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Endometriosis progression in tumor necrosis factor receptor p55-deficient mice: Impact on oxidative/nitrosative stress and metallomic profile

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ABSTRACT

The present study was conducted to investigate whether the deficiency of tumor necrosis factor receptor p55 (TNFRp55) modulates oxidative/nitrosative stress and metallomic profile into the peritoneal cavity during the experimental endometriosis progression in mice. Female C57BL/6 mice, *wild-type* (WT) and TNFRp55 *knockout* (KO) of two months were used. Endometriosis was induced experimentally by autotransplanting three pieces of the right uterine horn to the intestinal mesentery. After four weeks, endometriotic-like lesions and peritoneal lavage fluid were collected. The obtained peritoneal fluid was analyzed for nitrite levels using the Griess method and trace elements concentrations by ICP-MS. Both endometriotic-like lesions and cells isolated from peritoneal lavage were analyzed for the following oxidative/nitrosative stress markers: inducible nitric oxide synthase (iNOS) expression by Western Blot; total antioxidant capacity (TAC), the activity of two antioxidant enzymes (CAT and GPX) and thiobarbituric acid-reactive substances (TBARS) concentration, by spectrophotometric method; and protein carbonyl content and nitrotyrosine presence by ELISA. In comparison to WT group, KO mice exhibited larger lesion volume; higher levels of nitrite, copper (Cu) and strontium (Sr) in the peritoneal fluid; increased TAC, CAT, and GPX in peritoneal lavage cells; decreased concentration of TBARS in lesions and protein carbonyl in peritoneal lavage cells. Significant positive correlations between Cu and lesion volume, Sr and lesion volume, and Cu and Sr were obtained. Our results suggest that the TNFRp55 deficiency increases antioxidant protection and promotes high Cu-Sr concentrations in the peritoneal cavity, which favors the progression of experimental endometriosis.

1. Introduction

Endometriosis is a chronic inflammatory estrogen-dependent disease, characterized by implantation and growth of endometrial tissue outside the uterine cavity [1,2]. One of the main cytokines involved in this pathology is tumor necrosis factor alpha (TNF-α), which transduces its signal mainly through two distinct receptors referred to as type 1 (also called TNFRp55) and type 2 (also called TNFRp75) [3]. These receptors are expressed in different cell types, regulating the cellular processes of proliferation, differentiation, and apoptosis. Recently, using a murine model of induced endometriosis, we have demonstrated that TNFRp55 deficiency inhibits apoptosis and promotes cell proliferation in endometriotic-like lesions, in an environment where high levels of estradiol predominate [4].

A characteristic aspect of endometriosis is the activation of peritoneal macrophages that, in addition to increasing the TNF-α secretion [5], lead to an increased iNOS expression and nitric oxide (NO) production [6,7]. Increased iNOS expression has also been found in endometrial tissues of women with endometriosis [8]. NO acts as mediator and regulator of the inflammatory response [9] and of the endometrial angiogenesis [10].

Alternatively, large quantities of reactive oxygen species (ROS) are

Abbreviations: TNF-α, tumor necrosis factor alpha; TNFRp55, tumor necrosis factor receptor p55; WT, wild-type; KO, knockout; ROS, reactive oxygen species; iNOS, inducible nitric oxide synthase; NO, nitric oxide; TAC, total antioxidant capacity; $ABTS^{\cdot+}$, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; CAT, catalase; GPX, glutathione peroxidase; TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde

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released by chronic stimulation of macrophages [11]. In fact, oxidative stress, defined as an imbalance between ROS and the antioxidant defense system, is a potential factor involved in different reproductive pathologies such as endometriosis [2,12]. The antioxidant defense system, consisting of antioxidant enzymes and non-enzymatic antioxidants, appears to be less active in endometriosis. In addition, ROS levels are high in endometriosis, and their main effects on cells are translated into damage and proliferation. Lipids, proteins and nucleic acids are potential targets for ROS. For this reason, several authors suggest that oxidative stress is responsible for the local destruction of the peritoneal mesothelium, thereby creating adhesion sites for ectopic endometrial cells [2,13].

Trace elements can affect the incidence of endometriosis. Trace amounts of cobalt, copper, iron, manganese, molybdenum, selenium, strontium and zinc are essential for cells, and most are referred to as micronutrients [14]. Copper, iron, manganese, selenium and zinc have a significant influence on the activity of antioxidant enzymes [15]. However, excessive accumulation, deficiency or an imbalance of trace elements can promote oxidative stress and disrupt cellular functions, resulting in abnormal cell proliferation and malignant transformation, or cellular degeneration and apoptosis [16,17]. Lead, chromium, and thallium are considered toxic metals and can promote oxidative damage in cells [14,18,19]. Moreover, it has been reported that aluminum, barium, cobalt, nickel, lead, copper and chromium act as endocrine disruptors [20] and, noteworthy, the last three trace elements have been associated with endometriosis [21,22]. Low levels of zinc and high levels of nickel in the blood have also been found in women with this condition [22,23]. However, there are relatively few studies evaluating the association between trace elements and endometriosis.

Among the research priorities in endometriosis established after the 12th World Congress of Endometriosis (2014), it is recommended to deepen the role of oxidative stress in the development and potential treatment of this pathology [24]. Interestingly, the signaling of TNF-α through TNFRp55 mediates the generation of ROS in the plasma membrane of macrophages and in the vasculature, affecting NO bioavailability and the NO/reactive nitrogen species (RNS)-ROS balance [25,26]. These observations prompted us to analyze whether the deficiency of TNFRp55 modulates the oxidative/nitrosative stress and metallomic profile into the peritoneal cavity during the progression of experimental endometriosis in mice.

2. Materials and methods

2.1. Animals

Female mice of the C57BL/6 strain, WT and TNFRp55-/- (KO) of two months, weighing 19–21 g were used. The TNFRp55-/- mice were obtained from the Max von Pettenkofer-Institute, Munich, Germany. Breeding colonies were established in the Animal Facility at Universidad Nacional de San Luis (San Luis, Argentina) under rigorous light conditions (12 h light, 07:00-19:00, and 12 h darkness), controlled temperature (22 ± 2 °C) with water and sterile food ad libitum. Animals were handled following the Guidelines for the Care and Use of Laboratory Animals of the National Academy of Sciences (https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf). The experimental protocol was approved by the Comité Institucional de Cuidado y Uso de Animales de Experimentación (CICUA) of the Universidad Nacional de San Luis, Argentina (Protocol #B-225/16).

2.2. Surgical induction of endometriosis

Endometriosis was induced experimentally, as reported previously [4,27]. The animals were anesthetized with 100 mg/kg of ketamine (Holliday Scott, Buenos Aires, Argentina) and 10 mg/kg of xylazine (Richmond, Buenos Aires, Argentina) by intraperitoneal injection. A mid-ventral incision was then made to expose the uterus and the intestine. The right uterine horn was removed from the animal, placed in DMEM-F12 (Gibco), opened longitudinally and cut into three square pieces of approximately 4mm^2. Then, the three equal pieces of uterine horn were sutured onto the colonic mesentery with the endometrial layer facing the bowel serosa (autologous transplant) by means of a single stitch (supralong 6-0, Ethicon, NJ, USA). The abdomen was then closed with a 5-0 nylon suture. Mice were monitored daily in relation to body weight, food consumption, preening behavior, and daily activity. No alteration in their behavior was noted. After 4 weeks of treatment, animals were sacrificed by cervical dislocation. Then, a small medioventral hole was opened through which 1.5 ml of PBS was injected in the peritoneal cavity of each animal. The peritoneal lavage fluid was collected and centrifuged at 250 *g* for 10 min at 4 °C. The supernatant (peritoneal fluid) was separated from the precipitate (peritoneal lavage cells) and both were maintained at −80 °C until the corresponding determinations. Finally, the abdomen was completely opened to have access to the endometriotic-like lesions.

2.3. Macroscopic evaluation of the ectopic uterine tissue

The lesions were identified, counted and measured with caliper in two perpendicular diameters. The volume of the developed lesions was calculated with the following equation: $\boldsymbol{V = (4/3)\,\pi\, r_1^{\,2}\, r_2}$ (r_1 and r_2 are the radiuses and $r_1 < r_2$).

2.4. Nitrite assay

Levels of nitrite, a water-soluble and stable NO metabolite, were measured spectrophotometrically by Griess reaction [28]. Briefly, 50 µl of peritoneal fluid was mixed with Griess reagent (1% sulfanilamide with 0.1% *N*-l-naphthyl-ethylenediamine/HCl in 1% phosphoric acid). After 10 min incubation at room temperature, the absorbance was measured at 540 nm A solution of nitrite of known concentration was used to prepare a standard calibration curve. The method limit of detection was < 2.5 nmol/ml. The intra-assay coefficients of variation for all the assays were less than 10%.

2.5. Preparation of protein extracts

Ectopic uterine tissue and peritoneal lavage cells were homogenized separately in 100 µl RIPA buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. The homogenates were centrifuged at 21,912 *g* for 15 min at 4 °C to remove nuclei and cell debris. The supernatants were collected and used to determine the iNOS expression. The TAC, the enzymatic activities of catalase (CAT) and glutathione peroxidase (GPX) were also determined. Furthermore, TBARS concentration, carbonyl protein content levels, and nitrotyrosine presence were assessed. The total protein concentration in the supernatants was determined with Folin-Ciocalteu's Phenol reagent (Biopack, Buenos Aires, Argentina) as described by Lowry et al. [29]. Bovine serum albumin (Sigma, St. Louis, MO, USA) was used as a reference standard (0.1–1.0 mg protein/ml). The reaction mixtures were incubated for 30 min at room temperature and then read at 750 nm using a Beckman DU-640 B spectrophotometer (CA, USA).

2.6. iNOS expression

The expression of iNOS was analyzed by Western Blot. Aliquots containing 40 µg of total protein were subjected to electrophoresis in 10% SDS-PAGE gels and then electrotransferred to PVDF membrane (Millipore Corporation) at 100 V for 1 h in transfer buffer (25 mM Tris, 192 mM glycine and 20% v/v methanol, pH 8.3). The membranes were immersed in 5% non-fat milk in a PBST solution (KH_2PO_4 0.015 M, NaH_2PO_4 0.017 M, KCl 0.076 M, NaCl 0.14 M (pH 7.4), 0.5% Tween 20) for 1 h at room temperature, followed by an overnight incubation at

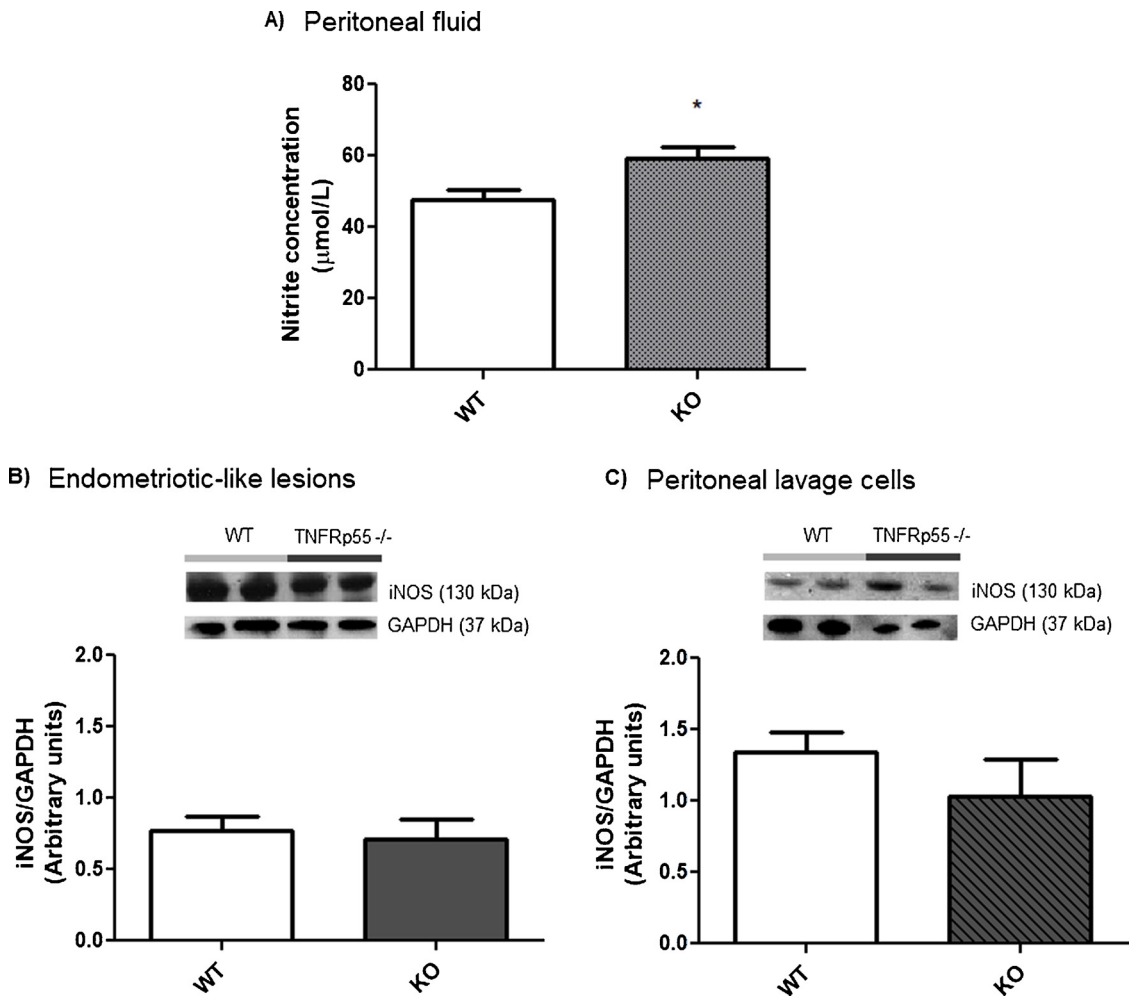

Fig. 1. Effect of TNFRp55 deficiency on nitrite concentration and iNOS protein expression. Nitrite concentration were measured spectrophotometrically by Griess reaction in peritoneal fluid **(A)**. Eight animals per group were used. iNOS protein expression was analyzed in both endometriotic-like lesion **(B)** and peritoneal lavage cells **(C)** by Western Blot. Four WT and four TNFRp55-/- mice were used. Statistical comparisons were performed by Student's *t*-test. *p < 0.05.

4 °C with either mouse monoclonal anti-iNOS antibody (1:1000, Cat # 610328; BD Transduction Laboratories, Mississauga, ON) or rabbit anti-GAPDH (D16H11) antibody (1:2000, Cat # 5174; Cell Signalling Technology, USA). After incubation with primary antibodies, membranes were washed in PBST and incubated with goat anti-mouse IgG-HRP antibody (1:5000, SC-2005; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or goat anti-rabbit IgG [H + L]-HRP antibody (1:10 000, Cat # 1706515; Bio-Rad Lab, Hercules, CA, USA) respectively, for 1 h at room temperature, respectively. Following washing in PBST, blots were developed using an enhanced chemiluminescence Western blotting detection system, Thermo Scientific SuperSignal West Pico Chemiluminescent (Pierce Biotechnology, Rockford, IL, USA) and exposed to X-ray films, Thermo Scientific CL-XPosure Film (Pierce Biotechnology Rockford, IL, USA). The mean of intensity of each band was measured using the NIH ImageJ software (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/). iNOS protein levels were normalized against GAPDH (endogenous control).

2.7. Total antioxidant capacity

TAC was measured by an improved method of quenching of the 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$) by both lipophilic and hydrophilic antioxidants present in the protein extracts [30]. The $ABTS^{\cdot+}$ was generated by oxidation of 7 mM ABTS with 2.45 mM potassium persulfate. The TAC was expressed as the percentage of reduction in the absorbance due to the $ABTS^{\cdot+}$, and it was determined as follows: $\%inhibition = [(A_0-A_f)/A_0] \times 100$, where A_0 and A_f were the absorbance at 734 nm of the reaction mixtures measured at t = 0 and after 5 min of sample addition, respectively. All measurements were performed in duplicate for each sample.

2.8. Antioxidant enzymes activity

The specific CAT and GPX enzymatic activities were determined following Aebi's [31] and Flohé and Gunzler's [32] methods, respectively. Briefly, the CAT activity was determined by measuring the decrease in the absorbance at 240 nm when 100 µl of 3 mM H_2O_2 were added to a reaction medium containing a 1/500 dilution of protein extracts in 50 mM phosphate buffer, pH 7.3. The decrease of the absorbance at 240 nm was acquired every 5 s during a total time of 30 s. During this time, the decomposition of H_2O_2 was observed as the first-order kinetics. The GPX activity was determined following NADPH oxidation at 340 nm in a reaction medium containing 0.2 mM GSH, 0.25 IU/ml yeast glutathione reductase and 0.5 mM *tert*-butyl hydroperoxide in 50 mM phosphate buffer, pH 7.2.

2.9. Measurement of TBARS-MDA

TBARS concentration was determined according to the method described by Draper and Hadley [33]. The TBARS assay measures malondialdehyde (MDA) production from lipid hydroperoxides. A calibration curve was performed using 1,1,3,3-tetramethoxypropane as

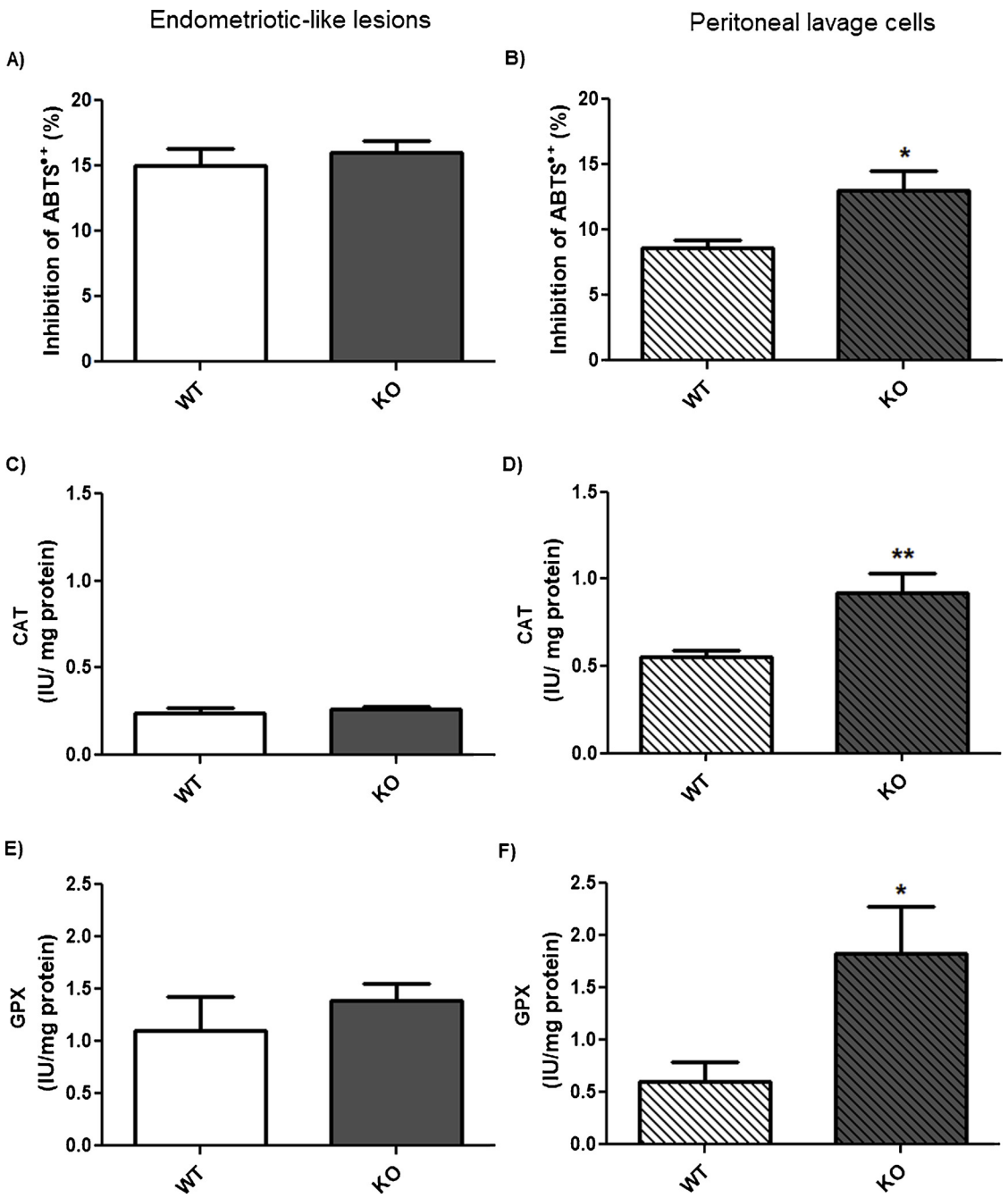

Fig. 2. Effect of TNFRp55 deficiency on the antioxidant defense system. Both endometriotic-like lesions and cells isolated from peritoneal lavage were analyzed for the total antioxidant capacity **(A and B)**, the activity of CAT **(C and D)**, and the activity of GPX **(E and F)** by spectrophotometric method. Results are expressed as mean ± S.E.M. of eight animals per experimental group. Statistical comparisons were performed by Student's *t*-test. *$p < 0.05$, •$p < 0.01$. $ABTS^{\cdot+}$, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; CAT, catalase; GPX, glutathione peroxidase.

standard. TBARS were determined by the absorbance at 535 nm.

2.10. Measurement of protein carbonyls

Protein carbonyls were analyzed as a measure of protein oxidation using the method proposed by Winterbourn and Buss [34], with a slight modification. Fifty microliters of each protein extract (50 µg) were derivatized to 2,4-dinitrophenylhydrazone by reaction of carbonyl groups in oxidized proteins with 2,4-dinitrophenylhydrazine in 2 M HCl. Ten microliters of the derivatized or nonderivatized sample were added to 190 µl of 0.1 M bicarbonate buffer, pH 9.6, in clear 96-*well* microplates (Corning Incorporated, Corning, NY, USA) and incubated overnight at 4 °C. After washing with PBS/0.05% Tween-20/5% and blocking with PBS/0.05% Tween-20/5% non-fat dry milk for 1 h at 37 °C, the microplates were incubated with 50 µl of rabbit polyclonal anti-dinitrophenyl antibody (1:2000 dilution; Thermo Fisher Scientific Inc., Waltham, MA, USA) for 1 h at 37 °C. After three washes, 50 µl of goat anti-rabbit IgG-HRP conjugate (1:5000 dilution; Jackson Immuno Research Laboratories, West Grove, PA, USA), was added to each *well* and incubated for 1 h at 37 °C. Finally, immunocomplexes were quantified using TMB. The oxidation reaction of the substrate was stopped with 2 M sulphuric acid and the absorbance was measured at 450 nm using a TECAN microplate reader (Infinite M200 PRO, Research Triangle Park, NC, USA). Standard curve was constructed by mixing varying proportions (0–100%) of HOCl-oxidized BSA with fully reduced BSA while maintaining a constant total protein concentration. The

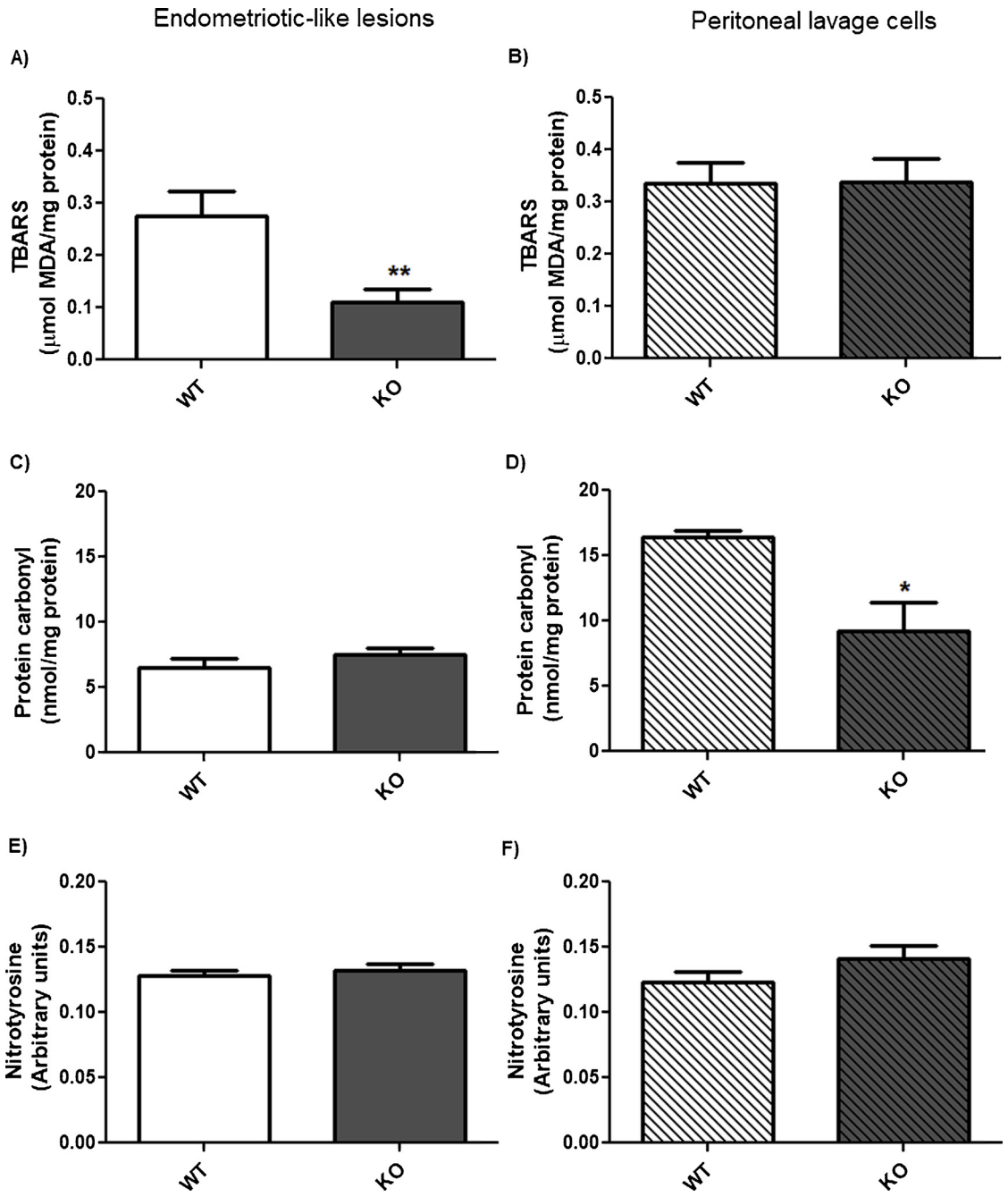

Fig. 3. Effect of TNFRp55 deficiency on markers of oxidative/nitrosative damage. Both endometriotic-like lesions and cells isolated from peritoneal lavage were analyzed for TBARS-MDA **(A and B)** by spectrophotometric method, and protein carbonyl content **(C and D)** and nitrotyrosine presence **(E and F)** by ELISA. Results are expressed as mean ± S.E.M. of eight animals per experimental group. Statistical comparisons were performed by Student's *t*-test. *p < 0.05, •p < 0.01. TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde.

carbonyl content of the oxidized BSA was determined colorimetrically.

2.11. Measurement of nitrotyrosine

The presence of nitrotyrosine, a biomarker for the damage induced by NO-derived reactive nitrogen intermediates, was analyzed in ectopic uterine tissue and peritoneal lavage cells by ELISA. Ten microliters of sample (10 µg of total proteins) were added to 190 µl of 0.1 M bicarbonate buffer pH 9.6, in clear 96-well microplates (Corning Incorporated, Corning, NY, USA) and incubated 1 h at 37 °C. After washing with PBS/0.05% Tween-20/5% and blocking with PBS/0.05% Tween-20/5% non-fat dry milk for 1 h at 37 °C, the microplates were incubated with 100 µl of rabbit polyclonal anti-nitrotyrosine antibody (1:1000 dilution; Sigma, St. Louis, MO, USA) for 1 h at 37 °C. After three washes, 50 µl of goat anti-rabbit IgG-HRP conjugate (1:5000 dilution; Jackson Immuno Research Laboratories, West Grove, PA, USA), was added to each well and incubated for 1 h at 37 °C. Finally, immunocomplexes were quantified using TMB. The oxidation reaction of the substrate was stopped with 2 M sulphuric acid and the absorbance was measured at 450 nm using a TECAN microplate reader (Infinite M200 PRO, Research Triangle Park, NC, USA).

2.12. Trace element analysis

All samples, standards and reagent solutions were prepared in metal-free 15-ml polypropylene tubes (Sarstedt, Germany) into a class-

Table 1
Concentration of trace elements (μg/L) in peritoneal fluid of C57BL/6 WT and TNFRp55-/- mice with surgically induced endometriosis.

Trace elements	WT	KO	*p-value*
Aluminum (Al)	288.9 ± 37.980	302.8 ± 20.080	ns
Barium (Ba)	2.520 ± 0.206	2.483 ± 0.176	ns
Bismuth (Bi)	0.820 ± 0.075	2.034 ± 0.686	ns
Chromium (Cr)	36.44 ± 2.693	30.88 ± 3.189	ns
Cobalt (Co)	0.614 ± 0.021	0.650 ± 0.058	ns
Copper (Cu)	35.04 ± 3.006	46.60 ± 2.744	< 0.0131*
Iron (Fe)	108.5 ± 8.593	132.1 ± 9.386	ns
Lead (Pb)	0.8175 ± 0.162	1.835 ± 0.504	ns
Manganese (Mn)	3.429 ± 0.228	4.045 ± 0.520	ns
Molybdenum (Mo)	2764 ± 527.30	1615 ± 80.74	ns
Nickel (Ni)	18.94 ± 0.961	16.90 ± 2.255	ns
Strontium (Sr)	6.086 ± 0.318	7.763 ± 0.511	< 0.0146*
Thallium (Tl)	0.327 ± 0.065	0.445 ± 0.038	ns
Zinc (Zn)	48.09 ± 3.466	53.05 ± 4.322	ns

Results are expressed as mean ± S.E.M. of eight animals per experimental group. Statistical comparisons were performed by nonparametric Wilcoxon test.
* $p < 0.05$. ns: not significant.

1000 laminar flow cab. All reagents used were of trace analysis grade, and they included: Ultrapure water with a resistivity of 18.2 MΩ cm produced by an Easy pure RF system from Barnstead (Dubuque, IA, USA), Double distilled acids obtained with a PTFE sub-boiling acid distiller (Distillacid, Berghof Products + Instruments GmbH, Germany) and Hydrogen peroxide 30% Merck (Germany). Additionally, Multi-element calibration standards from Perkin Elmer Pure Plus, Atomic Spectroscopy Standard (Norwalk, USA), were used for instrument and method calibrations. For the external calibration against aqueous standards, the solutions were prepared in 1% v/v nitric acid. The concentrations of the elements for method calibration were 5.0, 10.0, 20.0, 40.0 and 80.0 μg L^{-1}.

Aluminum, barium, bismuth, chromium, cobalt, copper, iron, lead, manganese, molybdenum, nickel, strontium, thallium and zinc were determined by inductively coupled plasma-mass spectrometry (ICP-MS), according to the methodology described by Moyano et al. [35], with some modifications. For this purpose, an inductively coupled plasma mass spectrometer, Perkin Elmer SCIEX, ELAN DRC-e (Thornhill, Canada) was used. Samples of peritoneal fluid (500 μl) were mineralized with a mixture of 500 μl of concentrated nitric acid and 500 μl of hydrogen peroxide. After addition of the mixture, samples were heated up to 60 °C for 60 min in a water bath heated, obtaining transparent sample solutions. Parallel reagent blanks in triplicate were run in order to count for contaminations/sample loses. Finally, the solutions were taken to a final volume of 10 ml with ultrapure water (18.2 MΩ cm) and stored at 4 °C until analysis.

2.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism (Version 5, GraphPad Software Inc. San Diego SA). Values are presented as the mean ± SEM. Differences between the means of each group were analyzed using the Student´s *t*-test or nonparametric Wilcoxon *test* (where appropriate). Pearson's correlation coefficient was used to evaluate the relationships between trace elements concentration and lesion volume. Differences were statistically significant when $p < 0.05$.

3. Results

3.1. Effect of the TNFRp55 deficiency on the endometriotic-like lesions growth

To assess whether the TNFRp55 deficiency affects the growth of endometriotic lesions, a murine model of endometriosis induced surgically in WT and KO mice was used. Macroscopic evaluation of ectopic uterine tissue showed an approximate volume increase of 50 percent in lesions of TNFRp55 deficient mice compared to WT mice (33.13 ± 1.759 versus 19.38 ± 2.718, $p < 0.001$, N = 12). These data agree with observations previously published by our research group in this same experimental model [4].

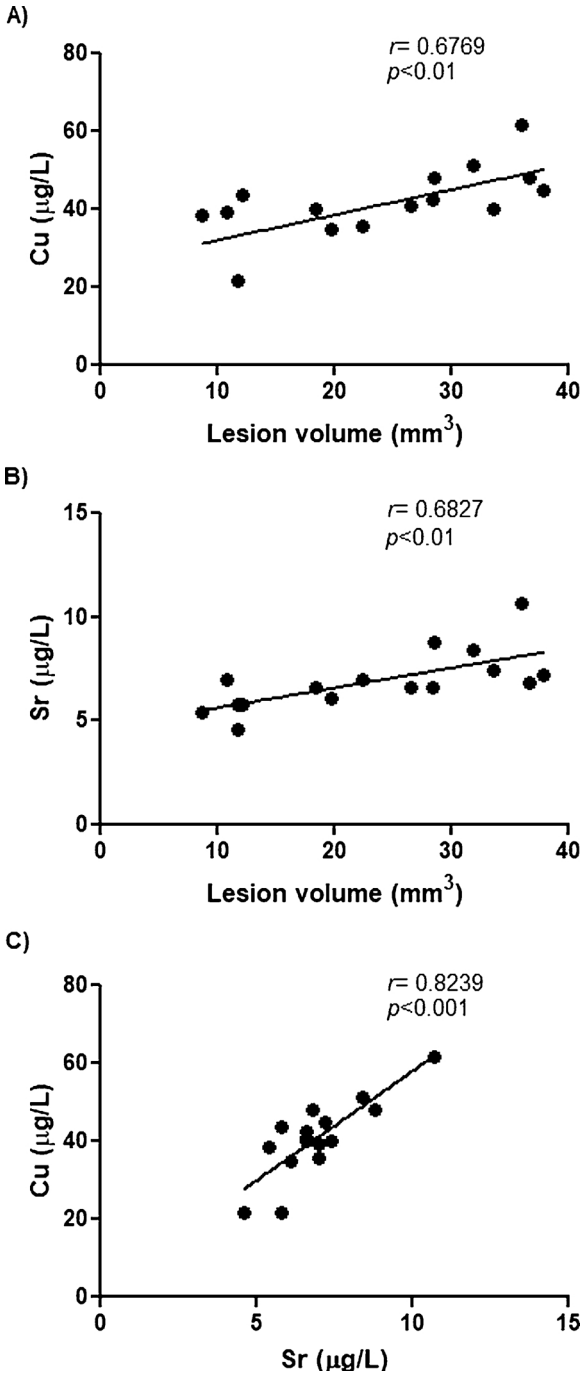

Fig. 4. Pearson correlations. This figure shows the correlations between lesion volume and Cu concentration **(A)**, lesion volume and Sr concentration **(B)**, Cu concentration and Sr concentration **(C)**. N = 16 for the entire group of mice.

3.2. Effect of the TNFRp55 deficiency on nitrite concentration and iNOS protein expression

To explore a possible link between the growth of endometriotic-like lesions and inflammation, nitrite concentration in the peritoneal fluid

and iNOS protein expression in the ectopic uterine tissue, as well as peritoneal lavage cells was analyzed. Higher concentrations of nitrite were found in KO mice compared to WT mice ($p < 0.05$) (Fig. 1A). In addition, the TNFRp55 deficiency did not significantly change iNOS expression in ectopic uterine tissue (Fig. 1B) or in peritoneal lavage cells (Fig. 1C).

3.3. Effect of TNFRp55 deficiency in the antioxidant defense system

Because inflammation is commonly related to changes in the redox balance, the effect of TNFRp55 deficiency on TAC as well as on CAT and GPX specific activities, both in endometriotic-like lesions and in peritoneal lavage cells, was measured. Interestingly, TNFRp55 deficiency did not change the antioxidant status in endometriotic-like lesions (Fig. 2**A, C,** and **E**). However, higher TAC was observed in the peritoneal lavage cells of KO mice ($p < 0.05$) (Fig. 2B), in agreement with higher specific activities of CAT ($p < 0.01$) (Fig. 2D) and GPX ($p < 0.05$) (Fig. 2F), with respect to WT mice.

3.4. Effect of TNFRp55 deficiency on markers of oxidative/nitrosative damage

NO and the antioxidant defense system can limit oxidative injury. In view of this fact, both the endometriotic-like lesions and the peritoneal lavage cells were analyzed for the following markers of oxidative or nitrosative damage: TBARS-MDA, carbonyl groups and nitrotyrosine. Lower TBARS-MDA content was found in endometriotic-like lesions of KO mice compared to WT mice ($p < 0.01$) (Fig. 3A). However, no change was observed in TBARS-MDA content in peritoneal lavage cells (Fig. 3B). Interestingly, TNFRp55 deficiency did not change protein carbonyl content in the ectopic uterine tissue (Fig. 3C) but did reduce it in peritoneal lavage cells ($p < 0.05$) (Fig. 3D). Nitrotyrosine content did not change in the endometriotic-like tissue (Fig. 3E) or peritoneal lavage cells (Fig. 3F).

3.5. Effect of TNFRp55 deficiency on the metallomic profile of the peritoneal fluid

The interruption of metal ion homeostasis can cause oxidative stress and inflammation. Therefore, the effect of TNFRp55 deficiency in the content of fourteen trace elements in peritoneal fluid of both WT and KO mice with induced endometriosis was measured by ICP-MS (Table 1). Interestingly, of the fourteen trace elements analyzed, only Cu and Sr concentrations were higher in TNFRp55 deficient mice than in WT mice ($p < 0.05$).

In addition, the correlation coefficients revealed a significant positive correlation between lesion volume with Cu concentration ($p < 0.01$) (Fig. 4A) and Sr concentration ($p < 0.01$) (Fig. 4B). Moreover, a positive correlation between Cu concentration and Sr was found ($p < 0.001$) (Fig. 4C).

4. Discussion

In the present study, we demonstrated that TNFRp55 deficiency increases antioxidant protection and promotes high Cu-Sr concentrations in the peritoneal cavity, which favors the progression of experimental endometriosis.

The first results revealed that the concentration of NO in peritoneal fluid is higher in deficient TNFRp55 mice compared to WT mice and does not imply iNOS expression changes. An increase in the peritoneal NO level appears to be a common alteration in endometriosis [6,7]. Many cells of the immune system, including macrophages that predominate in peritoneal fluid in both humans and mice [7,36], produce and respond to NO [37]. However, the results obtained would indicate that the production of NO would be mainly of endometrial origin, by the action of the constitutive isoforms of NOS.

Interestingly, the expression of iNOS has been implicated in the antitumor activity mediated by macrophages [38,39]. The fact that the expression of iNOS was not modified in uterine ectopic tissue or in peritoneal lavage cells, could explain the increased lesion volume observed in mice deficient TNFRp55.

NO plays a deleterious or protective role in tissue injury [27,40], modulating the severity and dynamics of endometriosis in a complex manner. The specific effect of NO usually depends on its bioavailability, target cell and interactions with proteins, metal ions and ROS [39].

Oxidative stress, defined as an imbalance between ROS and the antioxidant defense system, is also involved in the development and progression of endometriosis [41,42]. From the theoretical point of view, the activation of macrophages in the peritoneal cavity of women with endometriosis is an event that could potentially lead to oxidative stress, resulting in protein oxidation and lipid peroxidation. However, the real association between oxidative stress and pelvic endometriosis is unknown due to the lack of uniformity in the results obtained from different studies [43].

In our murine model of TNFRp55 deficient endometriosis, we observed higher TAC and increased CAT and GPX activity in peritoneal lavage cells. The activity of antioxidant enzymes usually increases in response to an increase in ROS production [44]. Hydrogen peroxide or other hydroperoxides appear to be key reagents in tumor suppression. Consequently, it has been shown that CAT and GPX, which catalyze the reduction of hydrogen peroxide in water or, in the case of GPX also the organic hydroperoxides in water or the corresponding alcohols, mediate the protective effects against oxidative stress in tumor cells promoting cell survival and growth by interrupting the apoptotic process [45,46]. Therefore, our findings suggest that in a TNFRp55 deficient environment, the enzymatic antioxidant system acts in an integral manner to ensure the protection of endometriotic-like lesions against cell death produced by ROS.

Although the main limitation of murine models is the lack of menstruation and spontaneous production of endometriosis, they have many similarities with the disease in humans, such as the development of endometriosis-like lesions in an estradiol-dependent manner [4]. Estrogens have anti-apoptotic effects in endometrial cells, and these effects appear to be exacerbated in women with endometriosis [47]. In addition, estrogens can also induce antioxidant enzyme expression [48]. These antecedents support the whole set of results obtained with our murine model of TNFRp55 deficient endometriosis, where the greater lesion volume is supported by lower apoptosis, increased endometrial cell proliferation, higher levels of estradiol [4] and increased antioxidant capacity in the peritoneal cavity.

To confirm antioxidant protection on endometriotic-like lesion and peritoneal lavage cells of TNFRp55 deficient mice, different markers of oxidative/nitrosative stress relevant to several chronic disorders were evaluated (MDA-TBARS, protein carbonyls and nitrotyrosine). The results revealed lower lipid peroxidation of ectopic uterine tissue and lower oxidative damage of proteins in free peritoneal cell of TNFRp55 deficient mice, compared to WT mice. On the one hand, it is well known that under physiological or low rates of lipid peroxidation (sub-toxic conditions), the cells stimulate their maintenance and survival through constitutive antioxidants defense systems or activation of signaling pathways that up-regulate antioxidants proteins [49]. This is well consistent with the results observed in our murine model of TNFRp55 deficient endometriosis. On the other hand, it has been reported that carbonyl modifications of extracellular matrix proteins favors macrophage activation [50]. Regarding the latter, our results suggest the opposite, that is low levels of the "classically activated" M1 macrophages in the peritoneal cavity of TNFRp55 deficient mice, resulting in a lower inflammatory response and lesser antitumor immunity [51], compared to WT mice. In addition, the absence of tyrosine nitration would corroborate that the concentration of NO in the peritoneal cavity of TNFRp55 deficient mice is not bioavailable or in toxic levels, which is to be expected when there is no activation of the M1 macrophages

that increase the activity of iNOS. Curiously, moderate levels of NO can also exhibit antioxidant effects by the attenuation of metal/peroxide oxidative chemistry [40].

Recent evidence suggests a critical role of trace elements in oxidative stress in pathophysiological processes as well as in endometriosis. However, the potential influence of trace elements on the redox system of the endometrial microenvironment remains unexplored. For this reason, the metallomic profile in peritoneal fluid was analyzed. Cu and Sr concentrations were higher in TNFRp55 deficient mice compared to WT mice. Early experiments have shown the presence of elevated levels of Cu in serum in many chronic diseases such as uterus myoma [52]. Elevated levels of Cu in urine of women with endometriosis have also been identified, increasing the probabilities of diagnosing this pathology [21]. Interestingly, Cu increased the proliferation of MCF7 human breast cancer cells dependent on estradiol for growth [53]. In fact, this metal is considered a metalloestrogen with the ability to activate the estrogen receptor alpha and exercise steroid agonist activity [20]. Therefore, a therapeutic modality to reduce copper levels in the peritoneal cavity could modulate the estradiol synthesis and have proapoptotic effects on endometriotic cells, this being a new approach for the treatment of endometriosis.

Currently, there is no history linking Sr with endometriosis. In an ovariectomized osteoporotic rat model, Sr increased the activity of CAT and GPX in liver and kidney, preventing oxidative damage [54]. Our findings in TNFRp55 deficient mice offer a key role for Cu and Sr in the sustained development of endometriotic lesions. These assumptions are supported by the fact that both trace elements showed a significant positive correlation between them and with respect to the lesion volume, which is higher in the TNFRp55 deficient group as reported by Vallcaneras et al. [4] and verified in the present study.

In summary, these findings suggest that the proliferation of the endometriotic-like lesion is favored in an immunosuppressed environment due to the TNFRp55 deficiency. It seems that the peritoneal environment is not a passive reservoir of trace elements and factors secreted by free peritoneal cells but actively promotes endometriosis. While the oxidative nature of peritoneal fluid is a growth mediator in endometriosis, antioxidant agents in association with Cu and Sr seem to promote the progression of this pathology in TNFRp55 deficient conditions.

The present study sheds light on the role of oxidative/nitrosative stress in the sustained development of endometriosis lesions under TNFRp55 deficiency, addressing one of the research priorities in endometriosis established after the 12th World Congress of Endometriosis (2014) [24]. It is evident that an alteration in the function of TNFRp55 would create possible scenarios for the development of atypical endometriosis.

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Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

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