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Antiproliferative and antiangiogenic effects of ammonium tetrathiomolybdate in a model of endometriosis

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Abstract

Aims: Copper (Cu) is involved in the endometriosis progression. Herein, an experimental endometriosis model was used to evaluate whether its chelation with ammonium tetrathiomolybdate (TM) affects the proliferation and angiogenesis in endometriotic-like lesions and the participation of oxidative stress in these processes.

Main methods: Female C57BL/6 mice were divided into three groups: sham-operated mice, endometriosis-induced mice, and TM-treated endometriosis-induced mice. Each animal in the third group received 0.3 mg of TM/day in their drinking water from the postoperative 15th day. The samples were collected after one month of induced pathology. In peritoneal fluids, Cu and estradiol levels were determined by electrothermal atomic absorption spectrometry and electrochemiluminescence, respectively. Endometriotic-like lesions were processed for the analysis of cell proliferation by PCNA immunohistochemistry, the expression of angiogenic markers by RT-qPCR, the presence of endothelial cells by immunofluorescent staining, and oxidative stress applying spectrophotometric methods.

Key findings: TM treatment decreased Cu and estradiol levels, which were increased by this pathology. In lesions, TM induced: (a) a decrease in tissue weight and volume, (b) a decrease in PCNA-positive cells, (c) antiangiogenic effects by decreasing the number of blood vessels, the mRNA expression of fibroblast growth factor 2 (*Fgf2)* and platelet-derived growth factor subunit B (*Pdgfb),* and the presence of endothelial cells, (d) a decrease in antioxidant activity and an increase in lipid peroxidation.

Significance: TM is a highly effective antiproliferative and antiangiogenic agent, modulating oxidative imbalance in endometriosis. Its anti-endometriotic potential is an attractive feature of TM as a possible non-hormonal treatment.

Keywords: endometriosis; copper; ammonium tetrathiomolybdate; cell proliferation; angiogenesis; oxidative stress

Introduction

Endometriosis is a chronic, estrogen-dependent systemic disease that affects 5%-15% of reproductive-aged women in the world [1,2]. This pathology is characterized by the growth of endometrial-like tissue outside the uterine cavity. Among the symptoms described, pelvic pain, dysmenorrhea, dyspareunia, and fertility problems stand out, which have a negative impact on the quality of life of patients [3].

Endometriosis is complex and there is currently no cure. Treatment strategies are limited to surgical resection of lesions or suppression of ovarian function and the action of estradiol. However, symptoms reappear in more than 50% of cases after surgery, and long-term ovarian suppression is often ineffective, suppresses fertility, and has unwanted side effects. There is, therefore, an unmet clinical need for new treatments, mainly non-hormonal, that make it possible to control the processes that favor the progression of endometriosis [4,5].

Elevated levels of copper (Cu) have been reported in serum and urine samples from women with endometriosis [6,7]. We recently reported elevated Cu levels in the peritoneal fluid of mice with induced endometriosis, which showed a positive correlation with the volume of the lesions [8]. Cu has been associated with oxidative stress in endometriosis [6]. In fact, it is an indispensable cofactor of several enzymes, including superoxide dismutase 1 (SOD1) [9]. This metal would also have the ability to stimulate the main signaling pathways of cell proliferation [5], and could even contribute to the malignant transformation within endometriosis [10]. It should be noted that Cu is considered a metalloestrogen and, therefore, can enhance estrogenic action favoring estrogen-induced tumor cell proliferation [11]. This metal is also required for the binding of hypoxia-inducible factor (HIF)-1α to

the hypoxia response elements, thus modulating the expression of vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), and cytokines [12], key angiogenesis mediators that stimulate endothelial cell migration and proliferation to generate new blood vessels. With the foregoing in mind, a therapeutic modality to reduce endometriosis Cu levels would be a novel approach for the medical treatment of this condition.

Ammonium tetrathiomolybdate (TM; $[NH_4]_2MoS_4$) is a rapidly absorbing Cu chelator with a good safety profile. TM interferes with the absorption of Cu at the intestinal level and, in addition, acts forming a stable tripartite complex with serum albumin and Cu, reducing the bioavailability of the metal. Among the most frequent side effects found, anemia, leukopenia, and increased transaminases have been reported, which are easily reversed by reducing the daily dose of the drug [13].

Several preclinical and clinical studies in various types of cancer have shown that TM reduces tumors growth and interferes with angiogenesis [14–21]. However, so far, no previous work has analyzed the Cu chelation effects in endometriosis. Like cancer, this pathology is characterized by cellular invasion and growth without restrictions, the development of new blood vessels, and a decrease in the number of apoptotic cells. Therefore, this work aimed to evaluate whether Cu chelation with TM affects the proliferation and angiogenesis in endometriotic-like lesions and the participation of oxidative stress in these processes.

Materials and methods

Two-month-old female mice of the C57BL/6 strain and weighing 19-21 g were used. Breeding colonies were established at the Animal Facility of the 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 sterile water and food *ad libitum*. All experimental procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory

Animals (NIH Publications No. 8023, revised 1978). The animal study was reviewed and approved by the Comité Institucional de Cuidado y Uso de Animales (Acronym: CICUA) of the Universidad Nacional de San Luis (Protocols No. B-304/18 and B-304/20).

Surgical induction of peritoneal endometriosis in mice

Twenty-four female mice were randomly divided into three groups: (1) sham-operated mice (Sham surgery), (2) endometriosis-induced mice (EDT), and (3) TM-treated endometriosis-induced mice (EDT+TM). The induction of experimental endometriosis was carried out at random phases of the estrous cycle [21]. The animals were anesthetized with an intraperitoneal injection of 100 mg/kg of ketamine (Holliday Scott, Buenos Aires, Argentina) and 10 mg/kg of xylazine (Richmond, Buenos Aires, Argentina). Then a mid-ventral incision was made to expose the uterus and intestines. The right uterine horn was removed from the animal and placed in DMEM-F12 (Gibco, Life Sciences, Great Island, NY, USA), opened longitudinally, and cut into three square pieces of approximately 4 mm^2 each. The three equal parts of the uterine horn were sutured to the mesentery of the intestine (autologous transplant) so that the endometrial layer faced the bowel serosa. A single suture (Supralon 6–0, Ethicon, Somerville, New Jersey) was used for each piece of uterine tissue. In sham-operated mice, three sutures were attached to the mesentery of the intestine without implants. The area was hydrated with sterile physiological solution supplemented with antibiotic-antimycotic before closing the abdominal wall with the same suture material, with continuous stitches. The bodyweight, food consumption, and grooming behavior of the mice were monitored daily. One month after the surgery, the animals were euthanized by cervical dislocation during diestrous stage. Immediately, a small medioventral hole was opened through which 1.5 mL of phosphate-buffered saline (0.015 M KH_2PO_4, 0.017 M NaH_2PO_4, 0.076 M KCl, and 0.14 M NaCl, pH 7.4) was injected into the peritoneal cavity of each animal. The peritoneal lavage fluid was collected and centrifuged at 250 *g* for 10 min at 4 °C min. The supernatants (peritoneal fluid) were collected and stored at -80 °C

until the determination of Cu and estradiol. Finally, the abdomen was completely opened to have access to the endometriotic-like lesions present in the induced animals.

Administration of TM

Before the animal experiments, water consumption was checked daily and recorded for 10 days to determine the daily water consumption of each mouse [15]. With this information, each animal in the EDT+TM group received 0.3 mg of TM (cat # 323446; Sigma-Aldrich, St Louis, MO, USA) daily in their sterile drinking water, from the postoperative 15th day (period required for the proper establishment of lesions) to the euthanasia day. The animals in groups 1 and 2 received just drinking water. To control that severe Cu deficiency did not occur in TM-treated mice, weekly it was verified that the weight of each did not decrease more than 10% of the initial value and that the hematocrit did not decrease below 80% of the baseline [16,24]. No evidence of toxicity was observed at the administered dose based on body weight, food consumption, or grooming behavior compared to controls. Furthermore, no histopathological alterations were observed in the liver of treated mice, compared to the EDT group (**Supplementary Figure S1**).

Evaluation of the endometriotic-like lesions

Lesions were identified, counted, and measured with a caliper in two perpendicular diameters. The volume of the developed lesions was calculated from the following equation: $\boldsymbol{V = (4/3)\ \pi\ r_1^2\ r_2}$ (r_1 and r_2 are the radiuses and $r_1<r_2$). Subsequently, the lesions were removed and weighed. One lesion per animal was fixed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4 °C and embedded in paraffin. At least two 4 µm-sections per specimen were stained with hematoxylin-eosin to confirm the presence of glands and stroma endometrial-like in the ectopic tissue and to identify and count the number of blood vessels. Other 4 µm-sections were prepared for cell proliferation and

angiogenesis studies. The rest of the lesions were kept at -20 °C in RNAhold® (TransGen Biotech Co., Ltd.) or at -80 °C until use.

Copper assay

Copper concentration in the peritoneal fluid was determined by electrothermal atomic absorption spectrometry (ETAAS) with a graphite furnace, a methodology with good selectivity and sensitivity [23]. 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 adding the solutions, the samples were heated at 60 °C for 1 h in a thermostated water bath, obtaining transparent sample solutions. All samples and reagent solutions were prepared in metal-free 15-mL polypropylene tubes (Sarstedt, Germany). 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).

The measurements were performed with a Shimadzu Model AA-7000 atomic absorption spectrometer (Tokyo, Japan), equipped with a GFA-EX7 atomizer and an ASC-6100 autosampler. Integrated platform (L'vov), graphite tubes, Shimadzu (Tokyo, Japan), were used in all experiments. A Cu hollow-cathode lamp (Hamamatsu, Photonics, K.K., Japan) was employed as a radiation source at 324.8 nm with a slit of 0.5 nm.

Estradiol assay

Electrochemiluminescence immunoassay (ECLIA) kit specific for estradiol (Elecsys Estradiol III, Roche Diagnostics International Ltd, Mannheim, Germany) was used according to the manufacturer's instructions. The lower and upper detection limits were 5 and 3000 pg/mL, respectively. Estradiol levels in the peritoneal fluid were expressed in pg/mL.

Immunohistochemistry of proliferating cell nuclear antigen (PCNA)

PCNA, a key factor in DNA replication and cell cycle regulation, was evaluated in endometriotic-like lesions of untreated and TM-treated mice. Tissue sections from six different animals per experimental group were deparaffinized in xylene and rehydrated in a graded series of ethyl alcohols. The slides were immersed in antigen-retrieval solution (0.01 M sodium citrate buffer, pH 6.0) in a plastic Coplin jar and placed in a microwave oven for 10 min on the highest power. Endogenous peroxidase was blocked by treatment with 3% H_2O_2 for 30 min. All the sections were then blocked with 4% BSA in phosphate-buffered saline for 2 h at room temperature and incubated with a rabbit polyclonal PCNA antibody (1:200; FL-261, Santa Cruz Biotechnology, CA, USA) overnight at 4°C. Subsequently, the sections were incubated with biotinylated goat-anti-rabbit IgG (1:200; B-7389, Sigma-Aldrich, St Louis, MO, USA) for 1 h at room temperature, followed by incubation with HRP-conjugated streptavidin (VectorLabs, Burlingame, CA, USA) for 30 min at room temperature. The signal was developed with diaminobenzidine (DAB) as substrate (Cell Marque, CA, USA). Finally, tissue sections were counterstained with hematoxylin, dehydrated through graded alcohols, clarified in xylene, and properly mounted. As a negative control, one section from each slide was run omitting the primary antibody. Cells positive for PCNA were identified by the presence of brown nuclear reactivity. The PCNA-positive cell count was established at 1000x magnification under an optical light microscope (Olympus, Japan), and 4-6 representative fields per section were considered. The results were expressed as a percentage of PCNA-positive cells by calculating the ratio between the number of positive cells and the total number of cells, for both epithelial and stromal cells. This percentage was calculated per slide and was used to obtain the mean of each experimental group.

Mean blood vessel count

Counting of blood vessels (small, medium, and large-caliber) was performed in sections of endometriotic-like lesions stained with hematoxylin-eosin, using an optical light microscope (Olympus, Japan) with a magnification of 400x. Tissue sections from six different animals per experimental group and between 6 and 8 fields per section were observed, in the areas with the highest vascular density. The results were expressed as the mean blood vessel count per field.

Quantitative reverse transcription PCR (RT-qPCR) analysis of angiogenic factors

Total RNA was isolated from endometriotic-like lesions using TRIzol® reagent (Life Technologies, Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNA integrity was confirmed by identifying 28S and 18S ribosomal rRNA by gel electrophoresis. Samples were quantified using an EPOCH™ microplate spectrophotometer (BioTek Instruments, Inc.), and RNA purity was determined by measuring optical density at 260/280 nm. All RNA samples were treated with RQ1 RNase-Free DNase (Promega). One microgram of total RNA was reverse-transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) and stored at -20 °C, following the manufacturer's guidelines. For qPCR, cDNA was amplified in an ABI PRISM® 7500 Instrument (Applied Biosystems, Life Technologies, Thermo Fisher Scientific, Inc.), using FastStart™ Universal SYBR® Green Master (Roche Diagnostics). qPCR reactions were processed in a final volume of 20 mL containing 2× FastStart™ Universal SYBR® Green Master Mix (10 µL), cDNA (2 µL), forward primer (10 µM, 1 µL), reverse primer (10 µM, 1 µL), and nuclease-free water (6 µL). The PCR cycling conditions were as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. The primers information for *Vegfa*, *Fgf2* and platelet-derived growth factor subunit B (*Pdgfb*) are listed in **Table 1**. *Rn18s* (18S ribosomal RNA) was selected as the internal reference gene, and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method [24].

Table 1 Primer gene symbols, sequences, GenBank access numbers, and sizes of amplicons

Gene	Sequences (5'-3')	GenBank access number	Amplicon
Vegfa	*Forward:* CACTTCCAGAAACACGACAAAC *Reverse:* TGGAACCGGCATCTTTATCTC	NM_001025250.3	95 bp
Fgf2	*Forward:* GGCATCACCTCGCTTCC *Reverse:* CGCCGTTCTTGCAGTAGAG	NM_008006.2	97 bp
Pdgfb	*Forward:* GAGTGTGGGCAGGGTTATTT *Reverse:* GAATCAGGCATCGAGACAGAC	NM_011057.4	105 bp
Rn18s	*Forward:* CTGAGAAACGGCTACCACATC *Reverse:* GCCTCGAAAGAGTCCTGTATTG	NR_003278.3	107 bp

Immunofluorescent staining of endothelial cells

Before staining, tissue sections from six different animals per group were deparaffinized and rehydrated. Slides were rinsed with phosphate-buffered saline and dye with DAPI 0.3 ug/mL plus fluorescein-labeled Griffonia (Bandeiraea) Simplicifolia Lectin I 20 ug/mL (GSL I; FL-1101; Vector Laboratories, Burlingame, CA, USA) that binds specifically to endothelial cells forming blood vessel in mouse tissues [25]. Subsequently, the sections were rinsed with phosphate-buffered saline and then mounted on microscope slides. Images of the stained sections were taken with a Nikon Eclipse E800 fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA). Images were analyzed using ImageJ software (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/).

Antioxidant enzyme activities

Endometriotic-like lesions were homogenized separately in 100 µL of radioimmunoprecipitation assay buffer (RIPA buffer; Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Suspensions were centrifuged at 21,912 *g* for 15 min at 4 °C to remove nuclei and cell debris. The supernatants were used to analyze different oxidative status markers. The total protein concentration was measured by the Bradford method [26].

The pyrogallol autoxidation method was used to measure SOD activity, monitoring the change in absorbance at 420 nm per min [27]. One unit of the enzyme was expressed as the amount of SOD

that inhibits 50% of pyrogallol autoxidation. Catalase (CAT) activity was evaluated by measuring the decrease in H_2O_2 absorption at 240 nm. One CAT unit is defined as the amount of enzyme required to decompose 1 μM of H_2O_2/min [28]. Glutathione peroxidase (GPX) specific activity was assessed by monitoring the NADPH oxidation rate at 340 nm [29]. The results were expressed as units of enzyme activity per milligram of protein (U/mg protein).

Assessment of lipid peroxidation

Lipid peroxidation in homogenates of endometriotic-like lesions was evaluated spectrophotometrically by measuring the levels of thiobarbituric acid reactive substances (TBARS) at 535 nm [30]. For malondialdehyde (MDA) standards, a stock of 1,1,3,3-tetraethoxypropane was used. The results were expressed as μmol of MDA per milligram of protein (μmol MDA/mg protein).

Statistical analysis

All the data were expressed as the mean ± standard error of mean (S.E.M.) and were analyzed using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). The comparisons between groups were analyzed using Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison test. Differences were considered statistically significant when $P<0.05$.

Results

Effect of TM on the development of endometriotic-like lesions

Macroscopic examination of the ectopic uterine tissue revealed that there were no significant differences in the number of established lesions per mouse between experimental groups (Figure 1A). However, the administration of the Cu chelator decreased the volume and weight of the lesions in the TM-treated mice, compared to the EDT group ($P<0.05$; Figure 1B, C).

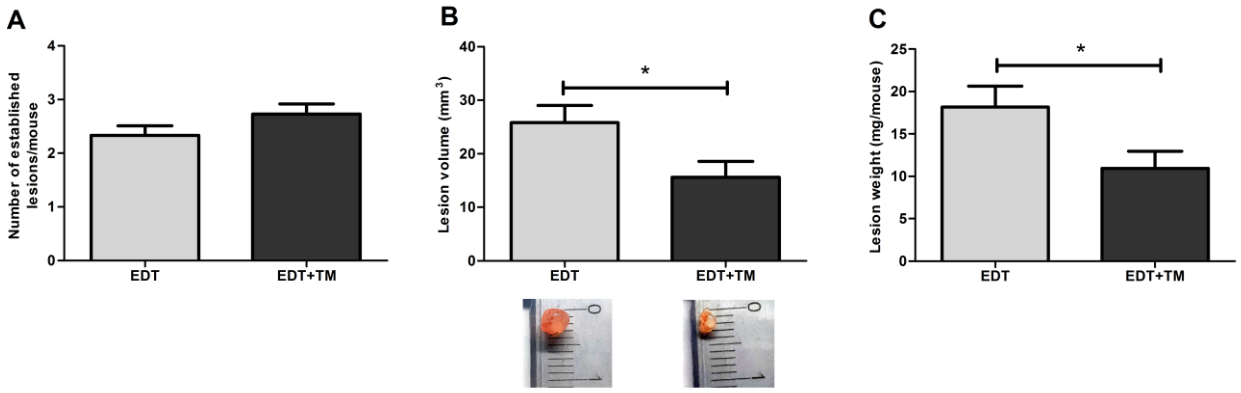

Figure 1 Effect of TM on the development of endometriotic-like lesions. The number of established lesions (A), their volume (B), and their weight (C) were evaluated in endometriosis-induced mice (EDT) and TM-treated endometriosis-induced mice (EDT+TM) after one month of inducing the pathology. This last group received 0.3 mg TM/day in their sterile drinking water, from postoperative 15th day (period required for the proper establishment of lesions) to the euthanasia day. Representative images of the morphology of endometriotic-like lesions are provided. Results are expressed as mean ± S.E.M. (n = 8 animals per group). Statistical comparisons were made using Student's *t*-test. *P<0.05.

Effect of TM on the concentration of Cu and estradiol in peritoneal fluid of endometriosis induced mice

As already mentioned, endometriosis is an estrogen-dependent gynecological condition, and Cu could favor the progression of the disease by increasing the estrogenic action. Therefore, we analyze the effect of the anti-Cu treatment on the concentration of the metal and the hormone in the peritoneal fluid. On the one hand, the establishment of the pathology produced an increase in the concentration of Cu and estradiol, compared with the sham surgery group (P<0.01; Figure 2A, B). On the other hand, the administration of the Cu chelating agent in our model of endometriosis effectively decreased the concentration of this metal (P<0.001; Figure 2A) and also the estradiol levels (P<0.01; Figure 2B), reaching values similar to those of the sham surgery group.

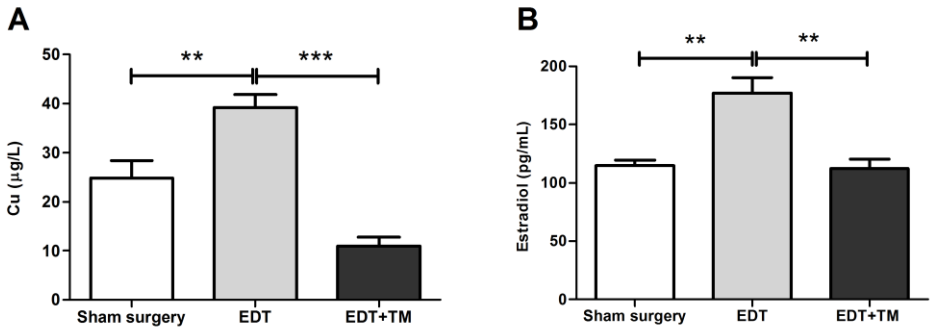

Figure 2 Effect of TM on the concentration of Cu and estradiol in peritoneal fluid. Concentrations of Cu (A) and estradiol (B) were determined by ETAAS and ECLIA, respectively, in sham-operated mice (Sham surgery), endometriosis-induced mice (EDT), and TM-treated endometriosis-induced mice (EDT+TM). Results are expressed as mean ± S.E.M. (n= 8 animals per group). One-way ANOVA followed by Tukey's test was used. $^{**}P<0.01$; $^{***}P<0.001$.

Effect of TM on the cell proliferation in endometriotic-like lesions

TM has been shown to have antiproliferative activity on tumors. Based on the above, we analyzed the PCNA labeling index in lesions sections. Histologically, endometriotic-like lesions from mice untreated showed abundant stroma and endometrial-type glands and TM administration reduced these histopathological marks of endometriosis (Figure 3A, B). In fact, TM reduced the proliferation of both epithelial and stromal cells ($P<0.001$; Figure 3C), compared to the lesions from untreated animals.

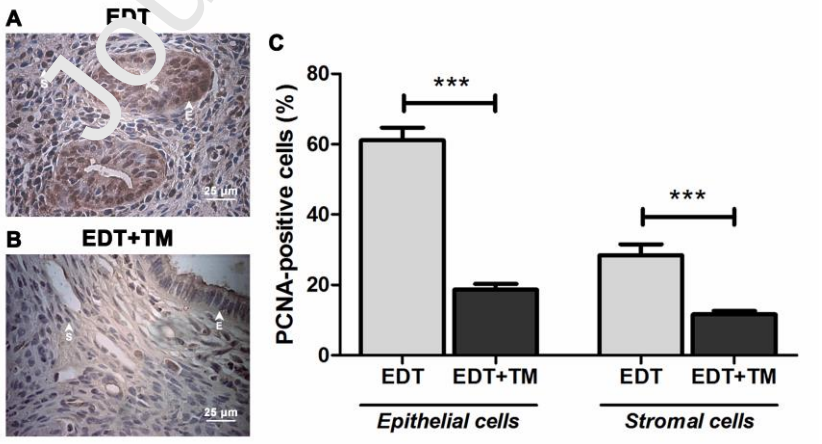

Figure 3 Effect of TM on cell proliferation in endometriotic-like lesions. The micrographs show representative histological sections of induced endometriotic-like lesions in untreated mice (EDT) (A) and TM-treated mice (EDT+TM) (B). Arrowheads indicate epithelial and stromal cells. Scale bar, 25 µm. Magnification: 400x. The percentage of proliferating cells was evaluated by immunohistochemistry for PCNA for both epithelial and stromal cells from endometriotic-like lesions of both experimental groups (C). PCNA-positive cells were identified by the presence of brown nuclear reactivity. As a negative control, one section of each slide was

tested without the primary antibody. Results are expressed as mean ± S.E.M. (n= 6 per group). Statistical comparisons were made using Student's *t*-test. ***P<0.001. E, epithelial cells; S, stromal cells.

Antiangiogenic effect of TM in endometriotic-like lesions

Previous reports indicate that TM interferes with angiogenesis, a process necessary for tumor progression. Therefore, this effect of the drug was analyzed in experimental endometriosis. In fact, TM significantly decreased the number of blood vessels (P<0.001; Figure 4A). It also showed a tendency to decrease the *Vegfa* mRNA expression, although the variation was not statistically significant (Figure 4B). In addition, the mRNA expression of *Fgf2* and *Pdgfb* reduced significantly (P<0.05; Figure 4C, D). The immunofluorescence intensity of GSL I-FITC also decreased in endometriotic-like lesions from TM-treated mice (P<0.001), indicating a lower number of endothelial cells in this tissue with respect to lesions removed from animals that did not receive the treatment (Figure 4E, F).

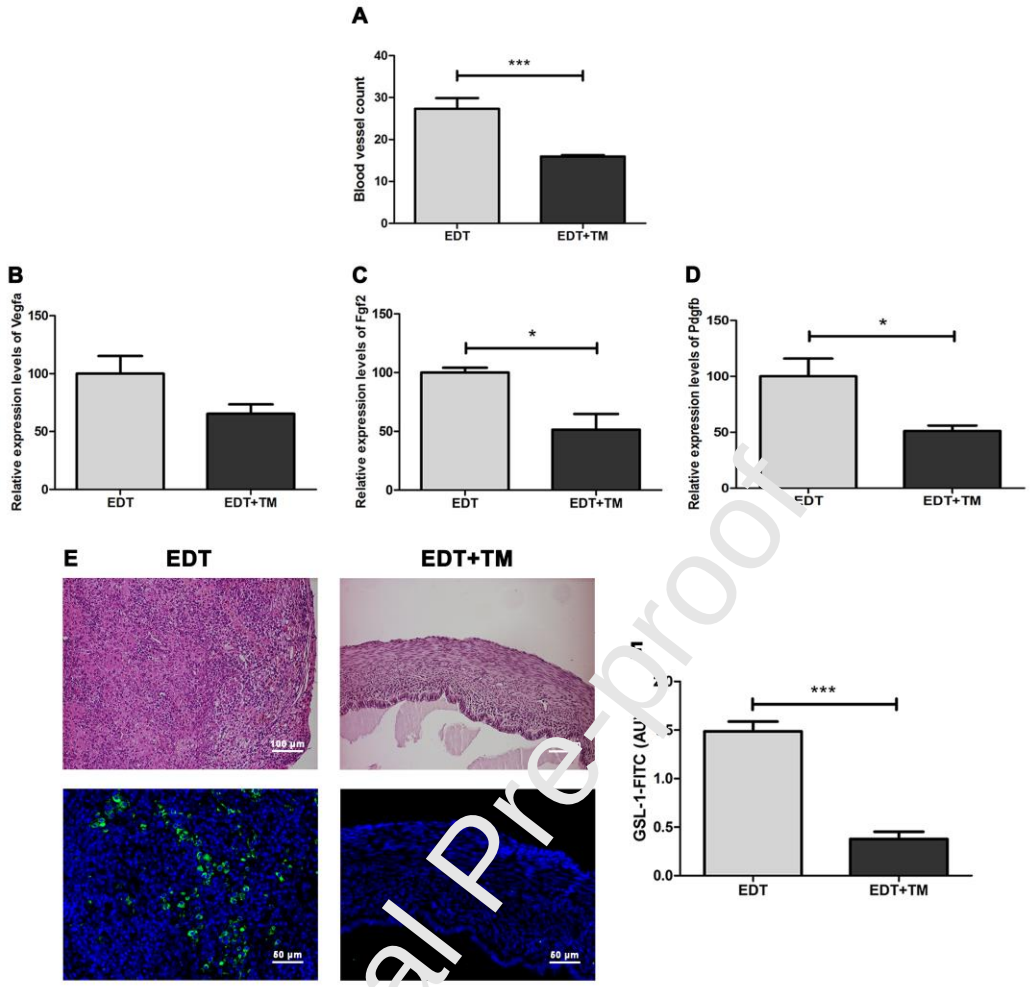

Figure 4 Antiangiogenic effect of TM in endometriotic-like lesions. The upper part of the panel shows the counting of blood vessels in sections of lesions in untreated mice (EDT) and TM-treated mice (EDT+TM). The results were expressed as the mean blood vessel count per field (A). In the middle part of the panel, the effect of TM on the expression of mRNA of *Vegfa* (B), *Fgf2* (C), and *Pdgfb* (D) in lesions for both experimental groups are shown. The relative quantification of each mRNA was calculated from the Cq values obtained for the gene of interest (*Vegfa, Fgf2, Pdgfb*) and the reference gene (*Rn18s*), using the $2^{-\Delta\Delta Ct}$ method. Endothelial cell immunofluorescent staining is exhibited at the bottom of the panel (E). The micrographs show representative histological sections of induced endometriotic-like lesions in the EDT and EDT+TM groups, stained with hematoxylin-eosin (scale bar, 100 µm) or with GSL I-FICT (green, endothelial cells) and DAPI (blue, nuclei) (scale bar, 50 µm). Magnification: 100x and 200x, respectively. Bars represent the average of GSL-1-FITC+/field ± S.E.M. from six representative visual fields (F). Statistical comparisons were made using Student's *t*-test. **P*<0.05; ****P*<0.001.

Effect of TM on oxidative stress in experimental endometriosis

Cu is associated with oxidative stress in endometriosis, therefore, we evaluate the effect of TM on antioxidant enzymatic defenses. The Cu chelator decreased SOD activity (*P*<0.05; Figure 5A) and CAT activity (*P*<0.05; Figure 5B), but did not alter GPX activity (Figure 5C) in endometriotic-like lesions, compared to the EDT group.

Since the treatment decreased antioxidant activity, we continued with the analysis of TBARS-MDA, a biomarker of oxidative stress widely used in endometriosis. TM increased the MDA concentration in endometriotic-like lesions with respect to the EDT group (P<0.05; Figure 5D), suggesting an imbalance between oxidants and antioxidants in favor of oxidants.

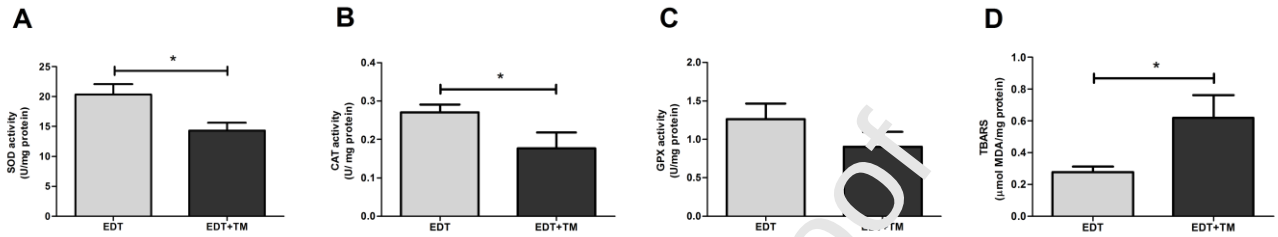

Figure 5 Effect of TM on oxidative stress in experimental endometriosis. Endometriotic-like lesions of untreated mice (EDT) and TM-treated mice (EDT+TM) were analyzed to determine the activity of the antioxidant enzymes SOD (A), CAT (B), and GPX (C), and the levels of MDA (a marker of lipid peroxidation) (D), by spectrophotometric methods. Results are expressed as mean ± S.E.M. (n= 8 per group). Statistical comparisons were made using Student's *t*-test. *P<0.05.

Discussion

Different studies have shown an association between endometriosis and Cu [6–8]. Therefore, in the present work, we evaluate the therapeutic potential of Cu chelation with TM in an experimental model of endometriosis using mice, focusing on certain processes that favor the progression of this gynecological disease.

Among the results obtained by the TM treatment, we observed that the estradiol concentration in the peritoneal fluid decreased, reaching values similar to those determined in the Sham group. This fact is consistent with a suppression in the development of established lesions. In peritoneal endometriosis, it has been suggested that endometriotic epithelial and stromal cells are responsible for the local secretion of estradiol because they overexpress the main steroidogenic enzymes that mainly use dehydroepiandrosterone (DHEA) and also cholesterol as a substrate [31]. Curiously, different works by other authors have shown the ability of Cu to modulate steroidogenesis. In a study in which bovine ovarian follicle granulosa cells cultured were treated with TM, estrogen production

decreased in a dose-dependent manner. Furthermore, the combined use of the equimolar Cu and TM media ameliorated the effect of TM on both estradiol production [32]. It has also been reported that while Cu at low levels can decrease serum DHEA [33], at high levels, it can stimulate the expression of enzymes involved in estradiol synthesis [34–36]. The aforementioned antecedents shed light on the possible effects that TM treatment could be exerting on the epithelial and stromal cells from ectopic implants to cause your estrogen levels to drop. TM treatment could affect the expression of steroidogenic enzymes and the availability of DHEA. Thus, as endometriosis is an estradiol-dependent disease, low levels of this hormone would affect the proliferation capacity of endometriotic cells, which, in turn, are an essential source of locally bioavailable estradiol. In fact, we analyzed the PCNA labeling index in lesions sections and observed that the TM treatment decreased cell proliferation.

It is well known that estradiol promotes cell proliferation in endometriosis [37]. Cu has also been attributed the pro-proliferative property in various pathologies [11,38]. A positive correlation has even been reported between Cu levels in peritoneal fluid and the volume of endometriotic-like lesions [8]. Both Cu and estradiol can mediate the activation of phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathways [5,37–40]. The two signaling pathways responsible for essential cellular processes, including cell proliferation and apoptosis, are active in endometriotic cells [5,37]. Furthermore, estradiol has been reported to decrease the nometastatic gene 23-H1 (NME1) expression, which limits cell proliferation mediated by signaling pathways mentioned above and down-regulates PCNA expression in endometrial stromal cells [41]. Therefore, these antecedents support our results on the decrease in both the peritoneal levels of Cu and estradiol and in the percentage of PCNA-positive cells, in agreement with the macroscopic changes observed.

It is important to highlight that the proliferation of both epithelial and stromal cells was affected by the anti-Cu drug, hindering the progression of the pathology. This fact is of importance considering that in both cell types, there are aberrant molecular pathways that orchestrate processes that favor the advancement of this devastating disease [42]. These endometriotic cells are involved in the synthesis of potent angiogenic factors [43,44], which promote the migration and proliferation of endothelial cells and recruit endothelial cells from the bone marrow necessary for the lesions to acquire new blood vessels [45]. In effect, the administration of TM in mice affected both cell proliferation and angiogenesis in endometriotic-like lesions. Angiogenesis is essential for the growth of endometrial-like tissue outside the uterine cavity because it establishes the necessary means for the adequate supply of oxygen and nutrients, immune components, hormones, and the removal of waste products [43,46]. In our experimental model, we found that the Cu chelator showed a tendency to decrease the *Vefga* mRNA expression and significantly affected the expression of *Fgf2* and *Pdgfb*. Consequently, it affected vascular sprouting and blood vessel stability, leading to vascular regression. Other authors reported similar results when they demonstrated that angiogenesis and vessel maturation in endometriotic-like lesions in hamsters was significantly reduced by SU6668, a multitargeted angiogenesis inhibitor [47]. The possibility of a differential inhibitory effect of TM treatment on these angiogenic factors is not ruled out, as observed in an *in vitro* study [48]. VEGF only showed a tendency to decrease and even so the presence of endothelial cells decreased considerably. In this sense, our results could indicate the existence of different mechanisms of blood vessel development in induced lesions in mice, where *Fgf2* plays a fundamental role. It is not a minor detail that experimental research with animals shows that FGFs are involved in enhancing the invasive capacity of endometriotic cells, and this has allowed their postulation as possible therapeutic targets for endometriosis [49].

According to Hosaka *et al*. [50], VEGF, FGF2, and PDGF contribute significantly to neovascularization and vascular remodeling in tumors. On the one hand, VEGF and FGF2 ensure that endothelial cell proliferation and budding processes occur simultaneously. On the other hand, endothelial cells produce PDGFb to recruit perivascular cells into the newly formed vasculature [50]. Furthermore, cancer background information suggests that Cu is necessary for the proliferation, migration, and organization of endothelial cells in capillary networks [15]. This would explain our results considering the decrease in the presence of endothelial cells accompanied by the decrease in the expression of *Fgf2* and *Pdgfb* in lesions of treated animals. Accumulating evidence indicates that a Cu deficiency negatively regulates inflammatory responses and angiogenesis, by inhibiting the expression of various angiogenic, growth-promoting, and pro-inflammatory cytokines through multiple mechanisms, including inhibition of nuclear factor-κB [51]. Based on the above and considering how the development of lesions is affected in our experimental model, the possible beneficial effects of reduced Cu concentration in endometriosis are significant.

The relationship between Cu, proliferation, angiogenesis, and oxidative stress cannot be ignored. Increased levels of superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), as well as increased SOD activity and GPX expression, and decreased levels of CAT, have been reported in endometriotic lesions compared to the controls [10,52]. In response to an oxidative and inflammatory microenvironment, endometriotic cells and macrophages secrete antioxidants that control excess oxidative stress and consequently cell toxicity and apoptosis [53]. In this scenario, although endometriosis is considered a benign pathology, an increase in antioxidant function in tissues could provide an environment that allows proliferation, giving cells an advantage for survival and growth, while the increase of oxidative species could stimulate the subsequent malignant transformation [10]. In our experimental model, the TM treatment produced an oxidative imbalance in the ectopic tissue by reducing the activity of SOD and CAT, which possibly increased the concentration of reactive

oxygen species to excessive levels associated with cell death. This would explain the lipid damage and the regression of the lesions. Our results agree with previous studies showing that TM treatment attenuates angiogenesis and tumor cell survival and proliferation by inhibiting SOD1. Among the possible mechanisms of action, the attenuation of ERK1/2 phosphorylation mediated by VEGF and FGF2 in endothelial cells, the accumulation of $O_2^{\bullet-}$ that induces oxidative damage, and down-regulation of the PDGF receptor have been reported [54]. Furthermore, the inhibition of SOD1 prevents the formation of H_2O_2 [54]. A decrease in the levels of this active species would explain the reduced CAT activity found in our experimental model because, unlike GPX, this enzyme acts only in the presence of toxic H_2O_2 levels [55]. SOD1 is more than an antioxidant enzyme and, therefore, its overexpression has been considered important for tumor formation, while its inhibition would cause apoptosis in cancer cells [56]. In the present work, TM treatment decreased SOD activity, supporting reduced cell proliferation, destabilization of blood vessels and reduced angiogenesis (both essential for sustained implants growth), and oxidative damage. Consistent with cancer studies, our findings provide evidence on the effects that TM treatment might exert in the management of endometriosis.

Conclusions

Our results show that TM is a highly effective antiproliferative and antiangiogenic agent in experimental endometriosis. This is supported by the fact that treatment with this Cu chelator was able to suppress the growth of established endometriotic-like lesions in mice through a mechanism that involves: decrease in estradiol levels, less expression of angiogenic factors, decrease in the number of blood vessels and endothelial cells, and modulation of oxidative imbalance. This ability to regress endometriotic-like lesions and its good safety profile are attractive features of TM as a possible non-hormonal treatment for endometriosis.

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Declaration of competing interest

The authors declare that there are no conflicts of interest. The publication of the manuscript was approved by all authors.

Author contributions

M.B.D.: Conceptualization, Investigation, Validation, Visualization, Writing - original draft. **R.A.C.**: Investigation, Methodology, Formal analysis. **D.L.V.**, **L.A.**, **P.P.**: Methodology, Resources. **S.A.**: Resources, Funding acquisition. **S.S.V.**: Investigation, Methodology. **M.C.**: Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

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Highlights

- Ammonium tetrathiomolybdate is an effective antiproliferative and antiangiogenic agent
- Copper chelation suppresses the growth of endometriotic-like lesions
- Ammonium tetrathiomolybdate reduces estradiol levels in a model of endometriosis
- Copper chelation modulates oxidative imbalance in experimental endometriosis