RESEARCH ARTICLE

Nitric oxide as a key regulator of extracellular matrix modulation during ovarian follicle development

Namita Naskar, Shampa Sarkar Biswas*

ABSTRACT

Background: Extracellular matrix (ECM) remodeling is essential for maintaining the structural and functional integrity of ovarian follicles. The turnover of ECM is controlled by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Nitric oxide (NO), produced by follicular cells, contributes to follicular development and may influence ovarian ECM dynamics. The present study aims to determine the regulatory role of NO in the expression of ECM components during follicle development under an altered NO milieu. *Methods*: Adult female rats in the estrous phase were subjected to intrabursal injections of the nitric oxide synthase (NOS) inhibitor L-nitro-arginine methyl ester (L-NAME) or the NO donor sodium nitroprusside (SNP) to modulate *in-vivo* NO levels. Ovaries were collected at the subsequent estrous phase. Paraffin-embedded ovarian sections were stained with hematoxylin–eosin and Masson's trichrome for histological and collagen analysis. ECM-related proteins, including MMP-9, TIMP-1, RECK, and SPARC, were analyzed using immunohistochemistry and Western blotting. *Results*: L-NAME–treated rats exhibited a significant reduction in the number of freshly formed corpora lutea and preovulatory follicles compared to the control group. Collagenase activity was reduced in L-NAME–treated ovaries, accompanied by increased collagen deposition, indicating impaired ECM remodeling. Furthermore, MMP-9 and SPARC expression levels were reduced, whereas TIMP-1 and RECK expression levels were elevated in NO-inhibited ovaries. *Conclusion*: These findings indicate that NO is an important regulator of ovarian ECM remodeling as it adjusts the proportion of MMPs to their inhibitors. NO signaling disruption changes the ECM dynamics, which may adversely affect folliculogenesis and luteal activity, influencing female reproductive health and fertility.

Keywords: Nitric oxide, Extracellular matrix, Folliculogenesis, Ovulation, Matrix metalloproteinases

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INTRODUCTION

Nitric oxide (NO) is a short-lived, highly reactive gaseous signaling molecule that plays a critical role in regulating a wide range of cellular and organ functions in the body. In mammals, NO is synthesized intracellularly by Nitric oxide synthase (NOS), which oxidizes the amino acid L-arginine to generate L-citrulline and nitric oxide in the presence of NADPH and oxygen. Three distinct isoforms have been identified: neuronal NOS (nNOS, NOS1), endothelial NOS (eNOS, NOS3), which are constitutively expressed, calcium-dependent enzymes responsible for producing physiological levels of NO, and inducible NOS (iNOS, NOS2), which is calcium-independent and generates large amounts of NO, in response to inflammatory cytokines. In addition to enzymatic synthesis, NO can also be generated non-enzymatically via nitrite reduction, mainly under ischemic conditions.[1,2]

NO exerts its biological effects primarily by binding to the Fe–heme moiety of soluble guanylyl cyclase, leading to the production of cyclic GMP, which regulates gene expression, inflammation, vascular smooth muscle relaxation, and angiogenesis. NO also readily reacts with protein containing iron-heme, iron-sulfur clusters, and thiol groups.[1,3] At higher micromolar concentrations, NO forms reactive nitrogen species (RNS), which are associated with cellular stress and cytotoxicity. In the ovary, NO is produced locally by different cell types, including follicular cells and the ovarian vasculature, in rats, humans, and other mammals.[1] NO has been shown to regulate follicular development, oocyte meiotic maturation, and ovulation,[1,4-7] follicular apoptosis[4], fertilization, embryo development, and implantation.[8]

Ovarian development in rats is orchestrated through distinct phases of the estrous cycle, during which follicular development, ovulation, corpus luteum formation, and luteal regression occur as continuous and tightly regulated processes.[9] Extracellular matrix (ECM) degradation and reorganization, along with connective tissue remodeling, are essential for the progression of these reproductive processes. ECM remodeling is also a hallmark of angiogenesis and inflammatory responses. The ovarian ECM is composed of structural proteins, including laminin, collagen, plasminogen, and fibronectin. Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, are responsible for degrading ECM components. The activity of MMPs is tightly regulated by their endogenous tissue inhibitors (TIMPs), forming a coordinated network that governs structural

Department of Life Sciences, Presidency University, Kolkata, India

***Corresponding author:** Shampa Sarkar Biswas, Department of Life Sciences, Presidency University, Kolkata, India, Email: shampa.dbs@presiuniv.ac.in

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remodeling of ovarian extracellular architecture throughout the estrous cycle.[9-12] This MMP system is regulated by gonadotropins, steroid hormones, and growth factors, including TNF-α, IGF-1, TGF-β, IL-1β.[11,13]

MMPs are synthesized as latent pro-enzymes, in which a conserved cysteine residue in the pro-peptide domain interacts with a catalytic zinc ion to block substrate access. Activation of MMPs occurs through the 'cysteine switch' mechanism, involving disruption of the cysteine-zinc bond and exposure of the active site. Notably, NO can interact with both zinc ions and cysteine residues, suggesting that NO and RNS may directly regulate MMP activation and ECM remodeling during physiological ovarian events.

The expression and activity of MMP-2, MMP-9, MMP-14, and MMP-19 in follicular cells are linked to ECM degradation during follicular growth. CL formation is accompanied by gelatinolytic activity that facilitates cellular migration and neovascularization. During folliculogenesis, gonadotropins upregulate the expression of MMPs and TIMP-1.[14-16] In rats, increased TIMP-1 expression in the corpus luteum maintains proteolytic balance and ECM homeostasis by inhibiting excessive MMP activity.[16,17] MMP-9 activity has been reported to be biphasically regulated by nitric oxide through both guanylyl-cyclase-dependent and independent pathways in murine macrophages[18].

SPARC (Secreted protein, acidic and rich in cysteine) is a significant member of the extracellular modulators that bind to collagen[19–21]. SPARC expression has been detected in developing follicles and CL of the rat ovary[16,22]. It regulates the activity of growth factors like VEGF [20], TGF-β [23], and has been proposed as a potential inducer of MMP expression in gonadotropin-treated rat ovaries throughout the estrous cycle[16]. NO dependent regulation of SPARC synthesis has been demonstrated in rat mesangial cells[24]. RECK (Reversion-inducing-Cysteine-rich protein with Kazal motifs) is another key modulator of ECM integrity, which inhibits the proteolytic activity of MMPs[25,26], and has been suggested to function as a physiological inhibitor of MMPs during ovarian matrix remodeling[16].

Although NO is known to exert biphasic effects on ovarian physiology, its precise regulatory role during estrous-associated ECM remodeling remains unclear. To better understand the dual actions of NO in the ovarian function, the present study aimed to examine the effects of local NOS inhibition and NO generation on ECM remodeling during follicle development at the estrous phase, following completion of one estrous cycle *in-vivo*.

MATERIALS AND METHODS

Chemicals

L-NAME (L-Nitroarginine methyl ester), Poly L-lysine, radio-immunoprecipitation assay (RIPA) buffer, and sodium citrate were obtained from Sigma-Aldrich (St. Louis, MO, USA). SNP (Sodium Nitropruside), celestine blue, acid fuchsin, methyl blue, phosphomolybdic acid, Tween-20, Phosphate-buffered saline (PBS), bovine serum albumin (BSA), and 3,3'-Diaminobenzidine (DAB) were purchased from SRL, India. Hematoxylin, eosin, polyvinylidene fluoride (PVDF) membranes, hydrogen peroxide, and sodium dodecyl sulfate (SDS) were procured from Merck, India. NBT-BCIP from Promega. Antibodies: β-Actin (Biobharti, cat # BB-AB0024), MMP-9 (Biolegend, cat #819701), TIMP-1 (product no. #8946; Cell Signaling Technology), RECK (product no. #3433; Cell Signaling Technology), SPARC (product no.#5420; Cell Signaling Technology).

Animal Model Preparation

All animal experiments were conducted under the supervision of the institutional Animal Ethics Committee, in accordance with the guidelines of the Committee for the Control and Supervision of Experiments on Animals (CCSEA), Ministry of Culture, and the Government of India. All procedures were approved by the Presidency University IAEC, PU/IAEC/SSB/38 dated 26.02.2021. The animals were maintained under standard husbandry conditions, with controlled light (12-h light/dark cycle) and temperature (23±1^{0}C). Standard laboratory food and filtered tap water were provided *ad libitum*. Female Wistar rats were monitored daily for estrous cyclicity using vaginal lavage, and only rats exhibiting regular 4-day estrous cycles were included in the study. The rats were randomly assigned to three groups (n=5 per group). L-NAME and SNP were used as nitric oxide synthase(NOS) inhibitors and nitric oxide (NO) donors, respectively[27]. The doses and routes of administration were selected based on previously published studies[8,28]. All animals received a 50 µL intrabursal injection of L-NAME at 2.5 mg/mL, SNP at 100 µg/mL, or sterile saline (0.9% NaCl) as a control.

Tissue Samples

Both ovaries were collected from all groups during the subsequent early estrous phase following sacrifice. One ovary from each animal was processed for histological and immunohistochemical analysis, while the contralateral ovary was used for protein extraction and Western blot analysis (Figure 1).

Corpus Luteum (CL) Count

After dissecting out, the ovaries were washed in 1x PBS and examined under a stereozoom microscope (Dewinter). Large, red coloured, round follicles were counted as freshly formed corpora lutea, representing the current estrous cycle.

Histological Evaluation

Ovarian tissues were fixed in 10% neutral buffer formalin, dehydrated through graded alcohols, and embedded in paraffin. Paraffin blocks were serially sectioned at 5 µm thickness using a Leica rotatory microtome. Sections were mounted on glass slides and stained with hematoxylin and eosin (H&E). To avoid double-counting, every tenth or twelfth section was used for counting preantral and antral follicles

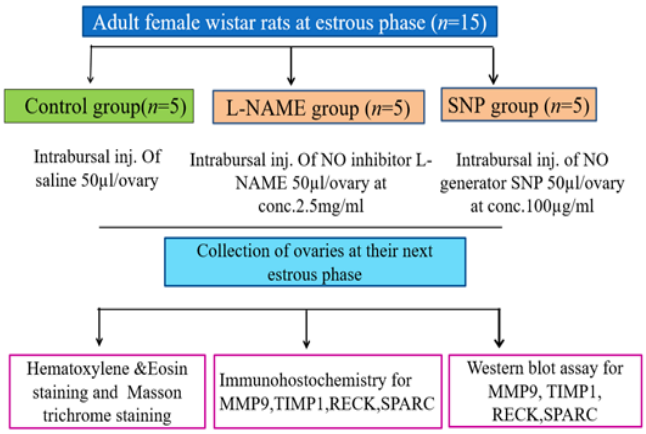

Figure 1: Schematic diagram for the experimental design. Fifteen adult Wistar rats (Body Weight 150-200 g) were divided into 3 experimental groups (N=15): Control, L-NAME, and SNP. Both treatments and ovary collection were performed during the estrous phase.

per ovary. Follicles were counted only if the oocyte and nucleus were clearly visible. Follicles surrounded by two or more layers of granulosa cells without an antral cavity were classified as pre-antral follicles, whereas follicles containing a visible antral cavity were classified as antral follicles.

Masson's Trichrome Staining

To assess collagen deposition, ovarian sections from each experimental group were stained using Masson's Trichrome. Stained slides were examined under a light microscope at 10x magnification. In this staining method, collagen fibers appear blue. Quantification of collagen deposition was performed using Image-J software, and results were expressed as the Masson's Trichrome-positive area.

Immunohistochemistry

Ovarian sections were deparaffinized in xylene, rehydrated through a series of decreasing ethanol concentrations, and rinsed in 1x PBS. Antigen retrieval was performed in sodium-citrate buffer (pH=6.0) at 88 to 90°C for 25 minutes, followed by gradual cooling to room temperature. Sections were washed in PBS-T (0.05% Tween-20 in 1x PBS) and endogenous peroxide activity was blocked with 3% hydrogen peroxide. After washing, sections were incubated with 5% bovine serum albumin (BSA) for 1 hour to reduce nonspecific binding. Sections were then incubated overnight at 4°C with primary antibodies diluted in PBS containing 5% BSA: rabbit polyclonal antibodies against MMP9 (1:100), and SPARC (1:100), or rabbit monoclonal antibodies against TIMP-1(1:100) and RECK (1:200). After washing with PBS-T, sections were incubated with HRP-conjugated secondary antibody (1:200). Immunoreactivity was visualized using DAB substrate, followed by counterstaining with hematoxylin. Sections were dehydrated, mounted with permanent mounting medium, and examined under a light microscope. Antibody specificity was validated based on the manufacturer's data and prior published studies. Negative controls were included in each experiment by omitting the primary antibody.

Western blotting

Ovarian samples were homogenized in RIPA buffer containing a protease inhibitor cocktail. Equal amounts of protein lysate were separated by 12% SDS-polyacrylamide gel electrophoresis and electro-transferred onto PVDF membranes. Membranes were blocked with 5% BSA for 1 hour at room temperature, followed by overnight incubation at 4°C with primary antibodies: β-Actin (1:1000), MMP-9 (1:1000), TIMP-1 (1:1000), RECK (1:1000), and SPARC (1:1000). After washing with 1x TBST (Tris-Buffered Saline with 0.05% Tween-20), membranes were incubated with alkaline Phosphatase-conjugated goat anti-rabbit IgG secondary antibody (1:1000) at room temperature for 2 hours. Membranes were washed three times with TBST, and protein bands were detected using NBT-BCIP substrate.

Statistical Analysis

All experiments were performed at least three times, and data are presented as mean ± SD. Statistical analysis was carried out using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to determine significant differences among groups. Results were considered statistically significant at $p < 0.05$.

RESULTS

Effect of NO on Follicle Numbers

Histological evaluation of adult rat ovaries at the estrous phase using H & E staining revealed significant differences in follicle numbers. The number of freshly formed CLs was significantly lower in L-NAME-treated ovaries compared to controls ($p < 0.05$; Table 1). In contrast, there was no significant difference in CL numbers between control and SNP-treated ovaries ($p > 0.5$) or between L-NAME and SNP-treated ovaries ($p > 0.19$). Similarly, the number of preantral follicles showed a significant decrease in L-NAME-treated ovaries compared to controls ($p < 0.05$; Figure 2). No significant differences were observed in the number of preantral follicles or antral follicles between control and SNP-treated ovaries or between L-NAME and SNP-treated ovaries. Morphological examination of freshly formed CLs in estrous revealed small, spindle-shaped luteal cells with basophilic cytoplasm, whereas

Table 1: Number of freshly formed Corpus luteum in the rat ovary.

Group	*No. of Fresh Corpus luteum per ovary*
Control	8.2 ± 1.3
L-NAME	6.6 ± 1.3a
SNP	7.6 ± 1.1

Data are collected from 10 ovaries from each group of rats. Data are presented as Mean ± SD. a indicates $p < 0.05$ compared to the control group.

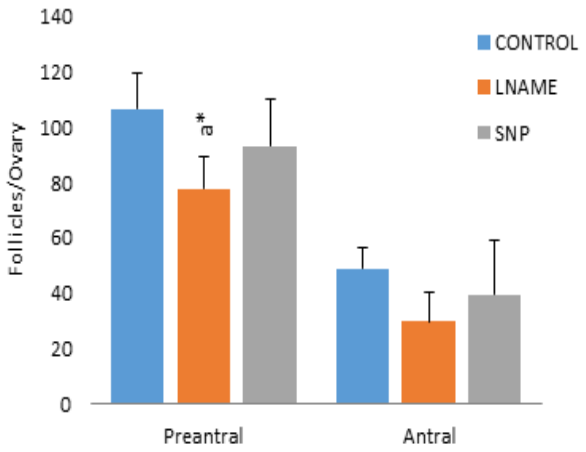

Figure 2: Role of NO on ovarian follicular development. Follicle counts in developmental (preantral and antral) stages in rat ovaries (n=5). The number of preantral follicles is significantly reduced in the L-NAME-treated group. Letter a represents statistically significant differences between the groups a=control vs. L-NAME; *p<0.05

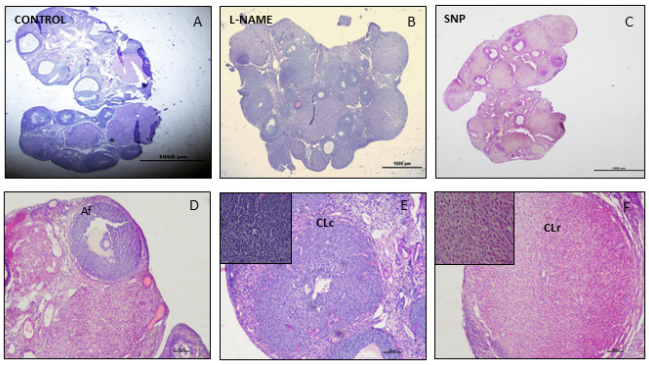

Figure 3: Histological sections of (A) Control, (B) L-NAME, and (C) SNP ovaries with H&E staining in 2x magnification. (D) antral follicle [Af] (E) CL of current cycle: characterized by small spindle-shaped luteal cells with basophilic cytoplasm (F) regressive CL of previous cycle: contains a necrotic area surrounded by macrophages and a fibrotic area in 10x magnification (40x magnification in box)

previously formed CLs displayed necrotic areas surrounded by macrophages and regions of fibrosis (Figure 3).

Effect of NO on Collagen Deposition

In control ovaries, Masson's Trichome staining revealed delicate collagen fibers in the extracellular matrix of the tunica albuginea and ovarian medulla with normal density and distribution. L-NAME-treated ovaries exhibited increased density of collagen fibers within the medulla, whereas SNP-treated ovaries showed normal collagen distribution and deposition similar to controls. Quantitative image analysis of Masson's Trichome- stained sections demonstrated that collagen deposition was significantly higher in L-NAME-treated ovaries compared to controls ($p < 0.05$). No significant difference in collagen deposition was observed between control and SNP-treated ovaries (Figure 4).

Effect of NO on the Localization of MMP and their Modulators

Immunohistochemical analysis revealed that MMP-9 was primarily localized in the theca layer, corpus luteum (CL), oocytes, and stromal cells of the ovary at the estrous phase. In L-NAME-treated ovaries, the MMP-9- positive area was significantly reduced compared to controls. TIMP-1immunoreactivity was observed in the stromal region, theca cells, and CL, with a significantly higher positive area in L-NAME ovaries relative to controls. Similarly, RECK expression was significantly increased in L-NAME-treated ovaries compared to controls (p <0.05). No significant differences in SPARC expression were observed among the three groups (Figure 5).

Effect of NO in Expression Level of MMP9, TIMP1, RECK and SPARC

Immunoblot analysis revealed that the fold change of pro-MMP-9 protein was significantly greater in L-NAME-treated ovaries compared with both control and SNP-treated groups (p<0.05). In contrast, levels of active MMP-9 were significantly reduced in both L-NAME and SNP-treated ovaries relative to control ovaries. TIMP-1expression was significantly increased

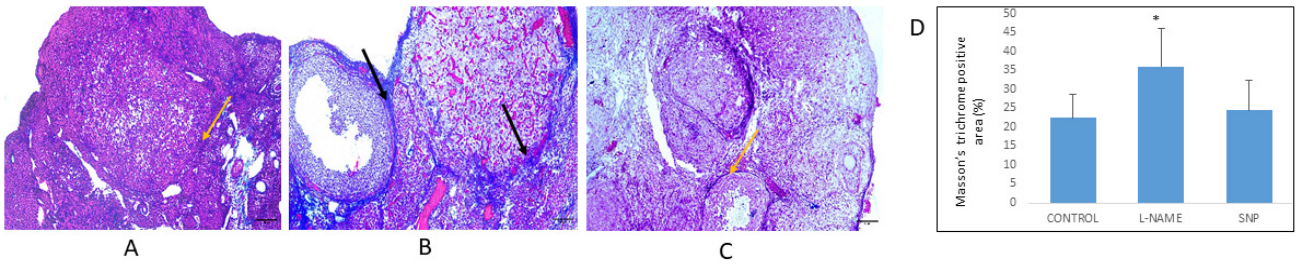

Figure 4: Effect of NO in collagen deposition by Masson's Trichrome staining in ovaries (n=5). (A) control and (C)SNP ovaries showed moderate collagen deposition (Yellow arrow), Significantly increased collagen deposition (black arrow) was observed in (B) L-NAME ovary (10X magnification). In (D), a higher % of Masson trichrome-positive area in L-NAME depicts a greater extent of collagen deposition in the ovary. The percentage of Masson trichrome-positive area was analyzed by ImageJ software (*p <0.05 vs. control).

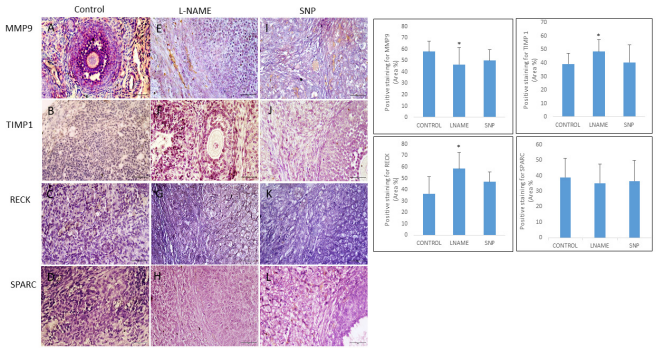

Figure 5: Immunohistochemical localization of MMP9, TIMP1, RECK, and SPARC in the ovary (*n*=5). (A, B, C, D) control (E, F, G, H) L-NAME and (I, J, K, L) SNP treated ovary with MMP9, TIMP1, RECK, SPARC, respectively (40x magnification). The percentage of DAB-positive area was analyzed using ImageJ. (**p* <0.05 vs. control)

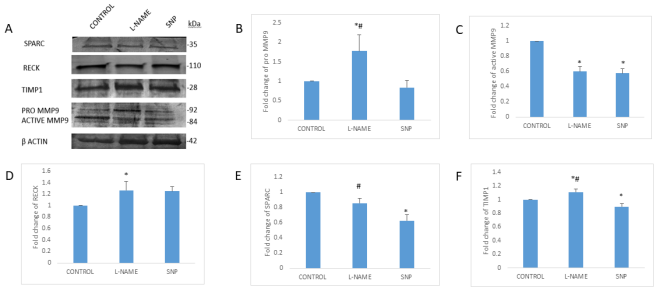

Figure 6: Western blot analysis of ECM protein expression in the ovary. (A)Representative western blots of ECM proteins. β-actin was used as a loading control. (B-F) Densitometric quantification of ECM proteins normalized to β-actin. The results are presented as fold changes relative to the control. Data were presented as mean ± SD from three independent experiments (*n*=3). Pro MMP9 (B), Active MMP9(C), RECK(D), SPARC (E) and TIMP1(F) in ovary. (**p*< 0.05 vs. control), (# *p*< 0.05 vs. SNP)

in L-NAME-treated ovaries compared with both control and SNP-treated groups, and a significant difference in TIMP-1 levels was also observed between control and SNP-treated ovaries. RECK protein expression was significantly elevated in L-NAME-treated ovaries compared to controls. Conversely, SPARC expression was significantly decreased in SNP-treated ovaries compared with both control and L-NAME-treated ovaries (Figure 6).

DISCUSSION

The role of nitric oxide (NO) in ovarian physiology has been investigated for more than three decades, and the cumulative evidence has established its critical involvement in multiple aspects of reproductive function. In the present study, we examined the effects of local modulation of NO on ECM remodeling and follicular development during the estrous phase, one cycle following intrabursal administration of an NOS inhibitor (L-NAME) or a NO donor (SNP), without exogenous gonadotropin (PMSG/hCG) stimulation. The present study demonstrates a critical role of nitric oxide (NO) in regulating folliculogenesis, luteal dynamics, and extracellular matrix (ECM) remodeling in the adult rat ovary. Inhibition of NO synthesis by L-NAME markedly altered ovarian structure and function, whereas NO donation *via* SNP largely preserved normal ovarian architecture, highlighting the importance of endogenous NO in maintaining ovarian homeostasis.

Ovulation in rats occurs during the estrous phase and is initiated by the LH surge, which triggers a cascade of events including follicular wall weakening, oocyte extrusion, and subsequent CL formation. This process requires the coordinated action of proteolytic enzymes such as matrix metalloproteinases (MMPs), which degrade follicular connective tissue and permit follicle rupture[29]. Nitric oxide production is an important physiological feature of normal ovulatory cycles, with its levels fluctuating across the estrous cycle to regulate follicular growth, ovulation, and luteinization[30]. Importantly, the effects of NO are concentration-dependent and often dualistic, exerting either stimulatory or inhibitory actions on follicular processes[4].

During follicular development, NO levels rise in parallel with increasing estrogen concentrations, a relationship observed both in the systemic circulation and in follicular fluid, particularly in dominant follicles [31]. Genetic studies further support the importance of NO signalling in cyclicity, as endothelial NOS knockout mice exhibit prolonged estrous cycles, whereas inducible NOS deficiency does not significantly affect cycle length [32]. Consistent with these findings, our data demonstrate that local inhibition of NO synthesis via L-NAME significantly reduces the number of freshly formed CLs and preovulatory follicles during estrus. In contrast, NO generation through SNP treatment did not significantly alter CL numbers or follicle counts compared to controls. The decreased number of CLs observed following NOS inhibition may therefore reflect disrupted ovulatory processes or compromised luteal formation. In contrast, the absence of significant changes in follicle numbers in SNP-treated ovaries indicates that exogenous NO supplementation does not adversely affect folliculogenesis under physiological conditions but rather supports normal ovarian function.

Reactive oxygen species (ROS) production within the ovary is essential for LH-induced cumulus expansion and ovulation, and antioxidant treatment has been shown to suppress these events[33]. Several studies have demonstrated the direct involvement of NO in ovulation; inhibition of NO synthesis blocks gonadotropin-induced follicular rupture and reduces ovulation rates in rats[28]. Similarly, continuous administration of L-NAME throughout the estrous cycle has been reported to impair follicular growth and delay hCG-induced ovulation in mares[34], effects that can be reversed by NO donors such as SNP [28]. Conversely, excessive NO production has also been

shown to be detrimental, leading to reduced ovarian size and ovulation rates through disruption of signaling pathways, including the Ron receptor tyrosine kinase, in immature mice[35]. These findings highlight the requirement for tightly regulated NO levels to maintain normal ovarian function.

Structural remodeling of the ovarian ECM, particularly within the tunica albuginea and thecal layers, is essential for ovulation and CL formation. As follicular development progresses, ECM degradation occurs through the action of collagenases, plasminogen activators, plasmin, and other proteases[28]. In the present study, collagen deposition was significantly increased in L-NAME-treated ovaries, consistent with reduced collagenase activity under NO-deficient conditions. The increased collagen content observed here is consistent with reduced ECM degradation and supports the concept that NO deficiency promotes a profibrotic ovarian environment. The normal collagen distribution in SNP-treated ovaries further reinforces the protective role of NO against abnormal ECM accumulation. Supporting this observation, inhibition of NO synthesis has been shown to increase collagen expression in human peritoneal fibroblasts[36]. Additionally, ovulation is associated with increased vascular permeability and leukocyte infiltration; as a potent vasodilator, NO plays a crucial role in regulating ovarian blood flow and vascular dynamics during follicular events[28]. L-NAME-mediated NO depletion likely disrupts these processes, contributing to impaired follicular rupture and CL formation.

At the molecular level, gonadotropins regulate the expression of both NOS isoforms (iNOS and eNOS) in the ovary. Disruption of NO signaling can arrest follicular growth at early stages by altering estrogen production. TNF-α- induced MMP-9 expression has been shown to promote degradation of the ovarian epithelial basement membrane during ovulation[13]. Immunohistochemical and immunoblot analyses revealed that NO imbalance profoundly affects the MMP system. Although pro-MMP-9 levels were elevated in L-NAME–treated ovaries, the active form of MMP-9 was significantly reduced, suggesting impaired activation rather than synthesis. This finding is further supported by the marked upregulation of TIMP-1 and RECK, both potent inhibitors of MMP-9 activity. Increased expression of these inhibitors likely suppresses MMP-9 activation, thereby reducing ECM degradation and leading to collagen accumulation. These molecular alterations provide a mechanistic explanation for the fibrotic changes observed in L-NAME–treated ovaries. A discrepancy was observed between immunohistochemical and western blot data for SPARC expression: no significant differences among groups were observed in IHC, but differences were observed in immunoblot data. This divergence may be attributed to the semi-quantitative nature of IHC, the heterogeneous stromal localization of SPARC, and its extracellular distribution, which may mask moderate changes in protein abundance that are more readily detected by western blotting. Previous studies using combined immunohistochemistry and western blot analyses in ovarian tissue demonstrate that spatial localization detected by IHC does not always align with the quantitative changes seen in western blotting.[7,37] Also discrepancy between the mRNA and protein levels for ECM modulators at the ovulatory phase was observed.[16] Further studies are needed to investigate the underlying mechanisms or reasons behind the observed discrepancy following NO modulation.

Interestingly, active MMP-9 levels were also reduced in SNP-treated ovaries despite normal collagen deposition, suggesting that NO may finely regulate MMP-9 activity to maintain ECM balance rather than promote excessive matrix degradation. The lack of significant changes in SPARC localization among groups, coupled with reduced SPARC protein expression in SNP-treated ovaries, suggests that SPARC may be less sensitive to NO depletion but responsive to elevated NO levels. Given SPARC's role in ECM organization rather than degradation, its downregulation in SNP-treated ovaries may represent a compensatory mechanism to prevent excessive tissue remodeling. SNP treatment decreased both pro- and active MMP9 levels, consistent with excess NO-mediated inhibition of MMP-9 expression and interference with activation via the cysteine switch and catalytic site.[38-40] Excess NO has previously been shown to inhibit interleukin-1β-stimulated MMP-9 induction via superoxide-ERK-dependent pathways in vascular smooth muscle cells.[41] Additionally, decreased NO concentrations have been associated with reduced MMP-1 and MMP-2 expression in infertile cows.[42]

Luteolysis of the corpus luteum is accompanied by extensive remodeling of the ECM and inflammatory responses, processes in which NO plays a central regulatory role. Through modulationof cyclic GMP and cyclic AMP signaling pathways, NO also inhibits estrogen production in rat granulosa cells.[43]

Collectively, these findings indicate that NO is essential for coordinating follicular development, luteal maintenance, and ECM remodeling in the ovary. NO deficiency disrupts the balance between MMP-9 and its inhibitors, leading to reduced matrix turnover, increased collagen deposition, and compromised ovarian function. In contrast, NO supplementation preserves ovarian structure and molecular homeostasis. These results highlight NO as a key regulator of ovarian physiology and suggest that altered NO signaling may contribute to ovarian pathologies associated with fibrosis and impaired fertility.

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CONFLICT OF INTEREST

Nil

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal procedures were performed in accordance with the Committee for Control and Supervision of Experiments on Animals (CCSEA) guidelines for the Humane Care of Laboratory Animals. All procedures were approved by the Presidency University IAEC, PU/IAEC/SSB/38 dated 26.02.2021.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.