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Effect of alpha-lipoic acid and myoinositol on endometrial inflammasome from recurrent pregnancy loss women

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Abstract

Problem: A significant increased expression/activation of one of the most well-characterized inflammasomes, the NAcht leucine-rich-repeat protein-3 (NALP-3), in the endometrium from idiopathic recurrent pregnancy loss women (RPL) has been previously found by our research group. We therefore, suggested this event as being one of the molecular mechanisms altering endometrial inflammatory status during early pregnancy. In the present research, we attempt to investigate whether molecules with anti-inflammatory activity, alpha-lipoic acid (ALA), and/or myoinositol affect the endometrial NALP-3 expression and activation.

Method of study: Women with a history of idiopathic RPL (n = 30) were included in the study and compared to a control group (n = 15). Endometrial tissues were collected by hysteroscopy during the mid-luteal phase. RPL women underwent a three-month prescription of tablets containing ALA plus myoinositol (Sinopol®). After treatment, hysteroscopic biopsies were repeated in RPL patients. Inflammasome expression was evaluated by immunohistochemical and Western blot analysis. NALP-3 activation was studied by quantifying the secretion of both caspase-1 and interleukin

Abbreviations: ALA, alpha-lipoic acid; IL, interleukin; NALP-3, NAcht leucine-rich-repeat protein-3; RPL, recurrent pregnancy loss.

Di Nicuolo and D'Ippolito contributed equally to this work.

(IL)-1ß and IL-18 through ELISA. In ex vivo experiments, the effects of each molecule on endometrial inflammasome were studied.

Results: Sinopol® significantly reduced the RPL endometrial inflammasome expression and activation. ALA, but not myoinositol, significantly reduced the endometrial inflammasome expression and activity.

Conclusion: Our data suggest a role for ALA on RPL inflammasome. Understanding the mechanisms involved in RPL and the observation that specific molecules are able to interfere with such complex at the endometrium might provide new rational design approaches to a personalized evaluation of endometrial status and, ultimately, a targeted medicine.

KEYWORDS

alpha-lipoic acid, inflammasome, myoinositol, recurrent pregnancy loss

1 | BACKGROUND

Recurrent pregnancy loss (RPL) is historically defined as three or more subsequent spontaneous pregnancy losses.[1,2] However, in 2012, the American Society for Reproductive Medicine Practice Committee and, more recently, the European Society of Human Reproduction and Embryology, "after a significant debate" tweaked the definition to the following: "a diagnosis of RPL could be considered after the loss of two or more pregnancies."[3-5] In reality, the new definition will have implications mainly for clinical research studies, and not for clinical practice. RPL occurs in about 2%-5% of clinically diagnosed pregnancies of reproductive-aged women. At present, accepted etiologies for RPL include parental chromosomal abnormalities, untreated hypothyroidism, uncontrolled diabetes mellitus, certain uterine anatomical abnormalities, antiphospholipid antibody syndrome, heritable and/or acquired thrombophilias, infections, environmental factors, and alterations in the quality of semen.[4-6] In addition, an increased risk of autoimmune and cellular immune abnormalities, such as an increased positivity for anti-nuclear (ANA) and/or anti-thyroid antibodies has been observed in RPL women.[7,8] After evaluation of these cases, approximately 30% of all cases of RPL remain idiopathic[4,9]: these cases represent, to date, the major challenge for researchers. An unsupportive endometrium is considered to be one of the key factors contributing to idiopathic RPL. During the early stage of pregnancy, the endometrium undergoes profound changes and develops into decidua, a newly formed tissue with a critical role for successful embryo implantation and regular fetal growth. The decidua provides a physical anchorage for the implanting trophoblast cells, which, in turn, being of fetal origin, represent a real stimulus for the maternal immune system.[10-13] Trophoblast invasion of decidua results in recruitment and activation of leukocytes and in the release of various cytokines, chemokines, and growth factors that promote tissue remodeling.[14,15] There is evidence for a dynamic balance between pro-inflammatory and anti-inflammatory mediators in normal pregnancy, with fluctuations between which signals predominate occurring during gestation.[14-16] Pro-inflammatory cytokines have been reported to induce the transforming growth factor-β (TGF-β),[17,18] able to stimulate endometrial secretion of matrix metalloproteinases (MMPs), main mediators of extracellular matrix turnover.[17-19] Embryo implantation shares, therefore, similarities with a "controlled" inflammatory process based on a highly regulated maternal immune response which, rather than having deleterious effects, helps promote fetal survival and allow normal progression of pregnancy.[14-16] A few studies have evidenced that an aberrant distribution and function in the physiological turnover of endometrial inflammatory molecules is associated with RPL: accordingly, an upregulated expression of pro-inflammatory cytokines has been reported in women with RPL.[15-27] Recently, we investigated the role of endometrial inflammasome in the RPL endometrium.[28] Inflammasome is a protein system representing the first line of defense against cellular stress: it is a crucial component of the innate immunity and, once recruited, enables the caspase-1-mediated proteolytic processing of pro-inflammatory cytokines, such interleukin (IL)-1β, IL-18, and IL-33.[29-32] The most well-characterized inflammasome is the Nacht leucine-rich-repeat protein-3 (NALP-3). NALP-3 can be activated by several stimulators through different signaling pathways, including, for example, reactive oxygen species (ROS) production and calcium mobilization.[33] We have recently found a significant increased expression/activation of NALP-3 inflammasome in the endometrium from idiopathic RPL and suggested this event as being one of the molecular mechanisms altering endometrial inflammatory status during early pregnancy.[28] As a subsequent step, we attempted to investigate whether molecules with anti-inflammatory activity might have an effect on the endometrial NALP-3. In this direction, in our research, we considered alpha-lipoic acid (ALA) and myoinositol.

Alpha-lipoic acid has been successfully used in patients with threatened miscarriage, even though the mechanisms by which it protects early pregnancy remain poorly understood.[34] What we know is that ALA is able to suppress the number and the percentage of T helper (Th) 1 and Th17 cells and the Natural Killer (NK) cell cytotoxicity and increase the splenic T-regulatory (T-reg) cells, involved in fighting excessive inflammation.[35] ALA also reduces the levels of pro-inflammatory cytokines such as tumor necrosis

factor (TNF)-α, ILs-1β, ILs-6, ILs-8, and ILs-17, interferon (INF)-ɤ as well as the production of vascular and intercellular cell adhesion protein-1 and MMP-9.[35,36] In addition, it induces the release of the anti-inflammatory IL-10 and the production of vascular endothelial growth factor.[37] By taking together all these observations, ALA seems to contribute strongly to counteracting many alterations involved in miscarriage pathogenic events.[34-37]

Myoinositol has been well studied for its involvement in biological processes, including insulin signal transduction, cytoskeleton assembly, and intracellular calcium concentration control.[38,39] By considering: (a) the anti-inflammatory effect of ALA, (b) the crucial role of calcium signaling on inflammasome activation,[35-37,40] and (c) the inositol-mediated effect on intracellular calcium concentration,[38,39] we intended to test the effects of such molecules on the endometrial NALP-3 and decided to consider a commercially available product containing both molecules (Sinopol®). To this end, we investigated the endometrial inflammasome expression and activity in idiopathic RPL women before and after treatment with a commercially available tablet containing both ALA and myoinositol. Then, to determine the role of each molecule, we performed ex vivo experiments on endometrial explants obtained from idiopathic RPL women.

2 | MATERIALS AND METHODS

2.1 | Patients

This study was performed at the Department of Woman and Child Health, Woman Health Area, Fondazione Policlinico Universitario A. Gemelli IRCCS, Università Cattolica del Sacro Cuore, Rome, Italy.

The study populations included 15 women with ≥2 previous uncomplicated term pregnancies (control group, CTR), and 30 women with history of idiopathic RPL with ≥3 spontaneous pregnancy losses (<12 weeks of gestation) clinically documented by ultrasonography and histopathology examination and no term pregnancies. The inclusion criteria for both groups were as follows: Caucasian, age ≤39 years, healthy, regular ovulatory cycles (28-32 days), serum levels of follicle-stimulating hormone <10 mIU/mL, luteinizing hormone <10 mIU/mL, and anti-mullerian hormone > 2 ng/mL on day 3 of the menstrual cycle, absence of abnormal ovarian and endometrial ultrasonographic findings. Exclusion criteria were as follows: use of any contraceptive drugs or intrauterine device in the last 6 months, use of anti-inflammatory, antibiotics, insulin-sensitizing drugs since the last 3 months before inclusion in the study; smoking, body mass index (BMI) ≥30, alcohol consumption, anatomical uterine abnormalities (endometrial polyps, uterine fibroids, and mullerian anomalies), length of the luteal phase <11 days, and/or serum progesterone levels on day 18, 21, and 24 of the menstrual cycle <10 pg/mL, hyperprolactinemia, insulin resistance,[41] serum thyroid-stimulating hormone >2.5 mIU/L, vaginal infections, hysteroscopic/histological evidence of chronic endometritis (CE),[42,43] parental karyotype abnormalities, previous numeric chromosome abnormalities of the product of conception, thrombophilic disorders, autoimmune disturbances (celiac disease, anti-thyroid, and anti-nuclear positivity), and anti-phospholipid syndrome.[14] All women gave their informed consent to use, anonymously, their data for research purposes, and the protocol was approved by the ethics committee of the Fondazione Policlinico Universitario A. Gemelli IRCCS, Italy (protocol number 36401/16 ID:1355).

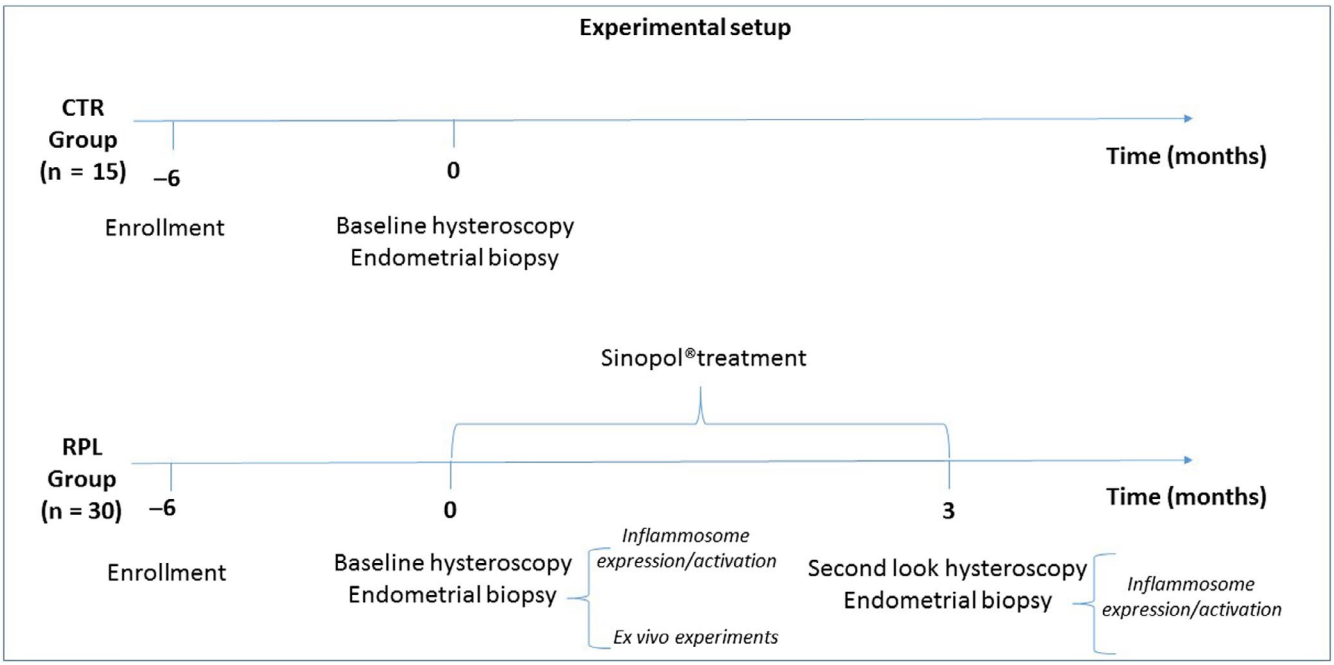

FIGURE 1 Experiment setup. Both control (CTR) and recurrent pregnancy loss (RPL) group underwent a first hysteroscopic biopsy at the baseline (time 0). The intervention consisted of a three months prescription of tablet containing 0.8 g of alpha-lipoic acid (ALA) and 1.0 g of myoinositol (commercially known as Sinopol®), twice a day to RPL women. To evaluate inflammasome endometrial expression after three months of treatment with ALA plus myoinositol, the RPL patients underwent a second-look hysteroscopic biopsy in the luteal phase

All the women were advised to avoid pregnancy in the months in which hysteroscopy was carried out.

Both CTR and RPL group underwent a first hysteroscopic biopsy at the baseline (time 0). The intervention consisted of a three months prescription of tablet containing 0.8 g of ALA and 1.0 g of myoinositol (commercially known as Sinopol®), twice a day to RPL women. To evaluate inflammasome endometrial expression after three months of treatment with ALA plus myoinositol, the RPL patients underwent a second-look hysteroscopic biopsy in the luteal phase (time 3; Figure 1). The adhesion to the prescription and possible side effects were registered through a weekly telephone call to participants made by a study coordinator throughout the study period.

2.2 | Endometrial samples

Both recurrent pregnancy loss women and the CTR group underwent hysteroscopic biopsy during the putative window of implantation (days 19th-24th) at baseline (Figure 1). The timing of the biopsy was dated according to the last menstrual period, monitoring the follicle size using a transvaginal ultrasound, the Luteinizing Hormone (LH) surge, and progesterone levels at LH + 7-LH + 11 days and was confirmed by histological assessment.[44] To evaluate inflammasome endometrial expression after three months of treatment with ALA plus myoinositol, the RPL patients underwent a second-look hysteroscopic biopsy in the luteal phase (Figure 1). Serum progesterone and β-hCG levels were determined in the day of the biopsy. Endometrial biopsies were performed using a 3-mm Novak curette for cultural, histological, and functional purposes. Extreme care was taken during endometrial sampling in order to avoid any contact between the curette and the vaginal walls. With regard to infectious agents, the presence of common bacteria such as Chlamydia trachomatis, Mycoplasma, Ureaplasma Urealyticum, Neisseria gonorrhea, and yeast was looked for. In case of detection of an infectious agent, an appropriate antibiotic treatment was prescribed and the related endometrial biopsies were excluded from the study. The samples were washed immediately in normal saline and stored at −80°C.

2.3 | Immunohistochemical analysis (IHC)

Paraffin sections (4 μm) were dewaxed in Histosol (Sigma Chemical Co) and rehydrated through descending grades of alcohol (95%-70%) to distilled water (dH_2O). Tissue histology was assessed following hematoxylin and eosin staining. At a minimum of three histological sections for each sample were assessed using 10x magnification and photographed using an Olympus BX50 (Olympus) photomicroscope. For IHC staining, paraffin sections were mounted on poly-L-lysine-coated slides. Endogenous peroxidase activity was blocked by incubation for 30 minutes with 0.3% (v/v) H_2O_2 in methanol. Tissue sections were subjected to antigen retrieval by boiling in 0.01 mol/L sodium citrate, pH 6, for 15 minutes in a microwave oven and incubated with the primary antibody (NALP-3, diluted 1:100) for 1 hour at room temperature. Slides were then incubated with biotinylated goat anti-rabbit antibody (diluted 1:250; Vector Laboratories) for 45 minutes washed and incubated with streptavidin-peroxidase (diluted 1:400; Immunotech, Beckman Coulter). Bound antibody was visualized by incubation with diaminobenzidine/H_2O_2. Slides were counterstained with hematoxylin and mounted. The nuclear positivity performed on histological samples was graded between 0 and 3+, and the 0 intensity was defined as negative, 1+ as positivity in less than 30% of cells, 2+ as positivity in more than 30 and <80% of cells, and 3+ as positivity in more than 80% of cells. For our convenience, we will report this arbitrary distribution in four categories (0, 1+, 2+, and 3+) of intensity of staining.

2.4 | NALP-3 expression: SDS-PAGE and immunoblotting

NAcht leucine-rich-repeat protein-3 expression was evaluated through Western blotting. For the analysis, total cellular lysates obtained from endometrial biopsies were separated by 10% SDS-PAGE electrophoresis under reducing conditions. After gel electrophoresis and transfer of proteins to a nitrocellulose membrane, nitrocellulose sheets were blocked at room temperature for one hour in 5% non-fat dry milk and incubated overnight at +4°C with a specific primary antibody (anti-NALP-3, ThermoFisher Scientific). The membranes were washed with PBST and incubated in specific horseradish peroxidase-conjugated IgG diluted 1:2000 in 5% non-fat dried milk in PBST. Bound secondary antibody was detected by chemiluminescence. Bands were analyzed with the use of a Gel Doc 200 Image Analysis System and quantified with the use of Quantity One Quantitation Software (both from BioRad). The level of NALP-3 was estimated vs the constant level of a 42-kDa protein present in the cytosolic extract (β-actin), which was identified with the use of a mouse monoclonal anti-human β-actin antibody (Sigma-Aldrich).

2.5 | NALP-3 activation: caspase-1, IL-18, and IL-1β immunoassays

To evaluate the NALP-3 inflammasome complex activation, the levels of caspase-1, IL-18, and IL-1β levels were measured in the lysates obtained from endometrial biopsies. To this end, an enzyme-linked immunoassay (ELISA) according to manufacturer's instructions (USCN Life Science Inc and Cloud-Clone Corp.) was used. Briefly, samples or standard (100 μL) was added to each well, coated with human monoclonal anti-caspase-1 or IL-18 or IL-1β antibodies. After 2 hours of incubation at 37°C, wells were washed and incubated with a specific enzyme-linked polyclonal antibody, horseradish peroxidase. Then, tetramethylbenzidine substrate solution was added to each well, and the color developed in proportion to the amount of the proteins bound in the initial step. The plate was read on a Titertek Multiscan plus Mk II plate reader (ICN Flow Laboratories) measuring the absorbance at wavelengths of 450 nm.

2.6 | Ex vivo experiments: endometrial explant culture

Endometrial biopsies from RPL patients at the baseline (time 0) were also used for the ex vivo experiments. One patient biopsy was used for each experiment. Endometrial fragments were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum and 100 IU/mL penicillin/streptomycin solution and treated with ALA (10 mmol/L), myoinositol (10 mmol/L), and ALA plus myoinositol (10 mmol/L) for 24 hours in an atmosphere containing 5% CO_2, air, and 100% relative humidity at 37°C.

2.7 | Statistical analysis

The sample was described through means and standard deviations (SD) in the cases of normally distributed variables. Median and interquartile range (IR) were used to present non-normally distributed variables. Qualitative variables were examined using absolute and relative frequencies. In order to detect differences between baseline RPL and CTR groups, *t* test or Mann-Whitney test was used. Differences before and after the treatment were detected by using paired *t* test or Wilcoxon matched pair test. Chi-square test (or Fisher's test) was used to detect any difference between categorical variables. Statistical analysis was carried out through STATA 13.0, and the *P*-value was set at 0.05.

3 | RESULTS

3.1 | Patients

The study population included 15 CTR women and 30 RPL women. The clinical characteristics of the patients are outlined in Table 1. Median age (IR), mean of the BMI, and absolute and relative frequencies of miscarriages or deliveries are reported for CTR and RPL women along with their differences. Our surveillance telephone calls revealed that 100% of the treated women took Sinopol® without any side effect.

3.2 | Endometrial samples

As previously reported, we evaluated inflammasome expression during the luteal phase (window of implantation, days 19-24). We obtained up to 98% of biopsies during the mid-luteal phase, and the remaining biopsies equally distributed during the early and late luteal phase. One patient biopsy was used for each experiment. We collected biopsies from both the CTR group (n = 15) and RPL group (n = 30) at the baseline (time 0). After a 3 months treatment with Sinopol®, RPL women repeated hysteroscopic biopsy (time 3). All biopsies were analyzed for NALP-3 expression and activation. NALP-3 expression was studied by Western blot analysis and immunohistochemical analysis; NALP-3 activation was evaluated by analyzing caspase-1 and IL levels by ELISA, as described above. For the ex vivo experiments, the biopsies obtained from RPL women included in the study were collected and analyzed.

TABLE 1 Clinical features of controls (CTR) and recurrent pregnancy loss (RPL) women

Characteristics of women	CTR (N = 15)	RPL (N = 30)	*P*-value
Age (y)	36 (32-38)	37.5 (37-30)	0.03
BMI (kg/m^2)	22.0 (2.25)	24.41 (3.00)	0.009
No. of spontaneous miscarriages			
3	0	16 (53.33%)	<0.001
4	0	8 (26.67%)	
≥5	0	6 (20.00%)	
No. of deliveries at evaluation			
2	12 (80.0%)	0	<0.001
3	2 (13.33%)	0	
≥4	1 (6.67%)	0	

Note: Median age (interquartile range), mean of the BMI (SD) and absolute and relative frequencies of spontaneous miscarriages and deliveries at evaluation are reported for both CTR and RPL along with their *P*-value.
Abbreviations: BMI, body mass index (kg/m^2); CTR, control; N, number; RPL, recurrent pregnancy loss.
P-values are indicated for the differences between groups.

3.3 | Immunohistochemical analysis

The immunohistochemical evaluation for NALP-3 showed the expression of the protein in epithelial glandular cells of human endometrium from fertile and RPL women. We noticed different (intensity and quantity) positive expressions in several stromal cells and neutrophils, particularly in the functional stroma around the glandular epithelium and a weak cytoplasm expression of NALP-3 (Figure 2A2). In particular, when subjectively quantifying the NALP-3 staining of the RPL group at baseline, we found that the glandular component generated a 2+ staining in 50% of the cases and a 3+ staining in 30% of the cases. The stromal component showed a 2+ staining in 50% of cases and 3+ staining in 20%, whereas the remaining cases were negative. After treatment with ALA plus myoinositol, we found that the glandular component generated a 2+ staining in 50% of the cases. The stromal component showed a 2+ staining in 20% of cases, whereas the remaining cases were negative. When analyzing the CTR biopsies, the glandular component showed a 2+ staining in 30% of cases and the stromal component a 2+ staining in 40% of cases; the remaining cases were negative for NALP-3 staining. A Fisher's exact test was performed to determine the statistical difference of NALP-3 immunostaining between the three groups of women. For statistical analysis of immunohistochemistry, the samples were grouped into negative (score < 2) or positive (score ≥ 2). We found a significant difference in glandular and stromal components between baseline RPL and control group (CTR; $P < 0.05$). In

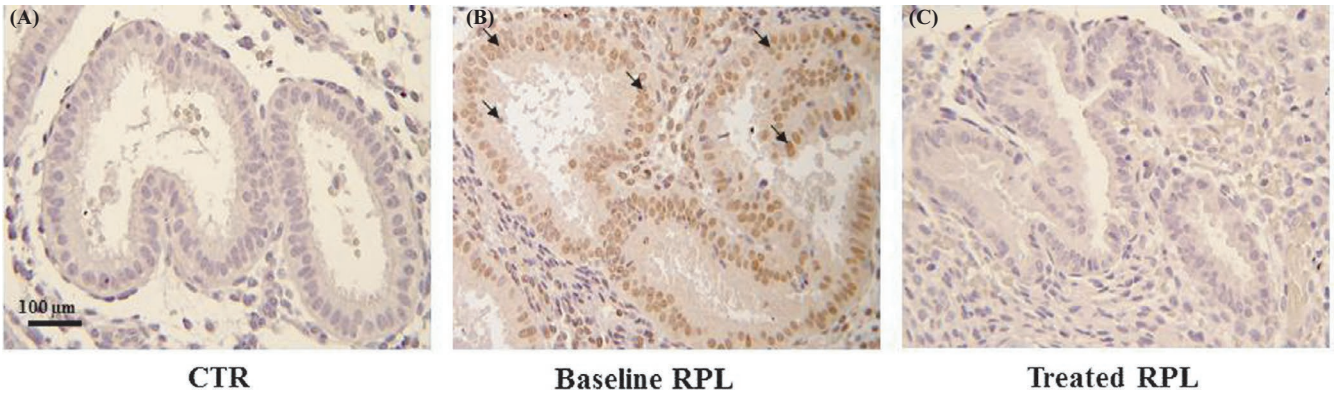

FIGURE 2 NAcht leucine-rich-repeat protein-3 (NALP-3) Immunohistochemical staining (IHC). Representative microphotographs of NALP-3 staining (scale bar = 100 µm). Three-µm sections of endometrial tissues obtained from fertile (control, CTR; A) and recurrent pregnancy loss (RPL) women at baseline (B) and after alpha-lipoic acid (ALA) plus myoinositol treatment (C) were stained with anti-NALP-3 antibody as described in Materials and Methods section. Endometrial tissues obtained from baseline RPL showed extensive NALP-3 staining (black arrows, B) with respect to CTR (A). Three months of ALA plus myoinositol treatment (C) reduces the endometrial staining of NALP-3 protein. (Original magnification 400x)

addition, ALA plus myoinositol significantly reduced the NALP-3 protein staining ($P < 0.05$; File S1).

3.4 | NALP-3 Expression

Through Western blot analysis of total endometrial cellular lysates, we found that, when compared to the control group (n = 15), the baseline RPL (n = 30) showed a higher expression of NALP-3 protein ($P < 0.001$). Furthermore, in endometrial RPL samples, treatment with Sinopol® significantly reduced endometrial NALP-3 expression when compared to baseline RPL ($P < 0.05$; Figure 3).

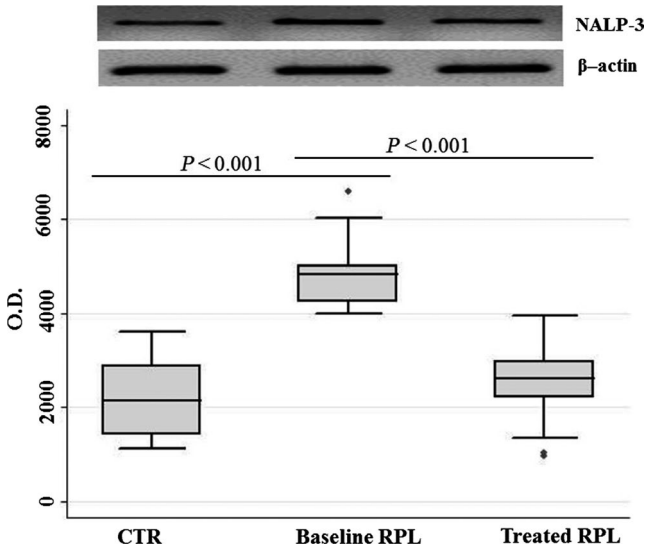

FIGURE 3 Box plot representation of NAcht leucine-rich-repeat protein-3 (NALP-3) expression in endometrial tissues analyzed by Western blot. Endometrial tissue lysates obtained from biopsies of women with uncomplicated term pregnancies (CTR) and women with idiopathic recurrent pregnancy loss (RPL) at baseline and after treatment. The levels of NALP-3 protein were compared to the constant level of β-actin (loading control) and are expressed as median values (interquartile range) of Optical Density (OD). A significant difference between baseline RPL and CTR was detected as well as between baseline RPL and treated RPL (both $P < 0.001$)

3.5 | NALP-3 activation: caspase-1 and cytokines levels

As shown in Figure 4, we found increased levels of caspase-1 (A; $P < 0.001$) and IL-1β (B; $P < 0.001$), but not of IL-18 (C; $P = 0.776$) in the endometrial tissues obtained from baseline RPL (n = 30) compared to the CTR group (n = 15). In RPL endometrial tissue, treatment with Sinopol® significantly reduced caspase-1, IL-1β, and IL-18 levels ($P < 0.001$ vs baseline RPL).

3.6 | Ex vivo experiments

In order to determine whether the restoring effect of ALA plus myoinositol on endometrial inflammation was dependent on the presence of ALA or myoinositol, we evaluated the effect of each molecule (alone or in combination) on NALP-3 expression. Endometrial biopsies, obtained from idiopathic RPL patients, were incubated with control medium, ALA, myoinositol, or ALA plus myoinositol as described in Materials and Methods. As shown in Figure 5, ALA and ALA + myoinositol treatment decreased the NALP-3 expression ($P < 0.05$); no changes in NALP-3 expression and activation were found in endometrial tissues in the presence of myoinositol alone.

Given the observation that only ALA affected the inflammasome expression/activity, in further experiments biopsies were treated with ALA (0.1-1-10 mmol/L) alone for 24 hours in an atmosphere containing 5% CO_2, air, and 100% relative humidity at 37°C. We found that ALA was able to reduce, in a dose-dependent manner, the expression of NALP-3 ($P < 0.05$; Figure 6), the activation of caspase-1, and the secretion of IL-1β ($P < 0.05$; Figure 7A,B).

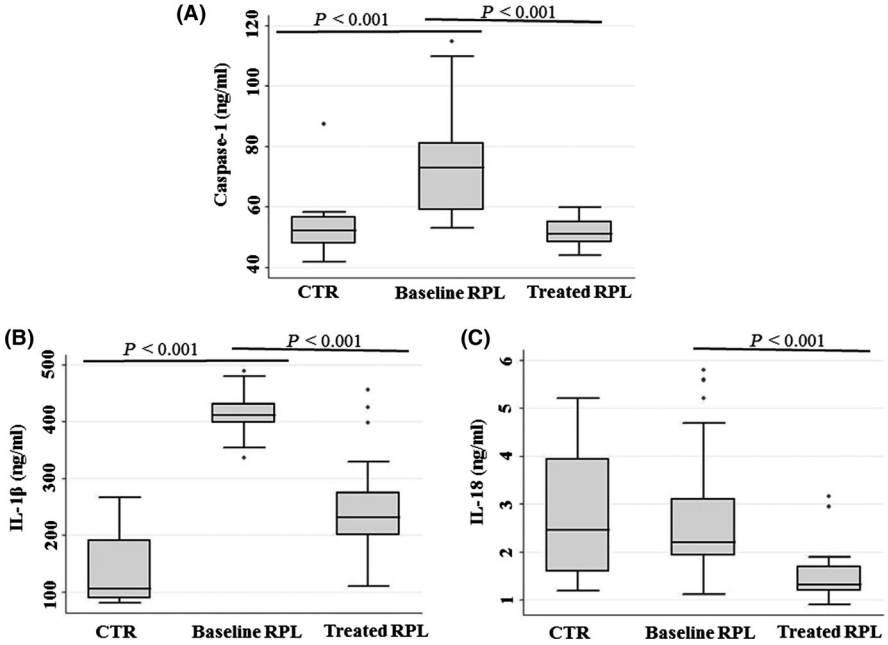

FIGURE 4 Inflammasome complex activation. Box plot representation: A, Caspase-1 levels in endometrial tissue lysates obtained from control group (CTR) or recurrent pregnancy loss (RPL), quantified by colorimetric ELISA. Results are median values (interquartile range) and are expressed as ng/mL of protein levels. As shown, significant difference between baseline RPL and CTR and between baseline RPL and treated RPL was found (both $P < 0.001$); B, interleukin (IL)-1β levels in endometrial tissue lysates obtained from CTR or RPL and quantified by colorimetric ELISA. Results are median values (interquartile range) and are expressed in as ng/mL of protein levels. As shown, significant difference between baseline RPL and CTR and between baseline RPL and treated RPL was found (both $P < 0.001$); C, IL-18 levels in endometrial tissue lysates obtained from CTR or RPL and quantified by colorimetric ELISA. Results are median values (interquartile range) and are expressed in as ng/mL of protein levels. As shown, no difference was found between CTR and baseline RPL ($P = 0.776$); significant difference between baseline RPL and treated RPL was found (both $P < 0.001$)

4 | DISCUSSION

The main finding of our research is that treatment of RPL women with Sinopol® (commercially product containing ALA + myoinositol) markedly inhibits endometrial expression of inflammasome NALP-3, caspase-1, and IL-1β ex vivo. To better determine the role of ALA and myoinositol on the endometrial inflammasome-dependent cascade, we analyzed in vitro the effects of ALA or myoinositol on endometrial explants obtained from idiopathic RPL women. Only ALA showed an inhibitory role on endometrial inflammasome, whereas no significant effects on NALP-3 levels, caspase-1, and IL secretion were observed in the presence of myoinositol.

The inflammasome component NALP-3 is the most versatile inflammasome subtype able to react to a multiplicity of potentially harmful biological and chemical agents.[29,30,45] It is the best characterized molecular trigger of IL-1β and IL-18 maturation/release, and its activation is due to different molecular mechanisms including ROS production and calcium mobilization. In the present research, we investigated the activity/expression of the inflammasome NALP-3 in the human endometrium by considering several steps and under different conditions. We firstly confirmed the expression of NALP-3 inflammasome in the human endometrium and its increased expression, and activation in endometrial tissues obtained from women with RPL when compared to a control group. Then, we intended to investigate the effect of molecules such as ALA and myoinositol, whose interference activity on various inflammatory processes has been well investigated.[34,36]

Alpha-lipoic acid is able to (a) suppress the number and percentage of Th1 and Th17 cells[35]; (b) impair the NK cell cytotoxicity[46]; (c) increase splenic T-regulatory (T-reg) cells count[47]; (d) reduce pro-inflammatory cytokines levels, such as TNF-α, IL-1β, IL-6, IL-8, IL-17, and INF-ɤ[46-50]; (e) induce the release of VEFG and of the anti-inflammatory IL-10[48,51]; and (f) decrease VCAM-1 and ICAM-1 and MMP-9 production.[52,53] All these activities may strongly contribute to counteract many alterations involved in miscarriage.

Myoinositol acts as a second messenger controlling the intracellular calcium mobilization. In addition, myoinositol is known for its positive effect on oocyte quality of women undergoing in vitro fertilization programs.[54-57] To date, we only have a few animal studies investigating the effect of myoinositol on the endometrium and no study on human endometrial tissues.[58]

In our study, we treated idiopathic RPL women with commercially available tablets containing ALA plus myoinositol (Sinopol®) for a three months period and also performed ex vivo experiments on endometrial explants from RPL women. NALP-3 levels appeared to be more heterogeneous in comparison to caspase-1 and ILs which showed larger variance. Possibly, it can be due to the small sample size and also to the fact that the activation of caspase-1 is the final result of multiple not mutually exclusive mechanisms that were not the subject of our research. We are still not able to explain why ex

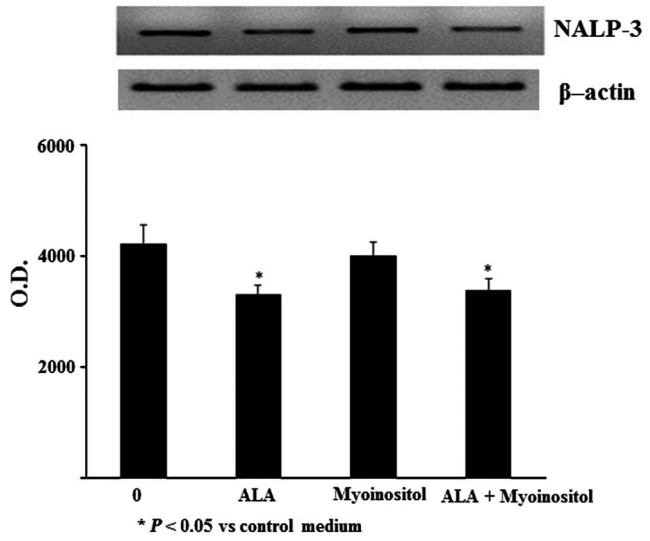

FIGURE 5 Alpha-lipoic acid (ALA) and/or myoinositol effect on endometrial NAcht leucine-rich-repeat protein-3 (NALP-3) expression. Endometrial biopsies obtained from recurrent pregnancy loss (RPL) women were exposed to ALA (10 mmol/L), myoinositol (10 mmol/L), and ALA plus myoinositol (10 mmol/L) and analyzed after 24 h of treatments. NALP-3 levels were quantified by Western Blot analysis and compared to the constant level of β-actin (loading control). Results are means ± SD of three independent experiments and are expressed as OD (Optical Density). *Statistical significance versus baseline RPL ($P < 0.05$)

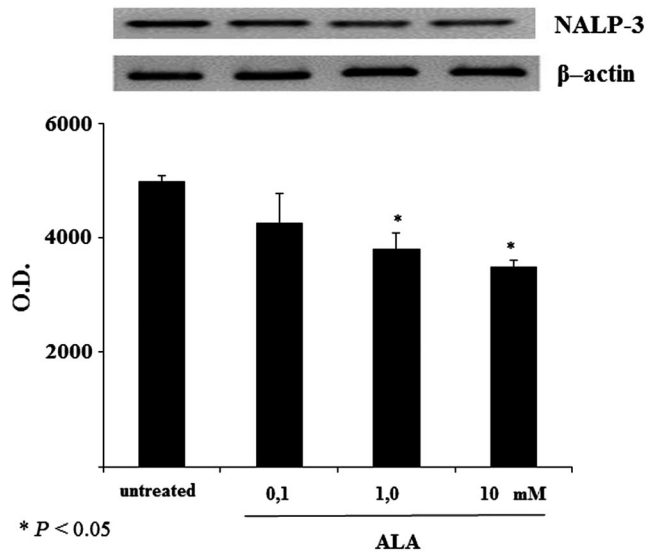

FIGURE 6 Alpha-lipoic acid (ALA) effect on endometrial NAcht leucine-rich-repeat protein-3 (NALP-3) expression. Baseline endometrial biopsies obtained from recurrent pregnancy loss (RPL) women were exposed to increasing doses of ALA (0-0.1-1.0-10 mmol/L) and analyzed after 24 h of treatments. NALP-3 levels were quantified by Western Blot analysis; the levels of NALP-3 protein expression were compared to the constant level of β-actin (loading control) and expressed as OD (Optical Density, means ± SD of six independent experiments). *Significant difference between treated versus baseline RPL biopsies ($P < 0.05$)

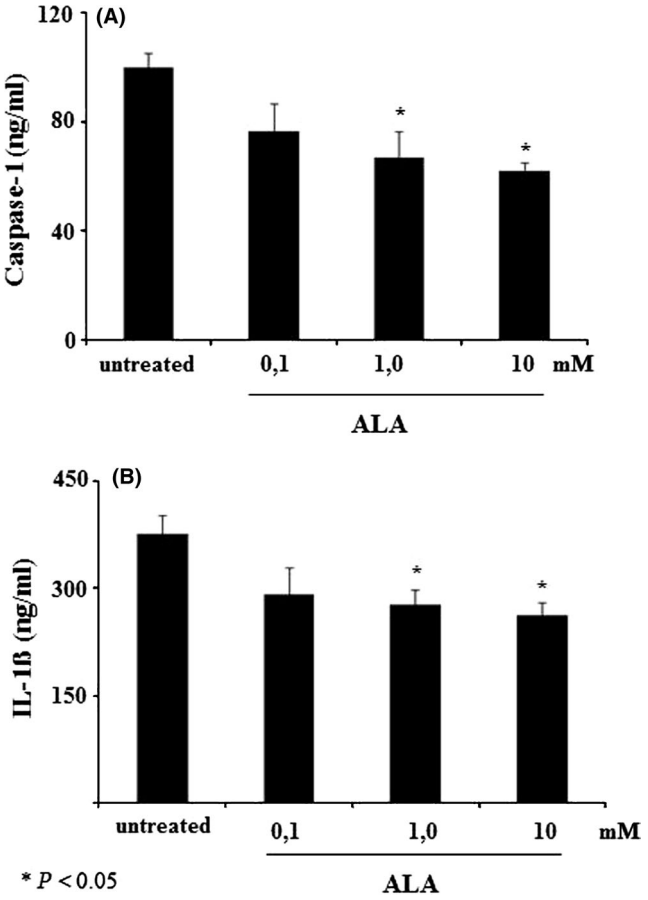

FIGURE 7 Alpha-lipoic acid (ALA) effect on endometrial NALP-3 activation: caspase-1 and IL-1β. Endometrial biopsies obtained from recurrent pregnancy loss (RPL) women at the baseline were exposed to increasing doses of ALA (0-0.1-1.0-10 mmol/L) and analyzed after 24 h of treatments. Caspase-1 and IL-1ß (A and B) were analyzed by colorimetric ELISA. Results are means ± SD of three independent experiments and are expressed as ng/mL of protein levels. *Significant difference between treated versus baseline RPL biopsies ($P < 0.05$)

vivo experiments showed an inhibitory role for ALA on endometrial inflammasome, while no significant effect was observed in the presence of myoinositol alone neither on NALP-3 levels nor on the activation of caspase-1 and ILs.

To our knowledge, this is the first study examining the role of ALA and myoinositol on the inflammasome system, suggesting the rational for its application in clinical practice. Imbalances in the immune system and failure to achieve immune tolerance to the fetus have been implicated as potentially modifiable causes of idiopathic RPL, as well as recurrent implantation failure. As such, in recent years, several trials evaluated the impact of immunomodulatory approaches in RPL women and reported borderline/significant benefits.[59-63] In addition, the methodological limitations of these studies and safety concerns prevent researchers to recommend these therapies in clinical practice.[64,65] Our results seem to suggest a promising role for ALA in the management of idiopathic RPL. We are aware

that a limitation of our study is the relatively small sample size and the lack of a placebo arm: further studies are needed to elucidate the effect of ALA on endometrial inflammation. In this regard, randomized clinical investigations are needed to evaluate the ability of ALA in restoring endometrial receptivity and improving the chance of a live-birth in idiopathic RPL women.

In conclusion, the pathogenesis of RPL is complex and after evaluation for known risk factors, approximately 30% of all cases of RPL remain idiopathic. RPL may involve not one but several disturbances or disruptions of the endometrial immune system: even subtle alterations in endometrial immune milieu have potentially a negative impact on the implantation process. Consistent with previous research,[28] our work confirmed the increased expression and activation of NALP-3 inflammasome complex in the human endometrium of women with RPL. Of interest, we found for the first time that ALA, a molecule with known anti-inflammatory activity, is able to interfere with such inflammatory pathway at the endometrial level. Understanding the endometrial mechanisms involved in RPL and the research on the effect of specific molecules able to interfere with such pathogenic mechanism may provide new rational design approaches to a personalized evaluation of endometrial status and, ultimately, a targeted medicine.

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CONFLICT OF INTEREST

All authors disclose any conflict of interest that could potentially influence the described research.

AUTHOR CONTRIBUTIONS

Study conception and design: FDN, NDS; executions of the experiments: EDR, FDN, RC; sample collection: MS, RC, VM; data acquisition: FDN, NDS; data analysis and interpretation: FDN, MM, NDS, SDI; writing of the paper: NDS, SDI; revision of the paper: AP, GS, NDS, SDI.

CONSENT FOR PUBLICATION

All subjects involved gave their informed consent to use, anonymously, their data for publication; all authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

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