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Potential Effects of Selenium and N-Acetylcysteine Supplementation in Ameliorating Cardinal Symptoms of Nω-Nitro-L-Arginine Methyl Ester Hydrochloride (L-NAME) Induced Preeclampsia in Wistar Rats

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Abstract

Background: Preeclampsia (PE) is a hypertensive disorder in pregnancy affecting multiple organ systems. This study hypothesized that oxidative stress and inflammatory responses contribute to the pathogenesis of Preeclampsia, and that selenium and N-acetylcysteine (NAC) could mitigate these effects.

Methods: The study was initiated after approval from the Institutional Animal Ethics Committee. Twenty-four female Wistar rats were divided equally into four groups. Group I served as controls, while Groups II, III, and IV received Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) to induce hypertension from day 10 to 20 of gestation. Additionally, Group III received selenium (240 µg/kg/day) and Group IV received NAC (160 mg/kg). On day 20, Blood Pressure (BP) monitoring and urine protein estimation were carried out to assess hypertension and proteinuria, while blood samples were collected to measure malondialdehyde (MDA) and interleukin-6 (IL-6) levels, as markers of oxidative stress and inflammation, respectively. Statistical analysis was performed using GraphPad Prism 10.2.

Results: Selenium improved L-NAME-induced hypertension (Mean BP 107.63±5.22 mmHg vs 140.9±8.38 mmHg in disease control (DC) and proteinuria (65.5±4.09 vs 140.2±11.85 mg/day in DC) and significantly reduced the inflammatory response (IL-6 23.4±1.06 vs 50.63±3.35 pg/mL in DC) but had little effect on oxidative stress (MDA 0.21±0.02 vs 0.24±0.02 nmol/mL in DC). NAC did not lower BP (Mean BP 129.33±7.96 mmHg) but significantly reduced proteinuria (92.7±6.37mg/day), IL-6 levels (18.24±0.42 pg/mL), and oxidative stress (MDA 0.16±0.01 nmol/mL).

Conclusions: These findings suggest that selenium and NAC play distinct protective roles in the pathophysiology of preeclampsia, potentially offering synergistic effects for cardiovascular and kidney health in hypertensive pregnancies.

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Introduction

Preeclampsia (PE) is a hypertensive disorder that manifests during pregnancy, affecting multiple organ systems and contributing to maternal and perinatal morbidity and mortality, affecting approximately 2-8% of pregnancies globally and 8% of pregnancies in India (1). This underscores the urgent need for effective risk stratification, preventive and therapeutic strategies. However, the mechanisms and mediators of PE are not well understood. PE has been classified as a relatively abrupt onset placental disease/ syndrome, which is characterized by abnormal placentation in early stages, followed by a maternal syndrome (2). The two-stage model of PE emphasizes the role of dysregulated and exaggerated inflammatory and oxidative damage response (3). Oxidative stress is marked by heightened generation of reactive oxygen species (ROS); in PE, the placenta produces excessive amounts of ROS, triggering endothelial dysfunction and ultimately leading to the clinical manifestations of the disease (4). Considering the above facts, there has been growing interest in the potential therapeutic strategy of utilizing antioxidants to mitigate the detrimental impacts of ROS in PE. By neutralizing ROS, antioxidants can restore balance and minimize the harmful effects of oxidative stress (5).

Several studies have investigated the use of various antioxidants in PE, such as Vitamins C, E, B12, and B6, as well as copper, zinc, iron, calcium, folic acid, selenium, and N-Acetylcysteine (NAC) among which selenium and NAC have s demonstrated the most significant potential (6,7). Selenium, a trace element and an essential component of several enzymes, has been implicated as a potent antioxidant. Studies have reported significantly lower circulating selenium levels in individuals with preeclampsia, further supporting the potential role of selenium supplementation in managing PE (8). NAC, a precursor of glutathione, one of the body's primary antioxidants, has been demonstrated to have potent antioxidant effects and is used in various clinical conditions characterized by oxidative stress, including PE (9).

While previous studies have established the antioxidant effects of selenium and NAC, no in vivo comparative studies evaluating these antioxidants have been conducted. Evidence from the literature highlights that Malondialdehyde (MDA) and Interleukin-6 (IL-6) are key circulating biomarkers of choice to evaluate the severity of oxidative stress and inflammation. MDA, a by-product of lipid peroxidation, is elevated in numerous pathological conditions, including PE (10). Similarly, IL-6 is a key pro-inflammatory cytokine implicated in the pathophysiology of PE, is often found at elevated levels in affected individuals (11).

This present study aims to assess the oxidative stress and inflammatory status by measuring the levels of malondialdehyde (MDA) and interleukin-6 (IL-6), respectively, in Wistar rats with Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME)-induced preeclampsia. It further seeks to compare the oxidative stress and inflammatory responses between untreated rats and those pre-treated or supplemented with selenium and NAC.

Materials and Methods

Experimental animals

The study commenced following approval from the Institutional Animal Ethics Committee (Approval No. 94/PO/ReBi/S/99/CPCSE) under the guidelines of the Committee for the Control and Supervision of Experiments on Animals (CCSEA). Animals were procured from the Central Animal Facility of the Institute. A total of twelve male and twenty-four female albino Wistar rats, aged 8–10 weeks and weighing 200–250 grams, were used for the study. The animals were housed under standard conditions, 12:12 12:12-hour light-dark cycle, 50% relative humidity, and 28 °C.

They were provided with standard food granules and water ad libitum.

Twenty-four female Wistar rats were randomly divided into four groups, with six rats in each group, as outlined in Table 1. Twelve male albino Wistar rats were used for mating. Before the experiment, the rats were acclimatized to the laboratory conditions for one week. For mating, two female and one male rat were housed together in a cage overnight. The following morning, vaginal smears were examined microscopically for the presence of spermatozoa to confirm successful mating. The day spermatozoa were detected in the vaginal smear was designated as gestational day 0. Upon pregnancy confirmation, each rat was weighed and placed in a metabolic cage for 24-hour urine collection. The male rats were then returned to the animal facility. Group I served as the control group. Groups II, III, and IV were
administered L-NAME (70 mg/kg/day; Sigma-Aldrich, Germany) from gestation at 10 to day 20 to induce preeclampsia. In addition:

- Group III received selenium supplement (240 µg/kg/day; Sigma-Aldrich, Germany) from gestational day 0 to day 20.
- Group IV received NAC (160 mg/kg/day; Sigma-Aldrich, India) on gestational day 20.

On day 20 of gestation, the rats were sacrificed, and approximately 1.5 mL of blood was collected from each animal. Serum was then separated and stored for biochemical estimations.

Table 1. The division of the groups (n = 6 in each group) with their details of L-NAME, Selenium and NAC dosage and duration.

Groups	Dosing details
Group I (CONTROL)	Control group: pregnant rats fed with standard food pellets and water.
Group II (CONTROL-PE)	Disease control group: Induced with L-NAME (70 mg/kg/day) orally (day 10 -20 of Pregnancy).
Group III (PE-Se)	Sodium selenite(240 µg/kg) with water orally during gestation (from day 1). L-NAME (70 mg/kg/day) orally (day 10 - 20 of Pregnancy).
Group IV (PE-NAC)	N-Acetyl Cysteine (160 mg/kg/day) with 0.9% NaCl during gestation (from day 1). L-NAME (70 mg/kg/day) orally (day 10 - 20 of Pregnancy).

Blood pressure measurements

In our study, we observed that in most of the groups, approximately two rats exhibited outlier blood pressure values that were significantly different from the rest of the group. These outliers were likely attributable to stress-induced variability associated with the tail-cuff method. To ensure the accuracy and reliability of our data, we excluded these outliers and reported the blood pressure values based on the remaining four rats per group. This approach allowed us to present a more consistent and representative assessment of blood pressure for each group.

Blood pressure was measured using the CODA non-invasive throughput blood pressure system, which is capable of accurately and noninvasively measuring systolic and diastolic blood pressure in mice, rats, and other rodents, along with heart rate and other blood flow parameters, thereby proving to be highly valuable in clinical research (15). The non-invasive blood pressure measurement method involves placing an occlusion cuff on the tail to restrict blood flow temporarily. As the occlusion cuff is gradually deflated, a sensor records the pressure of the flowing blood. In contrast, a second cuff incorporating the Volume Pressure Recording (VPR) sensor measures the physiological characteristics of the returning blood flow. The VPR sensor cuff assesses the swelling of the tail caused by arterial pulsations as the blood returns. The initial appearance of tail swelling indicates the automatic measurement of systolic blood

pressure (SBP). Once the rate of tail swelling ceases to increase, the automatic measurement of diastolic blood pressure (DBP) is obtained. The animal to be tested was gently placed into the holder, ensuring minimal force, and the rear gate of the holder was securely closed, allowing the animal's tail to extend beyond the hatch. The nose cone at the front end was adjusted to provide comfort to the animal while simultaneously restricting its movement to some extent. The holder containing the restrained animal was then placed on an infrared heating pad, ensuring the animal's tail rested on the pad for warming. The occlusion cuff was carefully inserted through the animal's tail and passed upwards until it encountered slight resistance, ensuring it sat close to the base of the tail and fit snugly but not too tightly. Subsequently, the VPR cuff was inserted into the tail and moved towards the occlusion cuff. Measurements from the VPR sensor can be taken from any part of the tail as long as an adequate section is inside the VPR cuff. Before commencing blood pressure measurements, the infrared thermometer was used to measure the temperature at the base of the tail, ensuring the temperature ranged between 32 and 35 degrees Celsius. Once the required tail temperature was obtained, the "start CODA" button was clicked to initiate the pressure recording measurements. The CODA software, connected to a desktop or laptop computer, monitored the blood pressure by observing pressure fluctuations in the VPR sensor and occlusion cuff during inflation and deflation. It presented the blood pressure readings as a waveform. For each animal, 15 cycles of inflation and deflation were performed, with the first five cycles serving as acclimatization cycles and the subsequent ten cycles considered as actual blood pressure recordings.

Evaluation of proteinuria

This investigation sought to determine the scope of urine sampling and assess the necessity of housing animals in metabolic cages for a complete 24-hour duration. Urine specimens were obtained from subjects from all 4 groups on days 4, 8, 12, and 16 of gestation over 24 hours, starting at 8:00 AM and concluding at 8:00 AM the following day, while the animals were maintained on a standard dietary regimen. Urine protein was estimated using the Biuret method. Urinary protein was precipitated using ice-cold ethanol and phosphotungstic acid, and its content was determined by a biuret assay. The protein concentration, measured by UV absorbance, was estimated from a standard curve of BSA at 540 nm (16).

Malondialdehyde assay (MDA) and IL-6 measurements

Malondialdehyde assay (MDA) was measured manually (Kei Satoh's Method) with the serum sample collected on day 20. MDA is the end-product of lipid peroxidation by reactive oxygen species. It is therefore used as a biomarker for the oxidative stress of an organism. MDA levels are estimated by the Thio barbituric acid (TBA) (Sigma-Aldrich, Switzerland) reaction. Serum Lipid peroxide is measured by precipitating lipoproteins with trichloroacetic acid (pH 2–3) and boiling with Thio barbituric acid, which reacts with MDA, forming an MDA-TBA to get a pink colour. The pink-coloured complex was cooled to room temperature and measured by using a spectrophotometer at 532 nm. Concentrations of MDA were expressed in nmol/mL (17).

To measure the IL-6 level, a Sandwich ELISA kit was used (ELISA MAX Deluxe Set Human IL-6, BioLegend, San Diego, USA).

Histopathology

At the end of the study, the placenta was dissected and stored in 10% formalin. Then, the placental bed cross-section was used for histological examination using paraffin-embedded specimens. The specimens were then stained with haematoxylin and eosin. The slides were then seen under the LX-500 LED trinocular research microscope (Labomed), and images were taken with the MiaCam CMOS AR 6 pro microscope camera connected to image AR pro software.

Statistical analysis

Results are reported as the mean ± standard error of the mean. Differences in outcomes were determined using one-way ANOVA and Tukey's post hoc tests for paired comparisons and were considered significant when P< 0.05. All statistical analyses were performed using GraphPad Prism version 10.2. Unpaired Student's t- test was used when one- way ANOVA showed a trend but was not significant for blood pressure changes.

Results

On the 16th day, blood pressure, MDA, IL-6, and extent of proteinuria were assessed to determine the extent of PE among different groups. Table 2 shows the comparison of target analytes/ parameters between different groups.

Table 2. Mean ± SEM for systolic BP, Diastolic BP, Mean BP, 24-Hour Urine Protein, MDA and IL-6 of all the groups.

Parameters (n = 6)	Units	Control	Control-PE	PE-Se	PE-NAC
Systolic BP	mmHg	133.57±5.33	156.91±7.99	124.99±6.45	142.86±6.32
Diastolic BP	mmHg	95.16±6.43	124.9±8.77	90.26±4	115.79±9.59
Mean BP	mmHg	114.36±5.88	140.9±8.38	107.63±5.22	129.33±7.96
24-Hour urine protein	mg/day	47.3±2.25	140.2±11.85	65.5±4.09	92.7±6.37
MDA	nmol/mL	0.13±0.01	0.24±0.02	0.21±0.02	0.16±0.01
IL-6	pg/mL	4.67±1.13	50.63±3.35	23.4±1.06	18.24±0.42

Blood pressure of L-NAME-induced rats

The L-NAME-induced rats showed moderate increase in their SBP compared to the control group. On the 16th day of Gestation, the preeclamptic group showed elevated SBP when compared to the control group. Rats on selenium supplementation during gestion (PE-Se) showed significantly lower SBP when compared to preeclamptic group while, NAC administration had lesser effect in decreasing the blood pressure (Fig. 1A and Table 2). Similarly, significantly lower DBP was recorded in rats supplemented with selenium supplementation when compared to disease control and healthy control groups (Fig. 1B and Table 2). Assessment of mean BP showed similar findings with the group on selenium supplementation showing significantly lowered mean BP (Fig. 1C and Table 2).

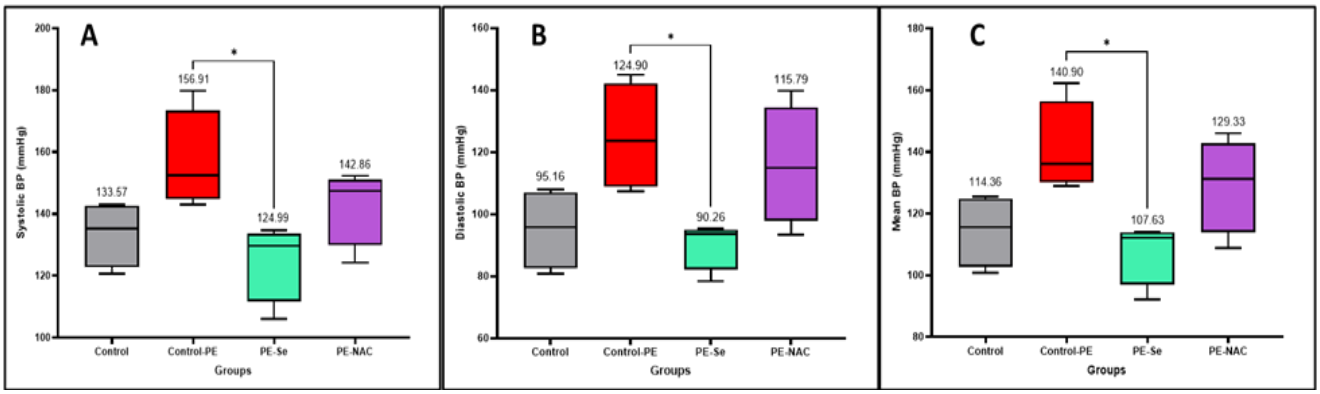


Fig. 1. Effects of selenium and NAC on L-NAME induced Systolic BP (Fig. 1A), Diastolic BP (Fig. 1B) and Mean BP (Fig.1C) in Control group, Control-PE, PE-Se and PE-NAC. Tukey's multiple comparisons test was performed, and the results are reported as Mean ± SEM.
The * indicates P< 0.05.

24-hour Urinary protein

Urinary protein excretion was higher in the disease group compared to healthy controls, while both the treatment groups (PE-Se: 65.5 ± 4.09 mg/day and PE-NAC: 92.7 ± 6.37 mg/day) showed significantly ($p< 0.001$ and $p< 0.0001$, respectively) lower proteinuria when compared to the disease control (Fig. 2 and Table 2).

Effect of Selenium and NAC on Oxidative Stress

Malondialdehyde assay (MDA) levels (assessed on Day 20 of gestation) were significantly lower in controls compared to disease control ($p< 0.001$), while rats supplemented with selenium (PE-Se: 0.16 ± 0.01 nmol/mL) showed lower MDA (non-significant) and supplementation with NAC showed statistically significant reduction ($p< 0.01$) compared to disease control with MDA levels closer to healthy controls (Fig. 3 and Table 2).

Selenium and NAC on Inflammatory Response

Serum IL-6 was significantly elevated in disease controls when compared to healthy controls ($p< 0.0001$), while both treatment groups (PE-Se: 23.4 ± 1.06 pg/mL; PE-NAC: 18.24 ± 0.42 pg/mL; $p< 0.0001$) showed significantly lower IL-6 (Fig. **4** and Table 2).

Effect of Selenium and NAC on the Placenta

The histopathological estimation of the placental spiral artery suggested that the spiral artery diameter was smaller in Group II dosed with L-NAME compared to that of controls and the treatment groups (Fig. 5).

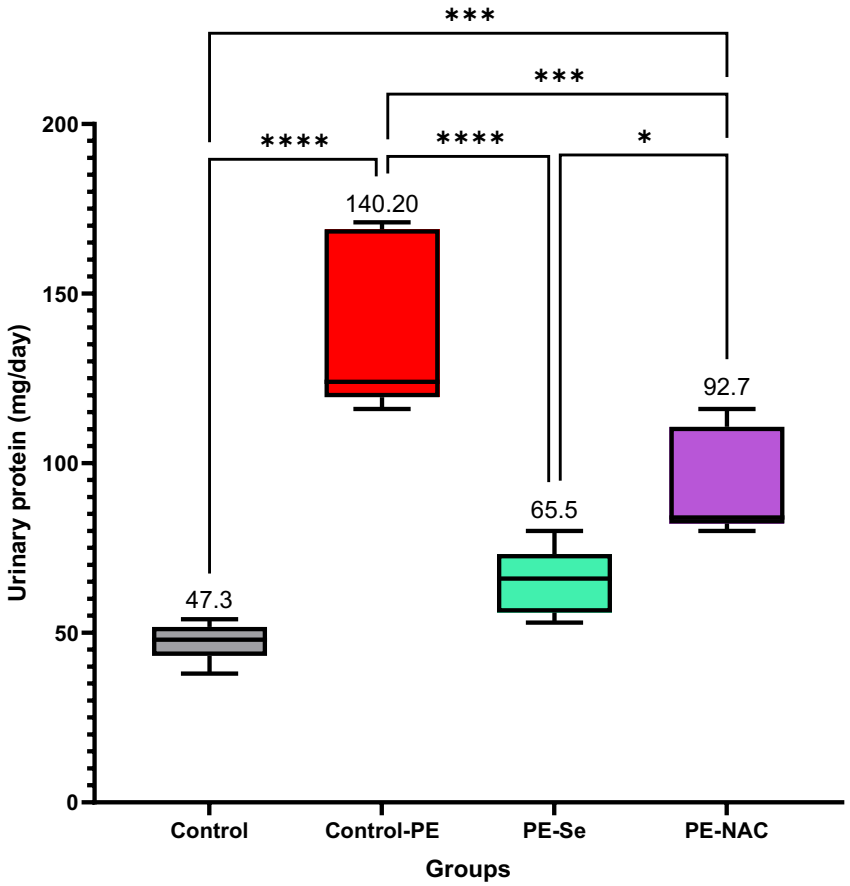


Fig. 2. Effects of selenium and N-acetylcysteine on L-NAME induced proteinuria in the Control group, Control-PE, PE-Se and PE-NAC. Tukey's multiple comparisons test was performed, and the results are reported as Mean ± SEM. The *, ***, and **** indicate $P< 0.05$, $P< 0.001$, and $P< 0.0001$, respectively.

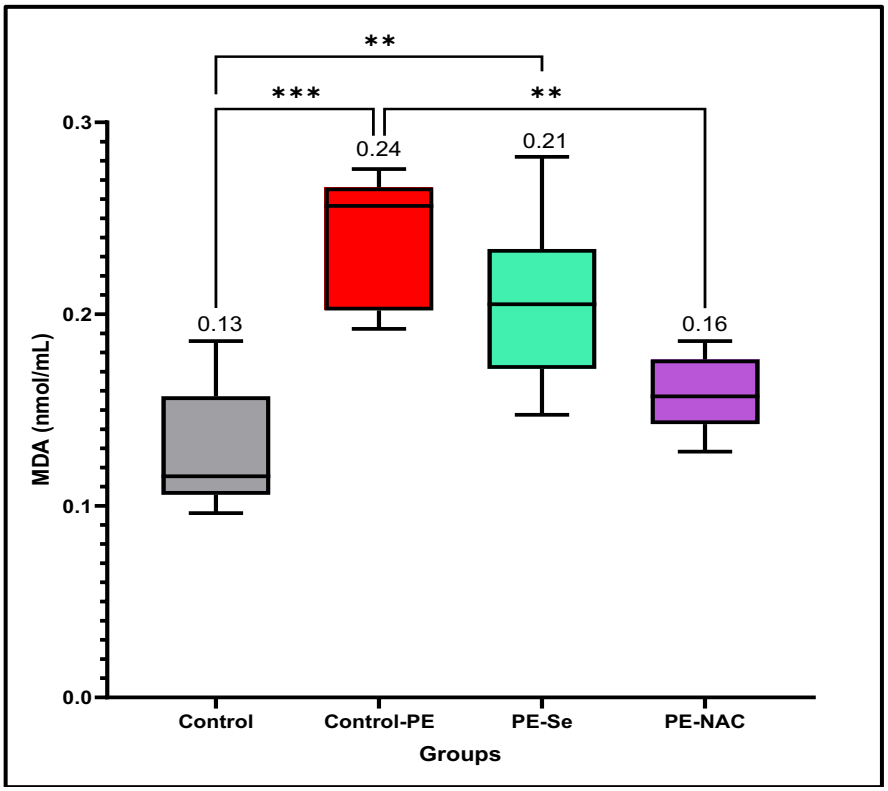


Fig. 3. Effects of selenium and N-acetylcysteine on oxidative stress in the Control group, Control-PE, PE-Se and PE-NAC. Tukey's multiple comparisons test was performed, and the results are reported as Mean ± SEM.
The *, **, ***, and **** indicate P< 0.05, P< 0.01, P< 0.001, and P< 0.0001, respectively.

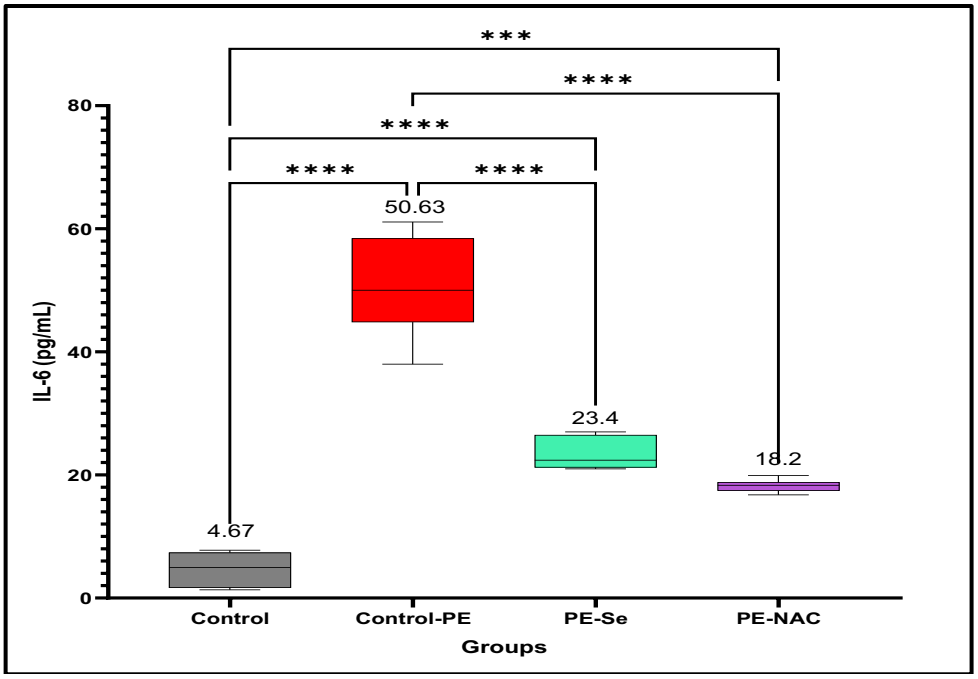


Fig. 4. Effects of selenium and N-acetylcysteine on inflammatory response in the Control group, Control-PE, PE-Se and PE-NAC. Tukey's multiple comparisons test was performed, and the results are reported as Mean ± SEM.
The *, ***, and **** indicate P< 0.05, P< 0.001, and P< 0.0001, respectively.

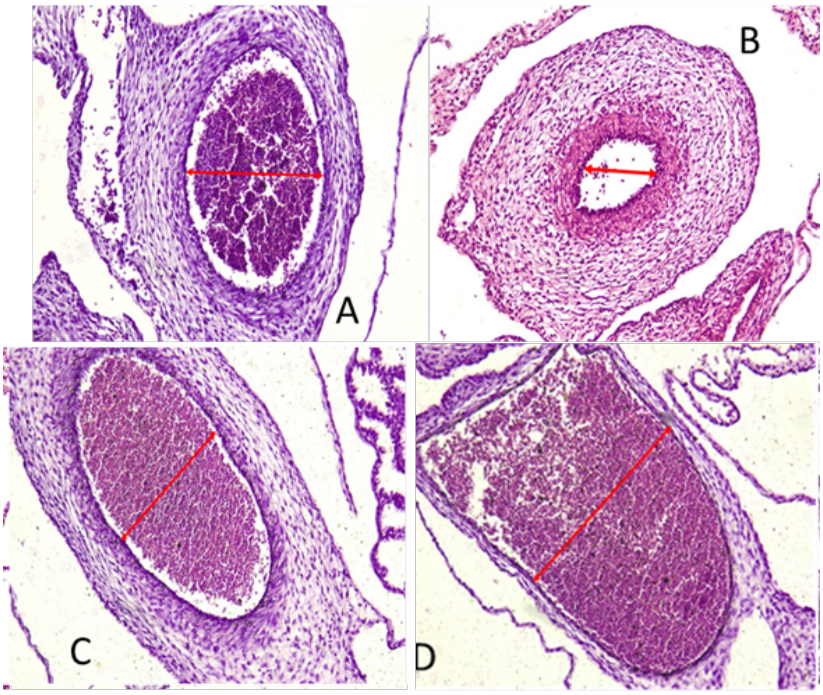


Fig. 5. Slides are stained with (from left to right) haematoxylin and eosin (H & E). Representative images of (A) a spiral artery from a normotensive control, and the arrow indicates the diameter of the spiral artery; (B) a spiral artery of the Diseased control group; (C) spiral artery of the selenium-supplemented group; (D) spiral artery of NAC NAC-supplemented group.

Discussion

Preeclampsia (PE) is considered to be an inflammatory systemic vascular disorder of pregnancy affecting multiple organ systems (primarily cardiovascular and neurological), resulting in considerable maternal, fetal, and neonatal morbidities and fatalities (18). It remains a sudden-onset, acutely progressing obstetric emergency with the exact mechanism and the pathophysiology of the disorder yet to be elucidated. However, the role of oxidative stress and inflammation remains central to the inception, progression, and prognostication of PE, and the current study explores the efficacy of Selenium and NAC supplementation in improving the inflammatory and oxidative stress-induced damage using an *in vivo* model. The study evaluates the effect of supplementation of selenium and NAC on the inflammation (IL-6) and oxidative stress (MDA) markers and cardinal clinical features (BP and proteinuria) of PE. The present study observed that L-NAME-induced rats receiving supplementation with selenium showed significantly lower SBP, DBP, and mean BP and significantly lower proteinuria compared to the disease control group. Circulating IL-6 was also observed to be significantly lower in the group supplemented with selenium, while lower MDA levels were observed in this group when compared to the disease control group. Similarly, it was observed that the intervention group supplemented with NAC showed significantly lowered circulating MDA, IL-6, and urinary protein excretion, while BP levels showed a marginal decrease (non-significant) when compared to the disease control group. Histopathological examination of the spiral artery showed increased diameter in both the treatment groups when compared to the disease control group.

Existing literature provides conflicting evidence on the role of antioxidant supplementation in combating hypertension. While antioxidants such as Vitamin C and Vitamin E did not demonstrate significant reductions in blood pressure, others like the superoxide dismutase mimetic tempol have demonstrated improvements in blood pressure,

endothelial function, vascular remodelling, and neuronal nitric oxide synthase (nNOS) activity (19–21). Previous research has indicated lower selenium levels in hypertensive pregnancies compared to normotensive pregnancies (22). Selenium acts as a cofactor for glutathione peroxidase; selenium deficiency during pregnancy impairs free radical scavenging, thus exacerbating placental dysfunction and pregnancy complications (23–25). N- Acetylcysteine (NAC), on the other hand, is a precursor to glutathione and exhibits antioxidant effects by directly scavenging free radicals and increasing glutathione levels, thereby preventing oxidative stress-related cellular damage (26). Studies have shown that NAC supplementation enhances uteroplacental blood flow, as demonstrated by ex vivo placental perfusion studies in preeclamptic placentas (9). Our study demonstrated the ameliorating effect of selenium on hypertension in preeclamptic rats; however, the effect of NAC supplementation was less pronounced. Conversely, NAC was more effective in reducing circulating MDA levels when compared to selenium supplementation.

In this study, urinary protein excretion was higher in the PE group compared to controls, with both selenium and NAC treatment groups showing significantly lower proteinuria compared to the preeclamptic group. Notably, selenium exhibited superior results over NAC in reducing proteinuria.

IL-6, a pivotal multifunctional cytokine of the immune-inflammation axis, is attributed to activated CD4 lymphocytes and deficient vascular remodelling, resulting in reduced placental perfusion and a hypoxic environment for placental and embryonic tissues (27,28). IL-6 also contributes to endothelial cell activation and vascular damage, further exacerbating the vascular syndrome, and is emerging as an important hallmark of the severity of PE (29). The present study reported significantly lower IL-6 levels in treatment/intervention arms when compared to preeclamptic rats; NAC demonstrated better efficacy than selenium in reducing IL-6. The above results are concordant with previous findings. Selenium has been inversely associated with IL-6 levels, and studies in the ApoE atherosclerosis mouse model have reported reduced IL-6 levels upon selenium treatment (30). The mechanism by which selenium suppresses IL-6 production is thought to involve its effect on STAT-3 phosphorylation (31,32). Additionally, NAC has been shown to decrease serum IL-6 levels (33). The anti-inflammatory action of NAC is believed to occur through multiple mechanisms, including the inhibition of nuclear factor kappa-B (NF-κB), which suppresses inflammatory biomarker production. NAC may also directly inhibit inflammatory biomarkers such as TNF-α and ICAM-1 (34,35).

The study clearly shows the protective effect of selenium and NAC supplementation in PE and indicates the action of parallel signalling pathways exerting protective/therapeutic effects. However, we acknowledge that assessment of a panel of additional cytokines and exploring the expression of the placental indicators could provide a more comprehensive understanding of the inflammatory milieu in PE. Future studies can aim to incorporate a broader panel of cytokines and other relevant biomarkers to provide a more detailed characterization of the inflammatory response and the therapeutic potential of antioxidant treatments in PE. Furthermore, the present data are not confirmatory proof of the effects of selenium and NAC on the spiral artery; owing to the small sample size, it is difficult to draw statistically significant conclusions. A larger sample size would be necessary to determine the true effects and reduce variability. The above findings indicate the potential therapeutic benefits of selenium and NAC in managing oxidative stress and proteinuria in PE, with the potential to evaluate synergistic effects of coadministration of the nutraceuticals in exerting protection against and management of PE.

Our research emphasizes the beneficial effects of selenium and NAC supplementation

in ameliorating oxidative stress in in vivo models of PE. This study will contribute to our understanding of the therapeutic potential of these nutritionally important antioxidants in PE and may pave the way for future clinical trials.

Ethical Consideration

The study commenced after the institutional animal ethics committee approval 94/PO/ReBi/S/99/CPCSE by the Committee for Control and Supervision of Experiments on Animals (CCSEA).

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Conflict of interest

The authors declare that they have no conflicts of interest.

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