

Subchronic Effects of Ethinylestradiol and Levonorgestrel on Hematological Parameters, Cytokine Signaling, and Oxidative Stress in Wistar Rats

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Published: 15 August 2025

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Abstract

Background Ethinylestradiol/levonorgestrel is a widely used contraceptive combination with various formulations and administration regimens. There is a hypothesis that the subchronic use of oral contraceptives containing EE and LNG will alter the blood profile by modifying cytokine signaling pathways and increasing oxidative stress levels, potentially contributing to systemic inflammation. The study aimed to investigate the subchronic effects of EE and LNG on hematological parameters, immune function, and oxidative stress in Wistar rats.

Methods Forty adolescent Wistar rats were divided into four groups (n = 10). The control (Group A) received only distilled water for 21 days. Groups B, C, and D were administered combined oral contraceptives (COC) containing EE and LNG at doses of 0.03 mg/kg and 0.15 mg/kg, respectively, for durations of 7, 14, and 21 days. Blood samples were analyzed using an autohematology analyzer, while plasma was used to determine the levels of SOD, MDA, and IL-1 β .

Results EE/LNG exposure (21 days) significantly increased oxidative stress, as evidenced by elevated MDA levels and reduced SOD activity. Furthermore, interleukin-1 β levels remained unchanged across all groups. Lastly, transient changes in RBC count, Hb, PCV, and WBC levels were observed, particularly in group B; however, these changes were not sustained or statistically significant, except for PCV recovery.

Conclusion While EE/LNG increases oxidative stress with prolonged use, it shows limited effects on cytokine signaling and blood profiles at the tested durations. Further research is needed to explore broader inflammatory markers and long-term effects.

Keywords Cytokine, Ethinylestradiol, Hematology, Levonorgestrel, Oxidative Stress

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1 Introduction

Oral contraceptives (OCs) are widely used for birth control and have been studied extensively for their effects on reproductive health. However, beyond their primary role in regulating fertility, OCs influence various physiological processes.^[1] Recent research has highlighted their impact on blood profile, immune system signaling, and oxidative stress, raising questions about their broader effects on women's health.^[2,3] These effects are particularly relevant given the widespread and long-term use of OCs. Understanding these influences is critical for clinicians to better advise patients and manage potential risks. Studies indicate that OCs can influence blood coagulation factors and hematological profiles.^[4] Tekle et al.^[3] emphasized the importance of monitoring hematological indices in users of oral contraceptives, suggesting that these medications can alter blood profiles. The question that requires an answer is what is the impact of different formulations of OCs on hematological parameters such as red blood cell count (RBC), hemoglobin (Hb) levels, and platelet counts in Wistar rats? Hematological profiles are measurable blood indices used to detect and monitor various pathological and physiological abnormalities. The use of OCs is a significant factor that can impact hematological test results and holds critical importance.^[3]

In addition to altering blood profile, OCs have been shown to modulate cytokine signaling, a crucial component of the immune system.^[5] Cytokines are small proteins that mediate inflammation and immune responses. Studies suggest that OCs can both suppress and enhance the production of specific cytokines, affecting inflammatory pathways.^[6] This modulation could influence susceptibility to infections and autoimmune conditions, making it important to understand how long-term contraceptive use alters immune function. The effects of hormonal contraceptives on immune parameters, including white blood cell counts (WBC), have been less explored. Osman and Al-Mutairi^[7] found no significant changes in WBC among contraceptive users, indicating a potential area for further research into how hormonal changes might influence immune function.

Oxidative stress, a state in which the balance between free radicals and antioxidants is disrupted, is another area affected by OCs.^[2,8] Hormonal fluctuations caused by OC use have been associated with increased oxidative stress, potentially leading to cellular damage and ageing.^[8] The relationship between OCs and oxidative stress is complex, with some studies indicating that OCs may decrease antioxidant levels, while others suggest a protective role through estrogen's antioxidant properties. Investigating this dual role is vital for understanding the long-term health effects of OCs and developing strategies to mitigate any adverse outcomes.

Ethinylestradiol (EE)/levonorgestrel (LNG) is a widely used contraceptive combination with various formulations and administration regimens. Continuous daily administration of this combination has shown high contraceptive efficacy. Both compounds are rapidly absorbed, with peak serum concentrations reached within 1-2 hours after administration.^[9] Pharmacokinetic parameters show dose-dependent variations and pronounced interindividual differences.^[10] Research suggests that OCs containing EE can increase oxidative stress.^[11] Studies have shown that OCs containing EE and LNG may affect blood coagulation, with studies showing a significant shortening of whole blood clotting time after 3-6 cycles of use.^[12]

The hypothesis is that the subchronic use of OCs containing EE and LNG will alter the blood profile by modifying cytokine signaling pathways and increasing oxidative stress levels, potentially contributing to systemic inflammation. This study aims to investigate the subchronic effects of EE and LNG on hematological parameters, immune function, and oxidative stress in Wistar rats.

2 Methods

Animals

Forty adolescent Wistar rats, averaging 144 g in weight, were obtained from Temilola Animal Husbandry in Osogbo, Osun State, Nigeria. The rats were acclimated for 14 days under a 12-hour light/dark cycle at a temperature of approximately 29°C in the animal housing facility of the Faculty of Basic Medical Sciences, Adeleke University, Ede. They were provided with food and water *ad libitum*. The animals were cared for in compliance with the guidelines for the care and use of laboratory animals in biomedical research.^[13,14] The study's experimental protocols received approval from the Adeleke University Ethical and Review Committee, under the approval number AUERC/1257.

Animal Grouping, Drug Preparation, and Administration Protocol

EE (0.03 mg) and LNG (0.15 mg) tablets (Levofem; manufactured by PT. Harsen Laboratories, Indonesia, and purchased from DKT Nigeria) were used. Forty rats were divided into four groups of 10. Group A served as the control and received only distilled water for 21 days. Groups B, C, and D were administered combined OCs containing EE and LNG at doses of 0.03 mg/kg and 0.15 mg/kg body weight, respectively, for durations of 7, 14, and 21 days. To ensure precise dosing, all rats were individually weighed using a digital weighing scale at the start of the experiment and weekly thereafter. The doses of EE (0.03 mg/kg) and LNG (0.15 mg/kg)

were calculated based on each rat's body weight. The combined OC tablets (Levofem, containing EE 0.03 mg and LNG 0.15 mg per tablet) were prepared by crushing the tablets and dissolving them in distilled water to achieve a stock solution concentration of 1 mg/mL for both active ingredients. The solution was then diluted to achieve the required concentrations for administration. For each rat, the appropriate volume of the prepared solution was calculated to deliver the target doses (e.g., a rat weighing 200 g would receive 0.2 mL of the solution for the required dose of 0.03 mg/kg and 0.15 mg/kg, respectively). A calibrated micropipette was used for precise volume measurement, ensuring accurate dosing. The doses administered are based on a pilot study done earlier by the authors.

Collection and Analysis of Blood

At the end of the administration period, the rats were anesthetized via intraperitoneal injection of ketamine hydroxide (50 mg/0.2 mL). Hematologic parameters, including RBC, WBC, Hb, platelets (PLT), and packed cell volume (PCV), were subsequently analyzed using an auto hematology analyzer (Seattle, USA).

Biochemical Assay

To analyze malondialdehyde (MDA), superoxide dismutase (SOD), and interleukin-1 beta (IL-1 β) in rat blood samples, plasma was collected and stored at -80°C to prevent degradation. MDA, a marker of oxidative stress, was assessed using the thiobarbituric acid reactive substances (TBARS) assay.^[15] The absorbance of the resulting complex was measured at 532 nm. SOD activity, indicative of antioxidant defense, was measured via colorimetric assays.^[16] The absorbance of the resulting complex was measured at 450 nm. IL-1 β , a pro-inflammatory cytokine, was quantified using ELISA.^[17] The absorbance of the resulting complex was measured at 450 nm.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 9 for Windows. Differences among groups were evaluated through one-way ANOVA followed by Tukey's post hoc test for all measured parameters. Data are presented as the mean $\pm$ standard deviation (SD), with n indicating the number of animals per experimental group. A significance threshold of $p < 0.05$ was applied.

3 Results

Comparison of Average Oxidative Stress Marker Levels across the Different Experimental Groups

Figure 1 shows that the MDA level in the EE/LNG 21 group ($0.75 \pm 0.11 \mu\text{M/mg}$) was significantly increased (p

< 0.05) compared to the control group ($0.55 \pm 0.071 \mu\text{M/mg}$). However, no significant differences were observed in MDA levels when comparing the control group with the EE/LNG 7 and EE/LNG 14 groups ($p > 0.05$). As shown in Figure 2, the SOD level was significantly decreased in the EE/LNG 21 group ($0.27 \pm 0.1 \mu\text{M/mg}$) compared to the control group ($0.63 \pm 0.15 \mu\text{M/mg}$) ($p < 0.05$). No significant differences in SOD levels were observed among the control, EE/LNG 7, and EE/LNG 14 groups ($p > 0.05$).

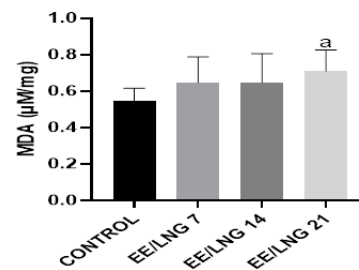


Figure 1 Malondialdehyde (MDA) level. Control = Control group; EE/LNG 7= Ethinylestradiol and Levonorgestrel 7 days administration; EE/LNG 14= Ethinylestradiol and Levonorgestrel 14 days administration; EE/LNG 21 = Ethinylestradiol and Levonorgestrel 21. Data expressed as mean $\pm$ SD (n = 10). ^a significant difference ($p < 0.05$) between the EE/LNG 21 group compared to the control group

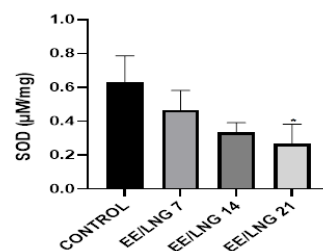


Figure 2 Superoxide dismutase (SOD) level. Control= Control group; EE/LNG 7= Ethinylestradiol and Levonorgestrel 7 days administration; EE/LNG 14 = Ethinylestradiol and Levonorgestrel 14 days administration; EE/LNG 21 = Ethinylestradiol and Levonorgestrel 21. Data expressed as mean $\pm$ SD (n = 10). *P value < 0.05 between the EE/LNG 21 group compared with the control group

Comparison of Mean Cytokine Levels across the Different Experimental Groups

Figure 3 shows that the IL-1 β levels did not differ significantly among the control ($126 \pm 7.3 \text{ pg/mL}$), EE/LNG 7 ($133 \pm 25 \text{ pg/mL}$), EE/LNG 14 ($126 \pm 33 \text{ pg/mL}$), and EE/LNG 21 ($140 \pm 23 \text{ pg/mL}$) groups ($p > 0.05$).

Comparison of Hematologic Parameters

Comparison of mean values of hematologic parameters in various groups of the experiment is shown in Table 1. The RBC count in the experiment demonstrates varying results across the different groups.

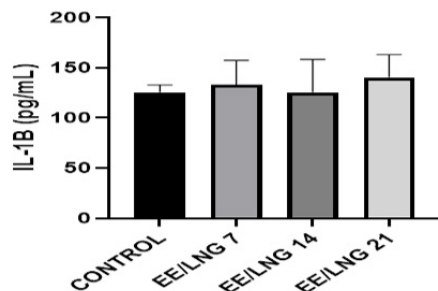


Figure 3 Interleukin- 1 β (IL-1 β) level. Control = Control group; EE/LNG 7 = Ethinylestradiol and Levonorgestrel 7 days administration; EE/LNG 14 = Ethinylestradiol and Levonorgestrel 14 days administration; EE/LNG 21 = Ethinylestradiol and Levonorgestrel 21. Data expressed as mean $\pm$ SD (n = 10). P value > 0.05 between experimental groups compared with the control group.

The control group shows an RBC count of 2.8 ± 0.55 ($\times 10^6/\mu\text{L}$), which is consistent ($p > 0.05$) with the EE/LNG 14 group ($2.8 \pm 0.35 \times 10^6/\mu\text{L}$) and EE/LNG 21 group ($2.8 \pm 0.62 \times 10^6/\mu\text{L}$). However, the EE/LNG 7 group exhibits a slightly lower ($p > 0.05$) RBC count ($2.2 \pm 0.28 \times 10^6/\mu\text{L}$), suggesting a potential transient reduction in RBC levels during this phase. The WBC count is slightly elevated ($p > 0.05$) in the EE/LNG 7 group ($8.7 \pm 0.92 \times 10^9/\text{L}$) compared to the control ($7.3 \pm 0.83 \times 10^9/\text{L}$). By days 14 and 21, the WBC levels ($7.6 \pm 0.46 \times 10^9/\text{L}$ and $7.2 \pm 0.78 \times 10^9/\text{L}$) return closer to control values.

Furthermore, in Table 1, Hb levels are slightly reduced ($p > 0.05$) in the EE/LNG 7 group (7.0 ± 0.97 g/dl) compared to the control (7.8 ± 0.84 g/dl). By day 14 (7.9 ± 0.81 g/dl), Hb levels recovered, though a modest reduction ($p > 0.05$) was observed again by day 21 (7.3 ± 0.17 g/dl). A notable reduction in PCV was observed in the EE/LNG 7 group (30 ± 4.2 %) compared to the control (39 ± 6.8 %). By day 14 (43 ± 6.7 %), significant ($p < 0.05$) recovery is seen, which remains stable through day 21 (41 ± 7.1 %). Platelet levels in the EE/LNG 7 group ($3.1 \pm 0.53 \times 10^9/\text{L}$) are slightly lower ($p > 0.05$) than those in the control ($3.6 \pm 0.19 \times 10^9/\text{L}$). By day 14 ($3.7 \pm 0.13 \times 10^9/\text{L}$), levels are slightly elevated ($p > 0.05$) before stabilising by day 21 ($3.5 \pm 0.57 \times 10^9/\text{L}$).

4 Discussion

Oxidative stress, characterized by an imbalance between reactive oxygen species (ROS) production and detoxification, significantly impacts blood cells, particularly RBCs.^[18,19] It is associated with various diseases, including diabetes, cancer, and neurodegenerative disorders.^[20] RBCs are uniquely vulnerable to oxidative damage due to their high oxygen-carrying capacity and lack of nucleus and mitochondria.^[21] Oxidative stress in blood cells can be induced by various factors, including extracorporeal procedures like apheresis, which increases ROS production and phosphatidylserine externalization while decreasing reduced glutathione levels.^[22] These changes can lead to cellular abnormalities, hemolysis, and platelet hyperactivation. Biomarkers of oxidative stress in RBCs, such as alterations in redox balance, are widely used for early diagnosis of various pathological conditions.^[21]

Oxidative stress profoundly affects blood cells, compromising their functionality, survival, and overall health. In RBCs, it causes hemolysis by damaging cell membranes, oxidizes Hb to form ineffective methemoglobin, and shortens their lifespan, impairing oxygen delivery. In WBC, excessive oxidative stress disrupts immune responses, induces apoptosis, and promotes chronic inflammation, which can contribute to immunodeficiency and inflammatory diseases.^[23] PLTs are also affected, with oxidative stress triggering premature activation and aggregation, thereby increasing the risk of thrombosis and cardiovascular issues, while altering their ability to adhere and aggregate, which in turn affects hemostasis and wound healing.^[24] This study reveals that EE/LNG treatment induces a transient impact on hematologic parameters, likely linked to oxidative stress. In the EE/LNG 7 group, reductions in RBC count, Hb levels, and PCV indicate early oxidative stress that impairs erythropoiesis or increases hemolysis, with recovery evident by day 14 and stabilization by day 21. A transient increase in WBC count points to an early inflammatory response, which resolves over time.^[25] Platelet levels also show a temporary reduction,

Table 1 Comparison of mean hematologic parameters among the various experimental groups

Parameters	Control group	EE/LNG 7 group	EE/LNG 14 group	EE/LNG 21 group
RBC ($\times 10^6/\mu\text{L}$)	2.8 ± 0.55	2.2 ± 0.28	2.8 ± 0.35	2.8 ± 0.62
WBC ($\times 10^9/\text{L}$)	7.3 ± 0.83	8.7 ± 0.92	7.6 ± 0.46	7.2 ± 0.78
Hb (g/dl)	7.8 ± 0.84	7.0 ± 0.97	7.9 ± 0.81	7.3 ± 0.17
PCV (%)	39 ± 6.8	30 ± 4.2	43 ± 6.7^b	41 ± 7.1
PLT ($\times 10^9/\text{L}$)	3.6 ± 0.19	3.1 ± 0.53	3.7 ± 0.13	3.5 ± 0.57

Data are represented as mean $\pm$ standard deviation (n = 10).

RBC: red blood cell, WBC: white blood cell; Hb: hemoglobin, PCV: packed cell volume, PLT: platelets, Control = Control group; EE/LNG 7 = Ethinylestradiol and Levonorgestrel 7 days administration; EE/LNG 14 = Ethinylestradiol and Levonorgestrel 14 days administration; EE/LNG 21 = Ethinylestradiol and Levonorgestrel 21.

^b significant difference ($p < 0.05$) between the EE/LNG 14 group compared with the EE/LNG 14 group

followed by stabilization and slight elevation by day 14, indicating initial oxidative effects on thrombopoiesis with subsequent recovery. These findings suggest an adaptive physiological response to mitigate oxidative damage over time.^[26-28]

These research findings reveal several significant insights into oxidative stress marker levels across different experimental groups. The MDA level, a marker of lipid peroxidation and oxidative stress,^[29, 30] was significantly elevated in the EE/LNG 21 group compared to the control group. This suggests that prolonged exposure (21 days) to EE/LNG may increase oxidative stress.^[31] No significant changes in MDA levels were observed between the control group and the EE/LNG 7 or EE/LNG 14 groups, indicating that shorter durations of exposure (7 or 14 days) may not significantly impact lipid peroxidation.^[32] Furthermore, the SOD level, a key antioxidant enzyme, was significantly reduced in the EE/LNG 21 group compared to the control group. This indicates a potential depletion of the antioxidant defence system after prolonged exposure (21 days) to EE/LNG. Similar to the MDA results, no significant differences in SOD levels were observed among the control, EE/LNG 7, and EE/LNG 14 groups, suggesting that shorter durations of exposure do not significantly alter antioxidant enzyme activity. The findings of oxidative markers in this study collectively suggest that prolonged exposure (21 days) to EE/LNG leads to increased oxidative stress, as evidenced by higher MDA levels and reduced SOD activity. The lack of significant changes in both markers in the EE/LNG 7 and EE/LNG 14 groups suggests a potential threshold or time-dependent effect, where oxidative stress only becomes evident after a certain duration of exposure.^[33,34] These results underscore the importance of duration in evaluating the oxidative effects of EE/LNG and suggest a potential risk of oxidative imbalance with prolonged use.

IL-1 β is a potent proinflammatory cytokine crucial for innate immunity and host responses to pathogens and tissue injury.^[35] While essential for immune activation, elevated IL-1 β levels are associated with various diseases.^[36] The complex mechanisms of IL-1 β release are not fully understood, but its secretion is thought to occur on a continuum depending on stimulus strength and extracellular requirements.^[35] These research findings indicate that exposure to EE/LNG, irrespective of the duration (7, 14, or 21 days), does not significantly affect IL-1 β levels. The lack of significant differences in IL-1 β levels across all groups suggests that exposure to EE/LNG does not induce measurable changes in this specific pro-inflammatory cytokine. This finding indicates that EE/LNG exposure, at the tested durations, may not provoke an inflammatory response mediated by IL-1 β .

5 Conclusion

The hypothesis that subchronic use of EE/LNG alters blood profiles, modifies cytokine signaling pathways, and increases oxidative stress is partially supported by the findings. Prolonged EE/LNG exposure (21 days) significantly increased oxidative stress, as evidenced by elevated MDA levels and reduced SOD activity. Secondly, interleukin-1 β levels remained unchanged across all groups, indicating no significant impact on this cytokine pathway or systemic inflammation. Lastly, minor, transient changes in RBC count, Hb, PCV, and WBC levels were observed, particularly in the EE/LNG 7 group; however, these changes were not sustained or statistically significant, except for PCV recovery. Further research is needed to explore broader inflammatory markers and long-term effects.

Declarations

Acknowledgments

The authors wish to express their gratitude to Rofiat Adekunle, Blessing Demilade, Beulah Salman-Idris, and Brianwill Laboratory Osogbo for the valuable technical assistance provided during this study.

Artificial Intelligence Disclosure

The authors confirm that no artificial intelligence (AI) tools were used in the preparation of this manuscript.

Authors' Contributions

Conceptualization and study design: Nathaniel Ohiemi Amedu; data collection: Nathaniel Ohiemi Amedu and Michael Obu; writing the original draft: Nathaniel Ohiemi Amedu and Habeebullahi Adelani Abdur-Rahman; review and editing: Nathaniel Ohiemi Amedu, Habeebullahi Adelani Abdur-Rahman, and Michael Obu.

Availability of Data and Materials

The datasets used in this study are available from the corresponding author upon reasonable request

Conflict of Interest

The authors declare no conflicts of interest with any entities.

Consent for Publication

Not applicable.

Ethical Considerations

The study's experimental protocols were approved by the Adeleke University Ethical and Review Committee, under approval number AUERC/1257.

Funding

This research did not receive any funding from public, commercial, or non-profit funding agencies.

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