Serum markers of oxidative stress and endometriosis

J.C. Rosa e Silva[1], V. Ferreira do Amara[1,2], J.L. Mendonça[2], A.C. Japur de Sá Rosa e Silva[1], L.S. Nakao[2], O.B. Poli Neto[1], R.A. Ferriani[1]

[1]Department of Gynecology and Obstetrics, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto
[2]Postgraduate Health Sciences Program, Pontifícia Universidade Católica do Paraná-CCBS/PUCPR, Curitiba (Brazil)

Summary

Purpose of investigation: To assess the changes secondary to chronic inflammation in women with and without pelvic endometriosis by the determination of serum thiols and carbonyls. *Materials and Methods:* Sixty-seven women with endometriosis consecutively submitted to laparoscopy and 41 women without endometriosis consecutively submitted to tubal ligation (control group) were selected. Serum levels of total thiols and carbonyls were determined in both groups. *Results:* Patients with endometriosis had significantly lower thiol levels than controls (342.37 ± 142.09 μM vs 559.60 ± 294.05 μM) ($p < 0.001$), as well as significantly lower carbonyl levels (8.97 ± 3.76 μM vs 16.40 ± 9.26 μM) ($p < 0.001$). Other clinical characteristics were not associated with changes in marker levels. The cut-off point established by the ROC curve was 396.44 μM for the thiols, with 73.1% sensitivity and 80.5% specificity, and 14.9 μM for the carbonyls, with 94% sensitivity and 51.2% specificity. *Conclusions:* The serum thiol levels revealed an increase in oxidative stress related to the development of pelvic endometriosis.

Key words: Endometriosis; Oxidative stress; Serum markers; Thiols; Carbonyls.

Introduction

Pelvic endometriosis is a benign gynecologic affection in which endometrial glands and/or stroma develop outside the uterus. The prevalence of endometriosis is ten to 15% among the female population of reproductive age and 20% to 50% among infertile women [1, 2]. Classic clinical signs and symptoms are dysmenorrhea, dyspareunia, acyclic chronic pelvic pain, and infertility [3].

The presence of endometrial cells in the peritoneal cavity leads to the recruitment of monocytes that provoke the release of cytokines, consequently favoring a pelvic inflammatory reaction [4]. On this basis, endometriosis can be considered to be a disease of an inflammatory nature, as confirmed by evidence showing elevated levels of cytokines and growth factors present in peritoneal fluid. Changes in B lymphocyte activity and increased antibody production are detected in women affected by this disease [1]. The inflammatory process is associated with elevated levels of oxidative stress [4].

Oxygen free radicals have been suggested to be involved in the pathogenesis of endometriosis and in the association of the disease with infertility [5]. Oxidative stress is induced when there is an imbalance between oxidant and antioxidant substances. This phenomenon is caused by overproduction of reactive oxygen species (ROS) associated with depletion of the antioxidant system [4]. ROS and reactive nitrogen species produced *in vivo,* which are not adequately metabolized by the antioxidant system may cause alterations in proteins, lipids, carbohydrates, and nucleic acids. The oxidative modification of these molecules by toxic levels of these species may have deleterious consequences. The production of subtoxic ROS levels can lead to a change in the intra- and extracellular redox state and has been demonstrated to signal changes in cellular functions. Thiols and carbonyls are recognized as key components of many of these events [6]. Thiols are a class of organic derivatives with a critical intra- and extracellular function as equilibrators of the redox state through the thiol/disulfide protein. Extracellular thiols are an important component of the antioxidant defense with relevance for cardiovascular diseases, representing a direct measurement of the *in vivo* redox state. In addition, they also reflect the ability of DNA repair or the possible accumulation of genetic damage to cells [7, 8].

ROS induce lipid peroxidation and modify amino acid and carbonyl derivatives, with the latter, in turn, reflecting protein oxidation [9]. Carbonyl concentration is important in the pathogenesis of atherosclerosis and its formation is a subproduct of phagocyte-derived reactions. Thus, carbonyl concentration is a marker of both phagocyte activation and inflammation [7].

Free radicals and oxidative stress are being extensively studied in different diseases and are believed to participate in their etiology and prognosis. Endometriosis is one of these diseases and the demonstration of a positive relationship between this disease and oxidative stress may provide a definitive, sensitive, and non-invasive method for the determination of its diagnosis [10-12].

The objective of the present study was to evaluate the changes secondary to chronic inflammation in women with

Revised manuscript accepted for publication June 24, 2013

and without pelvic endometriosis by means of the determination of serum thiols and carbonyls, as well as the influence of clinical characteristics on these determinations. The cut-off points for these serum markers with better sensitivity and specificity was determined by a ROC curve.

Materials and Methods

The authors selected 83 women of fertile age consecutively submitted to laparoscopy due to suspected endometriosis and with histologic confirmation of endometriosis in the endometriosis outpatient clinics of the Department of Gynecology and Obstetrics, Faculty of Medicine of Ribeirão Preto, and of "Santa Casa de Misericórdia" of Curitiba. Exclusion criteria were smoking, the use of anti-inflammatory medications up to two months before surgery, patients presenting ovarian tumor, pelvic inflammatory disease, myomas and adenomyosis as intraoperative or ultrasonographic findings, patients who had received hormonal therapy (oral contraceptives, progestogens or GnRH analogues) during the preceding three months, pregnant patients or patients who refused to participate in the study. The authors selected as controls 55 women of fertile age consecutively submitted to bilateral tubal ligation and attended in the family planning sector of the Maternity Victor Ferreira do Amaral and in the family planning outpatient clinic of the Department of Gynecology and Obstetrics, Faculty of Medicine of Ribeirão Preto. These women had no endometriosis as determined by laparoscopy and the same exclusion criteria as cited above were applied to this group. Ten ml blood were collected in each venipuncture and centrifuged at 5,000 rpm for ten minutes and the serum obtained was frozen at -80ºC until the time for analysis. Some samples were discharged because of hemolysis or high lipid concentration in the centrifuged serum, what could interfere in the markers' measurement and consequently could constitute a bias. Therefore, the number of analyzed patients was 67 and 41 in the endometriosis and control groups, respectively.

The project was approved by the Research Ethics Committees of the participating institutions and all patients gave written informed consent to participate in the study.

Analysis of thiol concentration

Thiols were determined using the dithiobis 2-nitrobenzoic acid (DTNB) method [13]. The samples were thawed and immersed in ice. The standard curve used for the calculations of unknown concentrations was obtained using glutathione at concentrations from 0.2 to 2 mM. Samples were analyzed in duplicate on 96-well microplates. Ten µl serum plus 190 µl of the DTNB solution and of diethylenetriamine-penta-acetic acid (DTNB) were added to each well. The plates were incubated for ten minutes at room temperature and the absorbance of the samples was read at 405 nm. The concentration (in µM) was calculated for each patient using a specific equation.

Analysis of carbonyl concentration

The analysis was carried out by the 2,4 dinitrophenyl hydrazine (DNPH) method [14]. Two test tubes per patient were used. Serum (200 µl) and one ml DNPH (one mM in two M HCl) were added to the tube identified as the sample, and 200 µl of serum in one ml two M HCl were added to the tube identified as the blank. All tubes were left in a water bath at 37ºC for 90 minutes. After cooling, each tube received one ml 28% trichloroacetic acid (w/v) and the tubes were vortexed for three minutes and then centrifuged at 6,000 rpm for an additional three minutes.

The supernatant was discarded and one ml ethanol: ethyl acetate (1:1) was added to the pellet. The material was again homogenized in a vortex for two minutes and the tubes were once again centrifuged at 6,000 rpm for six minutes. The procedure was repeated one more time.

The supernatant was discarded and one ml of six M guanidine was added to the pellet. Homogenization was carried out for one minute and the content of each tube was then transferred to microtubes which were centrifuged for three minutes at 6,000 rpm in a microcentrifuge. The absorbance of each sample was read at 360 nm and carbonyl concentration was obtained by a specific equation.

Statistical analysis

The GraphPad Prism 4.0 32-Bit Executable software and MedCalc statistical software, Version 7.2.1.0 were used for statistical analysis. The clinical variables were analyzed by the Fisher exact test. The nonparametric Mann-Whitney test was used for the evaluation of the serum markers and the cut-off points with highest sensitivity and specificity for these markers were determined by the ROC curve. The level of significance was set at $p < 0.05$ in all tests. Results of thiol and carbonyl levels were expressed in median (minimum-maximum).

Results

The mean age of the two groups was similar (33.22 ± 6.22 years for the endometriosis group and 32.49 ± 4.74 years for the control group). The endometriosis group had a greater prevalence of dyspareunia ($p < 0.001$), chronic pelvic pain ($p = 0.015$), infertility ($p < 0.001$), dysmenorrhea ($p < 0.001$), menstrual irregularity ($p = 0.001$), and cyclic intestinal changes ($p = 0.001$). There was no significant difference regarding the presence of midcycle pain, post-coital bleeding or cyclic urinary changes between groups.

The patients with endometriosis presented significantly lower serum thiol levels compared to control (median 325.25 (103.89 – 828.22) µM *vs* 530.29 (133.86 – 1892.00) µM) ($p < 0.001$) (Figure 1), as well as lower carbonyl levels (median 8.00 (1.10 – 20.60) µM *vs* 15.61 (5.23 – 44.13) µM) ($p < 0.001$) (Figure 2).

The presence of dysmenorrhea, midcycle pain, infertility, dyspareunia, post-coital bleeding, menstrual irregularity, and cyclic urinary or intestinal changes, when evaluated separately in each group, was not associated with significant changes in the levels of any marker.

The cut-off point suggested by the ROC curve for the thiols was 396.44 µM, with 73.1 sensitivity and 80.5% specificity, and the area under the curve equal to 0.806 (Figure 3). For the carbonyls, the suggested cut-off point was 14.9 µM, with 94% sensitivity and 51.2% specificity, and the area under the curve equal to 0.768 (Figure 4).

Discussion

The complaints and clinical symptoms observed in endometriosis were confirmed in the current study and, as expected, the presence of the tetrad dysmenorrhea, dyspareunia,

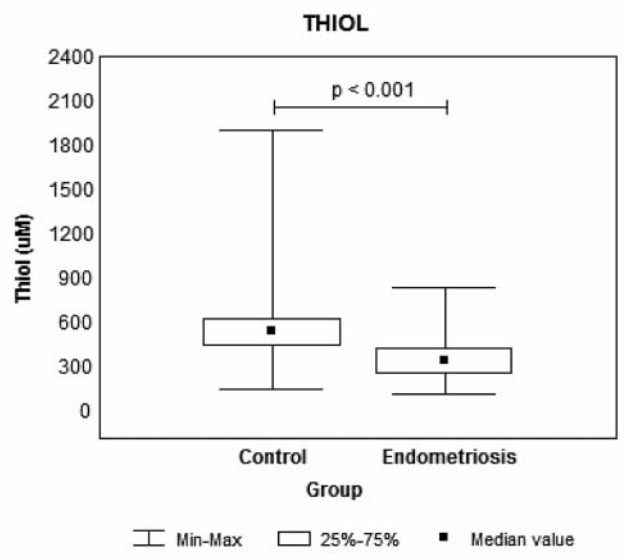

Figure 1. — Thiol levels in the endometriosis and control groups ($p < 0.001$).

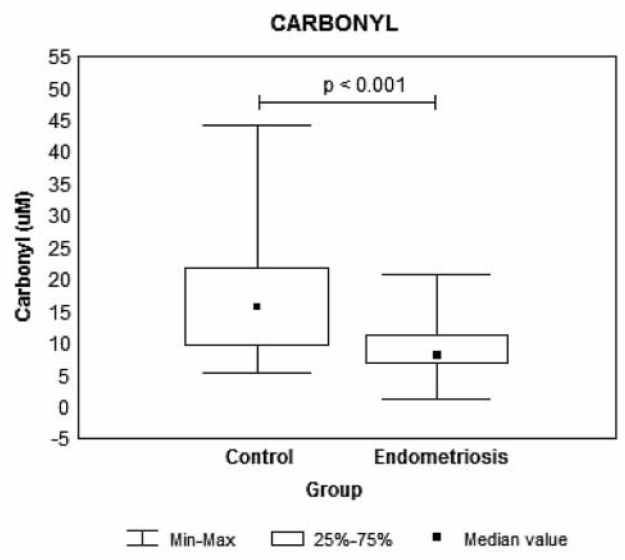

Figure 2. — Carbonyl levels in the endometriosis and control groups ($p < 0.001$).

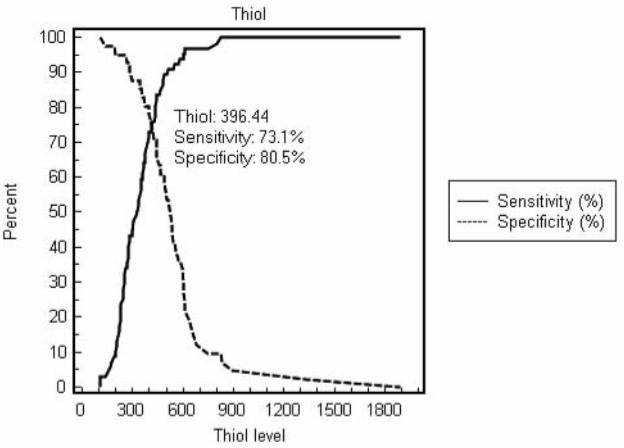

Figure 3. Sensitivity and specificity for cut-off point of thiols.

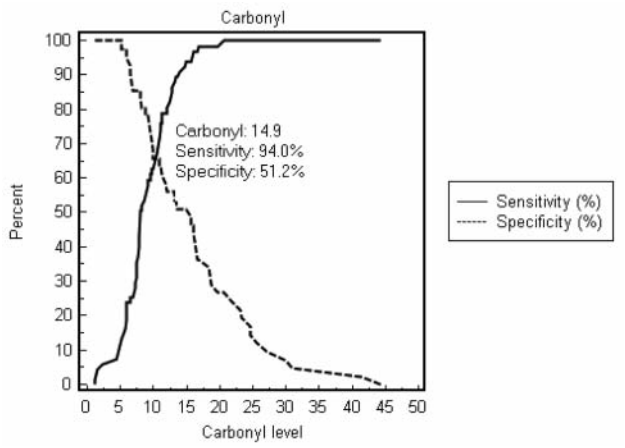

Figure 4. Sensitivity and specificity for cut-off point of carbonyls.

infertility, and chronic pelvic pain was prevalent in many of the women with the disease.

The markers chosen for the present investigation have already been extensively studied in other diseases, such as diabetes, chronic renal failure, atherosclerosis, hypertension, and psoriasis, having proved to be good markers for these diseases. However, they have not been studied previously in endometriosis.

Several studies have shown an association between endometriosis and oxidative stress. Jackson *et al.* [15] used four markers of oxidative stress to compare women with and without endometriosis on the basis of thiobarbituric acid reactive substances and detected an increase in these markers in women with pelvic endometriosis. In two different studies, Zhao *et al.* [16] and Sczepanska *et al.* [17] observed that the antioxidant status was reduced in women with pelvic endometriosis. Liu *et al.* [5] used peritoneal fluid as the biological medium to be evaluated and detected higher levels of oxidative stress in women with endometriosis [5]. de Foyouzi *et al.* [18] suggested that reactive oxygen species can modulate the growth of the endometrial stroma. In pathological conditions such as endometriosis, high levels of these unstable molecules and antioxidant depletion may contribute to the excessive growth of cells of the endometrial stroma [18]. Oner-Iyidogan *et al.* [19] concluded in their study that several factors, such as the cytokines released by macrophages activated in peritoneal fluid and hormones synthesized by the ovary, may locally affect the antioxidant status of the ectopic endometrium.

These results demonstrate the participation of oxidative stress in the development of the disease and suggest that treatment with antioxidant substances could be of benefit to women with endometriosis and protect healthy women against the disease.

However, some investigators did not detect oxidative stress in women with endometriosis. Ho *et al.* [20] compared women with and without endometriosis in terms of total antioxidant status and of the levels of nitric oxide-derived products and found no significant differences in these levels between the groups with and without the disease. Polak *et al.* [21] also did not obtain a positive result regarding the association of oxidative stress markers with the presence of endometriosis.

The carbonyl levels detected were lower in the endometriosis group, although, in contrast to the thiols, the higher the levels of this marker, the lower the oxidative stress, with these levels proving not to be good markers of oxidative stress in women with endometriosis.

It should be pointed out that the sensitivity and specificity of the two serum markers evaluated here were not high, that indicates a limitation of their use in clinical practice as serum markers of this disease when compared to other markers used such as CA125 [22, 23].

Conclusion

Based on the present data, we conclude that oxidative stress seems to play a role in the genesis of pelvic endometriosis, considering mainly the serum thiol levels. Nevertheless, it's levels should be evaluated with caution when used as a marker of the disease. Anyway other studies with bigger casuistic may be developed in order to definitely prove this relation.

References

[1] Vigano P., Parazzini F., Somigliana E., Vercellini P.: "Endometriosis: epidemiology and aetiological factors". *Best Pract. Res. Clin. Obstet. Gynaecol.*, 2004, *18*, 177.
[2] do Amaral V.F., Ferriani R.A., de Sa M.F., Nogueira A.A., Rosa e Silva J.C., Rosa e Silva A.C., *et al.*: "Positive correlation between serum and peritoneal fluid CA-125 levels in women with pelvic endometriosis". *Sao Paulo Med. J.* 2006, *124*, 223.
[3] Stevens A., Lowe J.: "Patologia". 1ª ed. São Paulo: Manole,1996.
[4] Gupta S., Argawal A., Krajcir N., Alvarez J.G.: "Role of oxidative stress in endometriosis". *Reprod. Biomed. Online,* 2006, *123*, 126.
[5] Liu Y., Luo L., Zhao H.: "Levels of lipid peroxides and superoxide dismutase in peritoneal fluid of patients with endometriosis". *J. Tongji Med. Univ.*, 2001, *21*, 166.
[6] Moran L.K., Gutteridge J.M., Quinlan G.J.: "Thiols in cellular redox signalling and control". *Curr. Med. Chem.*, 2001, *8*, 763.
[7] Himmelfarb J., Stenvinkel P., Ikizler T.A., Hakim R.M.: "The elephant in uremia: oxidant stress as a unifying concept of cardiovascular disease in uremia". *Kidney Int.*, 2002, *62*, 1524.
[8] Banne A.F., Amiri A., Pero R.W.: "Reduced level of serum thiols in patients with a diagnosis of active disease". *J. Anti Aging*, 2003, *6*, 327.
[9] Himmelfarb J., McMonagle E., McMenamin E.: "Plasma protein thiol oxidation and carbonyl formation on chronic renal failure". *Kidney Int.*, 2000, *58*, 2571.
[10] Evers J.L., Dunselmann G.A., Van der Linden P.J.: "Markers for endometriosis". *Beillieres Clin. Obstet. Gynaecol.*, 1993, *4*, 715.
[11] Broses I., Puttemans P., Campo R., Gordts S., Brosens J.: "Non-invasive methods of diagnosis of endometriosis. *Curr. Opin. Obstet. Gynecol.*, 2003, *15*, 519.
[12] Yang W.C.: "Serum and endometrial markers". *Best Pract. Res. Clin. Obstet. Gynaecol.*, 2004, *18*, 305.
[13] Levine R.L., Williams J.A., Stadtman E.R., Shacter E.: "Carbonyl assays for determination of oxidatively modified proteins". *Meth. Enzymol.*, 1994, *233*, 346.
[14] Himmelfarb J., McMonagle E., McMenamim E.: "Plasma protein thiol oxidation and carbonyl formation in chronic renal failure". *Kidney Int.*, 2000, *58*, 2571.
[15] Jackson L.W., Schisterman E.F., Dey-Rao R., Browne R., Armstrong D.: "Oxidative stress and endometriosis". *Hum. Reprod.*, 2005, *20*, 2014.
[16] Zhao L., Zhao W., Ou Y.: "Correlation of the metabolism of oxygen free radical in ovary, uterine muscle and vein blood with the pathogenesis of endometriosis and endometrioma". *Zhonghua Fu Chan Ke Za Zhi*, 2001, *36*, 299.
[17] Szczepanks M., Kozlik J., Zkrzypczak J., Mikolajczykm M.: "Oxidative stress may be a piece in the endometriosis puzzle". *Fertil. Steril.*, 2003, *79*, 1288.
[18] Foyouzi N., Berkkanoglu M., Arici A., Kwintkiewicz J., Izquierdo D., Duleba A.J.: "Effects of oxidants and antioxidants on proliferation of endometrial stromal cells". *Fertil. Steril*., 2004, *82*, 1019.
[19] Oner-Iyidogan Y., Kokac H., Gurdol F., Korkmaz D., Buyru F.: "Indices of oxidative stress in eutopic and ectopic endometria from women with endometriosis". *Gynecol. Obstet. Invest.*, 2004, *57*, 214.
[20] Ho H.N., Wu M.Y., Chen S.U., Chao K.H., Chen C.D., Yang Y.S.: "Total antioxidant status and nitric oxide do not increase in peritoneal fluids from women with endometriosis". *Hum. Reprod.*, 1997, *12*, 2810.
[21] Polak G., Wertel I., Kozid-Monteeka M., Tarkowski R., Kotarski J.: "Investigation of glutathione concentration in peritoneal fluid from women with and without endometriosis". *Eur. J. Obstet. Gynecol. Reprod. Biol.*, 2003, *109*, 206.
[22] Rosa e Silva A.C.J.S., Rosa e Silva J.C., Ferriani R.A.: "Serum CA-125 in the diagnosis of endometriosis". *Int. J. Gynaecol. Obstet.*, 2007, *96*, 206.
[23] Do Amaral V.F., Bydlowski S.P., Peranovich T.C., Navarro P.A., Subbiah M.T., Ferriani R.A.: "Lipid peroxidation in the peritoneal fluid of infertile women with peritoneal endometriosis". *Eur. J. Obstet. Gynecol. Reprod. Biol.*, 2005, *119*, 72.

Address reprint requests to:
J.C. ROSA E SILVA, M.D.
Department of Gynecology and Obstetrics,
Faculty of Medicine of Ribeirão Preto,
University of São Paulo,
14049-900 Ribeirão Preto, SP, (Brazi)
e-mail: juliocrs@convex.com.br