

More than antioxidant: N-acetyl-L-cysteine in a murine model of endometriosis

N-acetyl-L-cysteine exerts a complex action on endometrial cells, involving regulation of gene expression and protein activity and location, all converging into a decreased proliferation and a switch toward a differentiating, less invasive, and less inflammatory phenotype. Also considering the lack of undesired side effects, including unaffected fertility potential, this suggests a beneficial use of NAC in endometriosis clinical treatment. (Fertil Steril® 2010;94:2905–8. ©2010 by American Society for Reproductive Medicine.)

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Endometriosis is among most frequent benign gynecologic diseases, characterized by the implant and growth of viable endometrial tissue outside the uterine cavity, producing a general inflammatory response accompanied by pain (1). Although its pathogenesis is still debated, the invasive and proliferative properties of endometrial cells in the onset and progression of endometriosis are of relevance (2). The first-line intervention is the surgical removal of ectopic lesions, although with a relevant percentage of recurrence (3). In addition, hormonal therapies are currently available (4), all related to the suppression of ovarian estrogen biosynthesis. Due to severe secondary effects, these treatments cannot be used for prolonged periods. New and improved therapeutic solutions are definitely desirable to efficiently eliminate the endometriotic lesions and to hinder recurrences without interfering with fertility potential.

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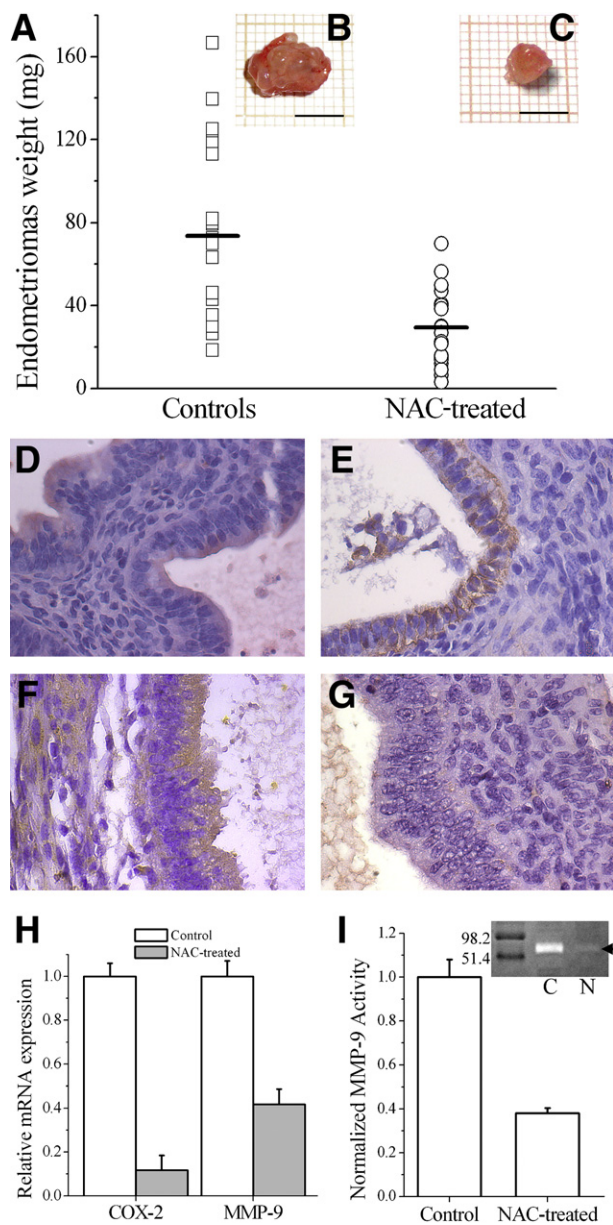
In both in vitro (5–6) and in vivo (7) models of endometriosis, the thiol-containing drug N-acetyl-L-cysteine (NAC) was successfully used to decrease endometrial cell proliferation, with attribution of this effect merely to its antioxidant properties. We recently demonstrated that NAC possesses a marked antiproliferative action on cancer cells of epithelial origin, the same origin of endometrial cells. This action was related to several morphologic, biochemical, and molecular changes all converging into a proliferation-to-differentiation switch, including a decreased invasiveness (8–11). Beyond its eventual antioxidant action, NAC thus emerges as a promising compound involved in the maintenance of redox homeostasis and able to interfere with thiol redox transitions in the proliferation/differentiation-signaling network (12, 13). In the present study, we explored NAC action in vivo by using a murine model of endometriosis. We examined lesion mass, cell proliferation, differentiation, and some inflammation- and motility-related genes and proteins.

Endometriosis was induced in 60 female, 6–8-week-old BALB/C mice (Charles River Italia, Calco, Italy) following a previously described surgical method (14) with the ratio of one donor to two recipients. Experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the European Communities Council Directive (86/609/EEC) and after receiving the institutional approval (Italian Ministry of Health no. 80/2009-B). Unless otherwise specified, all chemicals were from Sigma-Aldrich, Milan, Italy.

Recipient mice were randomly divided into two groups of 20 mice each: control and NAC-treated. Starting from the day after the endometrial implant, and for 5 d/wk for 3 weeks, mice were administered by gavage with 100 μ L of a 10 mg/mL NAC solution in water (only water for control group). Given the average weight of 22.5 ± 0.7 g, the NAC dose of 1 mg/mouse/d corresponded to an average of 44 mg/kg/d. No evidence of toxicity was noted based on unchanged body weight, food consumption, grooming behavior, or activity levels.

At the end of treatment, mice were killed through cervical dislocation, and lesions were excised from the surrounding tissue, weighed, and immediately either fixed in 10% formalin or frozen in liquid nitrogen and stored at -80°C . Formalin-fixed samples were paraffin embedded, and 5- μ m-thick sections were stained

FIGURE 1



Pittaluga. Correspondence. Fertil Steril 2010.

using hematoxylin-eosin. Slides were evaluated by an experienced pathologist blind to the study design who diagnosed endometriosis.

For antigen retrieval, sections were microwaved for 6 minutes in 0.1 mol/L citrate buffer (pH 6.0) and endogenous peroxidase activity blocked by 20 minutes' incubation in 3% H₂O₂/methanol. Sections were rinsed in phosphate-buffered saline solution (PBS)/Triton X-100, immersed for 15 minutes in PBS-bovine serum albumin, and then incubated with the selected primary antibody. For immunodetection, Ki-67, rabbit antihuman polyclonal, was from Abcam (Cambridge, United Kingdom); cooxygenase (COX) 2, rabbit antimouse polyclonal, was from Cayman

FIGURE 1 Continued

N-acetyl-L-cysteine (NAC) effect on endometrioma size, E-cadherin relocation, and cooxygenase (COX) 2 and matrix metalloproteinase (MMP) 9 decrease. **(A)** Weight of control (squares; n = 22) and NAC-treated (circles; n = 22) endometriomas after 21 days of treatment. Averages indicated by bold lines. Above histograms, representative pictures of endometriomas are shown for **(B)** a control and **(C)** a NAC-treated case (bar = 1 cm). A diffuse brownish staining of E-cadherin in **(D)** the cytoplasm of epithelial cells from control samples is **(E)** instead well confined at the level of cell-cell junctions in NAC-treated samples. A relevant COX-2 immunostaining is **(F)** visible in several areas of control samples, but **(G)** barely detectable in NAC-treated samples. **(D-G)**: original magnification $\times 40$. **(H)** Quantitative reverse-transcription polymerase chain reaction data averaged over five control samples and five NAC-treated samples, with SE. $P < .01$. **(I)** MMP-9 activity averaged over five control samples and five NAC-treated samples, with SE. $P < .02$. In the inset, a representative gelatin zymography gel is shown with arrow indicating MMP-9 in control samples (C) and NAC-treated samples (N).

Chemical (Milan, Italy); and E-cadherin was from BD Biosciences (Milan, Italy). Dako Cytomation LSAB2 System-HRP (Dako Italia, Milan, Italy) was used to reveal antigens. After counterstaining with hematoxylin, sections were observed with a Zeiss Axioplan microscope. Evaluation of Ki-67 staining was performed by two blind independent observers, each counting 200 cells in 10 randomly chosen fields for each sample (n = 8 for both control and treated groups), and the Ki-67 labeling index was expressed in percentage. Matrix metalloproteinase (MMP) 9 activity was tested by gelatin zymography as previously described (15), and gelatinases activity was evaluated by using the image software Quantity One (VersaDoc; Bio-Rad Laboratories, Milan, Italy).

From frozen endometriomas, lysed by TissueLyser (Qiagen, Milan, Italy), total RNA was extracted in Trizol Reagent (Invitrogen, Milan, Italy) and 1 μ g—treated with 1 U DNase I (Invitrogen)—was reverse transcribed with 200 U SuperScript III Reverse Transcriptase (Invitrogen) in 20 μ L reaction volume. Specific primer sequences were designed by using Primer-Blast, available at <http://www.ncbi.nlm.nih.gov>: NM_013599.2 MMP-9, 5'-CTGTTCTGTGGCAGCGCA-3' and 5'-CGCCTGTAGGGCCAACCGTG-3'; NM_011198.3 prostaglandin-endoperoxide synthase 2 (COX-2), 5'-TCACCCGAGGACTGGGCCAT-3' and 5'-CCCGAAGCCAGATGGTGGCA-3'; and NR_003278.1 18S RNA, 5'-CGCGGTCTATTTTGTGGT-3' and 5'-AGTCGGCATCGTTTATGGTC-3'. The 18S RNA housekeeping gene was the internal control. Real-time quantitative reverse-transcription polymerase chain reaction was performed on an ABI Prism 7000 (Applied Biosystems, Monza, Italy) by 5-Prime RealMasterMix Sybr Rox 2.5 \times (Eppendorf, Milan, Italy) in a 25- μ L reaction volume containing 1 μ L cDNA out of the 20 μ L of RT reaction volume, and 0.6 μ L (10 μ mol/L) each primer. A duplicate and a negative control (no template) were assayed, and amplification specificity was confirmed by melting curve analysis. Relative transcript abundance was determined by the $2^{-\Delta\Delta CT}$ method (16). All data were analyzed as averages with standard error, and significance was evaluated through the *P* parameter.

Ectopic lesions, preferentially found on the peritoneum of abdominopelvic cavity, were morphologically cystic and vascularized: They consisted of fluid-filled cysts white, red, or occasionally brown in color. Histologic observations did not reveal differences between the control and NAC-treated groups for the relative abundance of fluid in lesions. On the other hand, a significant weight reduction of endometriomas (by about -60%), directly related to their size, was observed in the treated group ($n = 22$) compared with the control group ($n = 22$), with an average weight of 29 ± 4 g in NAC-treated samples versus 74 ± 9 g in control samples ($P < .01$; Fig. 1A). Treatment did not alter the number of lesions per animal (one per animal, except one control with two lesions) nor of endometriomas per lesion, with an average of 1.2 ± 0.1 . We found that reduction in endometrioma size was not related to variation in the relative content of fluid but instead to decreased cell proliferation: The labeling index of Ki-67 antigen, only expressed in nuclei of proliferating cells, decreased from $1.88 \pm 0.28\%$ in control to $0.87 \pm 0.11\%$ in NAC-treated (-54% ; $P < .01$). Our results fit earlier reports on in vitro and in vivo models, but where a beneficial effect was observed only after a much higher NAC dose (5–7).

Analysis of E-cadherin immunohistochemical detection demonstrated that a decreased proliferation was associated with a switch toward differentiation. In control samples ($n = 11$) E-cadherin staining was prevalently diffused all over cell cytoplasm, whereas in endometriomas from NAC-treated mice ($n = 10$) an intense staining was mainly detected along cell-cell borders (Figs. 1D and 1E). The staining at cell-cell borders shifted from 35% in control samples to 60% in NAC-treated samples, with a 67% increase. Of note, E-cadherin relocation from cytoplasm to cell membrane is considered to be a marker of the switch from a proliferating to a differentiating phenotype and, when present at the cell borders, E-cadherin is also considered an inducer of differentiation (8, 17).

We examined COX-2 and MMP-9 proteins and genes as markers of inflammatory and invasive phenotype relevant in endometriosis (18, 19). Relatively to control samples ($n = 9$), samples from NAC-treated mice ($n = 9$) showed a reduction in the immunohistochemical staining of the inflammation-related COX-2 protein (Figs. 1F and 1G), present in 33% of NAC-treated samples versus 67% of controls. Consistently, COX-2 gene expression decreased, with a relative mRNA expression of 0.12 ± 0.07 in NAC-treated samples ($n = 5$; $P < .01$; Fig. 1H). The activity of MMP-9 decreased by $>60\%$ (relative activity 0.38 ± 0.02 ; $n = 5$; $P < .01$; Fig. 1I), with a consistent reduction in the MMP-9 gene expression resulting, in NAC-treated samples, in a relative mRNA expression of 0.42 ± 0.07 ($n = 5$; $P < .01$; Fig. 1H).

On a whole, our results demonstrate that NAC reduces endometrioma mass, by switching cell behavior from proliferation toward differentiation, and decreases both tissue inflammation and cell invasiveness. The decrease in MMP-9 expression and activity also suits a possible decrease in recurrences and adds to the shift toward differentiation. Actually, a direct role of MMP-9 in cell-differentiation has been reported (20). All of the above effects were achieved by using a relatively low dose of NAC, lower than that previously used in similar models (5–7), and without apparent undesired effects. In the perspective of a clinical treatment of endometriosis, the decrease in tissue inflammation predicts a beneficial effect of NAC in pain reduction, the first target of clinical treatments. The virtual lack of undesirable side effects, even after prolonged treatments, including interference with patients' fertility potential, envisages a potential use of NAC in the clinical treatment of endometriosis.

NAC therefore emerges as a thiol-containing compound whose beneficial effects in endometriosis are well beyond an eventual antioxidant capability, acting instead in the framework of the complex thiol redox signaling (13–14) and whose specificity must be still investigated.

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