity and the peripheral blood are immunologically different compartments. In both groups of women, the peritoneal milieu contained a higher percentage of total T and Tc cells and a lower percentage of Th, B and NK cells. This observation is consistent with the findings of Szyllo *et al.*¹⁶ (except for NK cells) and Opsahl *et al.*,¹⁴ who observed that the percentage of peritoneal activated T cells and monocytes was increased in both fertile and infertile

women and that there were decreases in the percentages of Th cells and NK cells.

With respect to the Th1 cytokine profile, we found reduced expression of intracellular IFN- γ by peripheral T lymphocytes in WEN, whereas Szyllo *et al.*¹⁶ did not find differences in this cytokine between groups. Furthermore, peritoneal T lymphocytes (helper and cytotoxic) displayed the same pattern of diminished IFN- γ and IL-2 expression. These cytokines are necessary to promote and enhance an adequate cytotoxic immune response.⁴ Altered synthesis of these cytokines may therefore be related to a functional rather than a numerical immune alteration in the women we studied. Gmyrek *et al.*⁶ compared the expression of

Table 1. Intracellular cytokines

	Women without endometriosis (n = 30)		Women with endometriosis (n = 32)	
	Peripheral	Peritoneal	Peripheral	Peritoneal
CD4 ⁺ /IFN- γ	9.01 \pm 4.02 (9; 1–18)	14.67 \pm 3.03*** (15; 10–22)	5.34 \pm 1.94* (6; 2–10)	8.26 \pm 3.25*** (8; 2–16)
CD4 ⁺ /IL-2	5.28 \pm 2.24 (5; 1–10)	18.06 \pm 3.72*** (18; 13–26)	4.74 \pm 2.51 (4; 1–12)	13.28 \pm 3.59*** (13; 5–22)
CD8 ⁺ /IFN- γ	6.59 \pm 1.69 (7; 3–9)	21.49 \pm 5.75*** (21; 14–39)	6.17 \pm 1.85 (6; 2–10)	10.37 \pm 6.01*** (10; 2–36)
CD8 ⁺ /IL-2	4.68 \pm 2.1 (5; 1–11)	13.59 \pm 3.52*** (12; 7–23)	4.03 \pm 2.24 (4; 1–9)	8.43 \pm 2.77*** (8; 4–15)

Values are mean \pm SD (median; minimum–maximum). Data are expressed as percentages of positive cells.

For peripheral cells from women without endometriosis versus women with endometriosis: Mann–Whitney rank sum test; * P < 0.001.

For peritoneal cells from women without endometriosis versus women with endometriosis: Student's t test; *** P < 0.001.

For peripheral cells versus peritoneal cells from each group: Mann–Whitney rank sum test; *** P < 0.05.

Table 2. Cell cycle and apoptosis stages

	Women without endometriosis (n = 30)		Women with endometriosis (n = 32)	
	Peripheral	Peritoneal	Peripheral	Peritoneal
G0/G1	93.10 \pm 11.12	90.23 \pm 13.92	91.2 \pm 12.5	88.52 \pm 12.5
S	4.22 \pm 1.16	4.61 \pm 1.93	6.79 \pm 3.02	7.34 \pm 3.44
G2/M	4.91 \pm 1.10	5.29 \pm 2.60	5.45 \pm 4.82	4.71 \pm 3.54
Apoptotic cells	5.68 \pm 2.14	8.70 \pm 3.21	6.34 \pm 1.94	16.3 \pm 4.41***

Values are mean \pm standard deviation. Data are expressed as percentages of cells in the area under the curve.

For peritoneal cells from women without endometriosis versus women with endometriosis: Student's t test; * P < 0.001.

For peripheral cells versus peritoneal cells from each group: Mann–Whitney rank sum test; *** P < 0.05.

Table 3. Oxidant and antioxidant markers

	Women without endometriosis (n = 30)		Women with endometriosis (n = 32)	
	Peripheral	Peritoneal	Peripheral	Peritoneal
Malondialdehyde	23.75 \pm 6.46 (33–8)	20.87 \pm 8.19 (53–15)	27.17 \pm 8.67 (42–14)	34.65 \pm 5.23*** (64–25)
Ascorbic acid	53.42 \pm 13.29 (106–28)	23.57 \pm 6.19** (33–13)	57.17 \pm 12.43 (122–24)	15.80 \pm 4.39*** (30–7)

Values are mean \pm standard deviation (median; minimum–maximum). Data are expressed as $\mu\text{mol/l}$.

For peritoneal fluid from women without endometriosis versus women with endometriosis: Student's t test; * P < 0.05.

For peripheral blood versus peritoneal fluid from each group: Mann–Whitney rank sum test; ** P < 0.05.

IFN- γ by peripheral and peritoneal lymphocytes in women with endometriosis and found that the percentages of CD3⁺, CD3⁺ CD8⁺ and CD3⁺ CD8⁻ cells stained for IFN- γ were higher in the peritoneum and that a higher percentage of peritoneal CD3⁺ CD8⁺ cells stained for IFN- γ compared with CD3⁺ CD8⁻ cells. We also observed that peritoneal CD3⁺ CD8⁺ cells from women with and without endometriosis expressed more IFN- γ than those from peripheral blood, and that CD3⁺ CD4⁺ cells expressed less IFN- γ than

CD3⁺ CD8⁺ cells. Moreover, when Gmyrek *et al.*²² compared the peripheral expression of IFN- γ between women with endometriosis (stages I to IV) and women with uterine leiomyomas as control group, they found slightly lower amounts of IFN- γ staining in CD3⁺ and CD3⁺ CD8⁻ cells in the former group, but the difference was not significant. However, when endometriosis stages were separated into mild (ASRM I–II) and advanced (ASRM III–IV), a statistically significant difference compared with the control

Table 4. Peritoneal cytokines and chemokine concentrations

	Women without endometriosis (n = 30)	Women with endometriosis (n = 32)
IL-6	649 (142–1736)	1261* (602–3735)
VEGF	399 (201–729)	1293* (259–3823)
MCP-1 (CCL2)	1076 (455–2794)	1571* (102–8514)
IL-8 (CXCL8)	131 (67–234)	526* (260–1440)
RANTES (CCL5)	327 (132–497)	502* (280–1654)
TNF- α	213 (146–477)	389* (135–476)
Eotaxin (CCL11)	233 (165–378)	360* (178–426)
IL-10	131 (67–274)	182* (103–499)
IL-1 β	72 (29–115)	135* (93–193)
IL-1 receptor antagonist	589 (386–971)	349* (135–476)
IFN- γ	746 (467–917)	280* (87–828)
IL-15	351 (144–605)	154* (109–245)
IL-2	54 (34–62)	45 (27–55)
IL-12	30 (12–52)	34 (7–48)
IL-4	7 (3–13)	6 (1–32)
IL-13	126 (83–244)	153 (104–207)

CCL2, C-C motif ligand 2; CXCL8, CXC motif ligand 8.

Values are median (minimum–maximum). Data are expressed as pg/ml.

For peritoneal fluid from women without endometriosis versus women with endometriosis: Mann–Whitney rank sum test; * $P < 0.05$.

group in the expression of this cytokine in CD3⁺ CD8⁻ cells was found for advanced but not for mild endometriosis. We found a similar reduction in the peripheral expression of IFN- γ by CD3⁺ CD4⁺ cells, but our comparison was of women who had stages I and II endometriosis with women without endometriosis.

Evaluation of the cytokine profile in the peritoneal fluid of WEN showed that IFN- γ production by Th and Tc cells was abrogated, whereas IFN- γ concentrations in the peritoneal fluid were diminished, as were concentrations of IL-15, a cognate of IL-2. Regarding the Th1-associated cytokines IL-2 and IL-12 and the Th2-associated cytokines IL-4 and IL-13, similar values were obtained between WEN and WWE; however, the concentration of IL-10 was increased in the peritoneal fluid of WEN. This finding is consistent with an immune imbalance towards the local Th2 response in this group of women.²³

In contrast, our results revealed, in the peritoneal fluid of WEN, higher concentrations of pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6) and a reduced concentration of IL-1 receptor antagonist,²⁴ a major anti-inflammatory cytokine involved in the regulation of inflammatory events by phagocytic cells. Consistent with the pro-inflammatory peritoneal environment found in WEN, angiogenic (IL-8

and VEGF) and chemotactic (MCP-1, RANTES and eotaxin) cytokines were found to be increased in concentration.^{25–29} These groups of molecules are synthesised and released by local peritoneal immune and nonimmune cells in response to stimulation by TNF- α and IL-1 β , and have been associated with the ectopic endometrial tissue adhesion and proliferation mechanisms involved in the aetiology of endometriosis. Similarly, pro-inflammatory and chemotactic cytokines play a central role in the recruitment and activation of phagocytic cells, which are the main producers of reactive oxygen and nitrogen species.

Evaluation of the peritoneal fluid of WEN revealed an imbalance in its antioxidant and oxidative markers favouring oxidative stress conditions, and also revealed a decreased concentration of ascorbic acid associated with higher values of malondialdehyde in comparison with samples from WWE. Conditions of higher oxidative stress could disturb several immunological mechanisms, including those investigated here. It has been reported that an excess of free radicals induces a reduction in the expression of CD3- ζ (zeta) chain on T and NK cells. The loss of CD3- ζ on T cells prevents its phosphorylation and blocks the initial activating signal transduction cascade necessary for the activation of T cells.^{30,31} This results in decreased production of IL-2, as a consequence of a reduced intracellular calcium response and altered activity of transcription factors required for IL-2 transcription. Also, nitric oxide acts at the transcriptional level, inhibiting IFN- γ synthesis by NK cells and Th lymphocytes in an autocrine and paracrine fashion, respectively, through a negative feedback inhibition mechanism.³²

The abrogation of Th1 immune cytokine synthesis is congruent with the apoptosis stage exhibited by T cells in WEN. The mechanisms involved in T-lymphocyte apoptosis induction may be related to the presence of cytokines, hormones or growth factors released by immune and non-immune cells located in the peritoneal environment. Selam *et al.*^{27,33} found a dose–response increase in Fas-L expression when cultured endometrial cells were stimulated with increasing doses of human recombinant IL-8. Selam *et al.*³³ also evaluated, *in vitro*, the induction of apoptosis in a T-lymphocyte cell line by direct cell–cell contact of T lymphocytes with cells that had previously expressed Fas ligand. Higher concentrations of IL-8 in the peritoneal fluid of women with endometriosis have previously been reported,³⁴ and this is thought to be related to the characteristic pro-inflammatory environment found in women with endometriosis. Previous studies have demonstrated that concentrations of TNF- α are increased in the peritoneal fluid of women with endometriosis. TNF- α is a powerful inducer of the expression and secretion of IL-8 by local peritoneal cells such as macrophages, T lymphocytes, neutrophils, mesothelial cells and endometrial gland cells,

which stimulate the proliferation of uterine stromal cells in an autocrine fashion. Iwabe *et al.*³⁵ demonstrated a positive correlation between TNF- α and IL-8 concentrations in the peritoneal fluid as well as a direct correlation between the size and number of initial peritoneal red endometrial lesions. In addition, TNF- α can directly induce apoptosis via the production of reactive mitochondrial species through xanthine oxidase and arachidonate metabolism,³⁶ which is consistent with evidence of oxidative stress in the peritoneal cavity of women with endometriosis obtained by others and by our group.^{37–39}

To date, the relationship between the immune response and oxidant/antioxidant characteristics presented by women with endometriosis has not been investigated sufficiently. Although the sample size in our study was small, the selection of women was rigorous, with inclusion/exclusion criteria being strictly applied to prevent selection bias and confounding by other factors (age, alcohol/cigarette use, other immunological and hormonal factors, use of medications, anatomical factors, weight and ethnicity) to ensure that the differences in biochemical and immunological factors found in the peritoneal milieu of women with endometriosis were attributable to this disease. The results of this study contribute to the elucidation of the aetiology of endometriosis.

Conclusions

No differences were found in peripheral and peritoneal lymphocyte subsets between women with and without endometriosis. The WEN showed decreased peritoneal IL-2 and IFN- γ synthesis by both Th and Tc lymphocytes in comparison with WWE. They also had higher peritoneal malondialdehyde and lower peritoneal ascorbate concentrations in comparison with WWE. Women in the WEN group had a higher percentage of peritoneal T-lymphocyte apoptosis than those in the WWE group. Concentrations of pro-inflammatory cytokines (IL-6, TNF- α and IL-1 β), angiogenic cytokines (IL-8 and VEGF), chemotactic cytokines (MCP-1, RANTES and eotaxin) and IL-10 were higher, and those of Th1 cytokines (IFN- γ and IL-15) and IL-1 receptor antagonists were lower in the peritoneal milieu of WEN.

Disclosure of interests

We declare that we have no financial or other contractual agreements that might cause conflicts of interest. All the authors read and approved the final submitted manuscript.

Contribution to authorship

JMC participated in the recruitment of women, carried out the main outcome determinations, the statistical analysis and the interpretation of results, and participated in discus-

sion of the data. LJZ and EGL participated in the analysis and discussion of the data. OCO participated in the recruitment of women and obtained the peritoneal fluid samples. CHG conceived and designed the study, participated in the analysis and discussion of the data, and drafted the manuscript. All authors read and approved the final manuscript.

Details of ethics approval

The research protocol was approved by the INPerIER's Academic Ethics bureau (212250-06081). The study procedures are in accordance with the Helsinki Declaration of Human Rights. All study participants signed an informed consent form before being enrolled in the study.

Funding

Financial support was obtained from the Consejo Nacional de Ciencia y Tecnología (CONACyT): Grant SALUD-2002-C-01-7615/A-1.

Acknowledgements

We acknowledge CONACyT for the grant awarded to J. Mier-Cabrera (fellowship register no. 200396, C.Q.B. PhD Program of the Escuela Nacional de Ciencias Biológicas, IPN.) ■

References

- 1 Kyama CM, Debrock S, Mwenda JM, D'Hooghe TM. Potential involvement of the immune system in the development of endometriosis. *Reprod Biol Endocrinol* 2003;1:123.
- 2 Somigliana E, Vigano P, Vignali M. Endometriosis and unexplained recurrent spontaneous abortion: pathological states resulting from aberrant modulation of natural killer cell function? *Hum Reprod Update* 1999;5:40–51.
- 3 Lebovic DI, Mueller MD, Taylor RN. Immunobiology of endometriosis. *Fertil Steril* 2001;75:1–10.
- 4 Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Altern Med Rev* 2003;8:223–46.
- 5 Antsiferova YS, Sotnikova NY, Posiseeva LV, Shor AL. Changes in the T-helper cytokine profile and in lymphocyte activation at the systemic and local levels in women with endometriosis. *Fertil Steril* 2005;84:1705–11.
- 6 Gmyrek GB, Sieradzka U, Goluda M, Gabrys M, Sozanski R, Jerzak M, *et al.* Differential flow cytometric detection of intracellular cytokines in peripheral and peritoneal mononuclear cells of women with endometriosis. *Eur J Obstet Gynecol Reprod Biol* 2008;137:67–76.
- 7 Peterson JD, Herzenberg LA, Vasquez K, Waltenbaugh C. Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns. *Proc Natl Acad Sci U S A* 1998;95:3071–6.
- 8 King MR, Ismail AS, Davis LS, Karp DR. Oxidative stress promotes polarization of human T cell differentiation toward a T helper 2 phenotype. *J Immunol* 2006;176:2765–72.
- 9 Revised American Society for Reproductive Medicine classification of endometriosis: 1996. *Fertil Steril* 1997;67:817–21.
- 10 Orlandi A, Ferlosio A, Ciucci A, Sesti F, Lifschitz-Mercer B, Gabbiani G, *et al.* Cellular retinol-binding protein-1 expression in endometrial

- stromal cells: physiopathological and diagnostic implications. *Histopathology* 2004;45:511–7.
- 11 Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979;95:351–8.
 - 12 Lee W, Hamernyk P, Hutchinson M, Raisys VA, Labbe RF. Ascorbic acid in lymphocytes: cell preparation and liquid-chromatographic assay. *Clin Chem* 1982;28:2165–9.
 - 13 Reichert T, DeBruyere M, Deneys V, Totterman T, Lydyard P, Yuksel F, *et al.* Lymphocyte subset reference ranges in adult Caucasians. *Clin Immunol Immunopathol* 1991;60:190–208.
 - 14 Opsahl MS, Hayslip CC, Klein TA, Cunningham DS. Characterization of peripheral blood and peritoneal fluid mononuclear cell subsets in fertile and infertile women. *Gynecol Obstet Invest* 1994;37:176–9.
 - 15 Zhang C, Maeda N, Izumiya C, Yamamoto Y, Kusume T, Oguri H, *et al.* Killer immunoglobulin-like receptor and human leukocyte antigen expression as immunodiagnostic parameters for pelvic endometriosis. *Am J Reprod Immunol* 2006;55:106–14.
 - 16 Szylo K, Tchorzewski H, Banasik M, Glowacka E, Lewkowicz P, Kamer-Bartosinska A. The involvement of T lymphocytes in the pathogenesis of endometriotic tissues overgrowth in women with endometriosis. *Mediators Inflamm* 2003;12:131–8.
 - 17 Hill JA, Faris HM, Schiff I, Anderson DJ. Characterization of leukocyte subpopulations in the peritoneal fluid of women with endometriosis. *Fertil Steril* 1988;50:216–22.
 - 18 Dmowski WP, Gebel HM, Braun DP. The role of cell-mediated immunity in pathogenesis of endometriosis. *Acta Obstet Gynecol Scand Suppl* 1994;159:7–14.
 - 19 Tariverdian N, Siedentopf F, Rucke M, Blois SM, Klapp BF, Kentenich H, *et al.* Intraperitoneal immune cell status in infertile women with and without endometriosis. *J Reprod Immunol* 2009;80:80–90.
 - 20 Ho HN, Chao KH, Chen HF, Wu MY, Yang YS, Lee TY. Peritoneal natural killer cytotoxicity and CD25⁺ CD3⁺ lymphocyte subpopulation are decreased in women with stage III–IV endometriosis. *Hum Reprod* 1995;10:2671–5.
 - 21 Oosterlynck DJ, Cornillie FJ, Waer M, Vandeputte M, Koninckx PR. Women with endometriosis show a defect in natural killer activity resulting in a decreased cytotoxicity to autologous endometrium. *Fertil Steril* 1991;56:45–51.
 - 22 Gmyrek GB, Sieradzka U, Goluda M, Gabrys M, Sozanski R, Jerzak M, *et al.* Flow cytometric evaluation of intracellular cytokine synthesis in peripheral mononuclear cells of women with endometriosis. *Immunol Invest* 2008;37:43–61.
 - 23 Podgaec S, Abrao MS, Dias JA Jr, Rizzo LV, de Oliveira RM, Baracat EC. Endometriosis: an inflammatory disease with a Th2 immune response component. *Hum Reprod* 2007;22:1373–9.
 - 24 Zhang X, Wen J, Deng L, Lin J. Decreased levels of peritoneal interleukin-1 receptor antagonist in patients with endometriosis and disease-related dysmenorrhea. *Fertil Steril* 2007;88:594–9.
 - 25 Hornung D, Dohrn K, Sotlar K, Greb RR, Wallwiener D, Kiesel L, *et al.* Localization in tissues and secretion of eotaxin by cells from normal endometrium and endometriosis. *J Clin Endocrinol Metab* 2000;85:2604–8.
 - 26 Kalu E, Sumar N, Giannopoulos T, Patel P, Croucher C, Sherriff E, *et al.* Cytokine profiles in serum and peritoneal fluid from infertile women with and without endometriosis. *J Obstet Gynaecol Res* 2007;33:490–5.
 - 27 Pizzo A, Salmeri FM, Ardita FV, Sofo V, Tripepi M, Marsico S. Behaviour of cytokine levels in serum and peritoneal fluid of women with endometriosis. *Gynecol Obstet Invest* 2002;54:82–7.
 - 28 Wang H, Gorpudolo N, Li Y, Feng D, Wang Z, Zhang Y. Elevated vascular endothelial growth factor-A in the serum and peritoneal fluid of patients with endometriosis. *J Huazhong Univ Sci Technolog Med Sci* 2009;29:637–41.
 - 29 Rong R, Ramachandran S, Santanam N, Murphy AA, Parthasarathy S. Induction of monocyte chemotactic protein-1 in peritoneal mesothelial and endometrial cells by oxidized low-density lipoprotein and peritoneal fluid from women with endometriosis. *Fertil Steril* 2002;78:843–8.
 - 30 Cemerski S, Cantagrel A, Van Meerwijk JP, Romagnoli P. Reactive oxygen species differentially affect T cell receptor-signaling pathways. *J Biol Chem* 2002;277:19585–93.
 - 31 Cemerski S, van Meerwijk JP, Romagnoli P. Oxidative-stress-induced T lymphocyte hyporesponsiveness is caused by structural modification rather than proteasomal degradation of crucial TCR signaling molecules. *Eur J Immunol* 2003;33:2178–85.
 - 32 Macphail SE, Gibney CA, Brooks BM, Booth CG, Flanagan BF, Coleman JW. Nitric oxide regulation of human peripheral blood mononuclear cells: critical time dependence and selectivity for cytokine versus chemokine expression. *J Immunol* 2003;171:4809–15.
 - 33 Selam B, Kayisli UA, Garcia-Velasco JA, Akbas GE, Arici A. Regulation of fas ligand expression by IL-8 in human endometrium. *J Clin Endocrinol Metab* 2002;87:3921–7.
 - 34 Calhaz-Jorge C, Costa AP, Santos MC, Palma-Carlos ML. Peritoneal fluid concentrations of interleukin-8 in patients with endometriosis depend on the severity of the disorder and are higher in the luteal phase. *Hum Reprod* 2003;18:593–7.
 - 35 Iwabe T, Harada T, Tsudo T, Nagano Y, Yoshida S, Tanikawa M, *et al.* Tumor necrosis factor-alpha promotes proliferation of endometriotic stromal cells by inducing interleukin-8 gene and protein expression. *J Clin Endocrinol Metab* 2000;85:824–9.
 - 36 Curtin JF, Donovan M, Cotter TG. Regulation and measurement of oxidative stress in apoptosis. *J Immunol Methods* 2002;265:49–72.
 - 37 Mier-Cabrera J, Aburto-Soto T, Burrola-Mendez S, Jimenez-Zamudio L, Tolentino MC, Casanueva E, *et al.* Women with endometriosis improved their peripheral antioxidant markers after the application of a high antioxidant diet. *Reprod Biol Endocrinol* 2009;7:54.
 - 38 Van Langendonck A, Casanas-Roux F, Donnez J. Oxidative stress and peritoneal endometriosis. *Fertil Steril* 2002;77:861–70.
 - 39 Mier-Cabrera J, Genera-Garcia M, De la Jara-Diaz J, Perichart-Perera O, Vadillo-Ortega F, Hernandez-Guerrero C. Effect of vitamins C and E supplementation on peripheral oxidative stress markers and pregnancy rate in women with endometriosis. *Int J Gynaecol Obstet* 2008;100:252–6.