RESEARCH ARTICLE

Oral contraceptive (Ethinylestradiol and Levonorgestrel) modulates the expression of proinflammatory and antioxidant genes in rat tissues

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Article info:
Received: 26 June 2025
Accepted: 01 December 2025

ABSTRACT
Ethinylestradiol/levonorgestrel (E/L), a commonly used hormonal oral contraceptive among women of childbearing age, has been linked to cardiovascular diseases (CVDs). Oxidative stress and inflammation collectively contribute to the initiation and progression of CVDs. This study investigated the impact of E/L on the expression of oxidative stress- and inflammation-associated genes (*PON2, TNF-α,* and *IL-1α).* Thirty-two female albino rats were randomly assigned to four groups (n=8 each): Group A (control) received DMSO and distilled water, while Groups B, C, and D were administered graded E/L dosages of 0.015, 0.03, and 0.060 mg/kg body weight, respectively. At the end of the experiment, tissues including the brain, liver, and kidney were harvested for total RNA extraction and purification. Gene expression was assessed by RT-PCR using specific primers. Notably, *PON2* was significantly upregulated in the brain but downregulated in the liver and kidney of all E/L-treated animals. Conversely, *TNF-α* and *IL-1α* were significantly upregulated in all organs except the liver. Collectively, this study provides preliminary evidence that E/L administration modulates the expression of key genes involved in oxidative stress and inflammation in a tissue-specific manner. These findings underscore the need for further research to elucidate the functional consequences of these changes and their relevance to cardiovascular health.

Key words: Ethinylestradiol, Levonorgestrel, Antioxidant, Inflammation, Cardiovascular diseases

Introduction

Cardiovascular disease (CVD) remains a leading cause of illness and death worldwide, driven by complex interactions among genetic, metabolic, environmental, and hormonal factors (Ciumărnean et al., 2021; Deepu et al., 2024). Oxidative stress and inflammation play central roles in CVD onset and progression, contributing to endothelial dysfunction and atherogenesis (Camps et al., 2021; Roth et al., 2020; Zuo et al., 2019). Hormonal regulation is known to influence these pathways, prompting investigation into how hormonal contraceptives may modulate cardiovascular risk (Suman et al., 2023).

Oral contraceptives (OCs), including widely used formulations with ethinylestradiol and levonorgestrel (E/L), are prescribed for birth control and hormonal regulation (Barton et al., 2024; Vrettakos & Bajaj, 2023). Beyond their reproductive effects, these synthetic hormones have been shown to influence systemic physiological processes, including oxidative stress and inflammation, which are key contributors to CVD pathogenesis (Bhullar et al., 2024; Cauci et al., 2021).

Oxidative stress arises when reactive oxygen species (ROS) overwhelm antioxidant defenses, causing cellular damage and endothelial dysfunction (Shaito et al., 2022). Antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase counteract ROS and prevent lipid peroxidation (He et al., 2017; Pignatelli et al., 2018; Taler-Verčič et al., 2020). Among these endogenous antioxidants, the paraoxonase family, particularly paraoxonase 2 (PON2) – a lactone hydrolyzing enzyme with a broad substrate range – has gained attention for its potent antioxidant properties (Costa et al., 2020; Kattoor et al., 2017; Li et al., 2017;

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Manco et al., 2021; Winnica et al., 2022). However, persistent oxidative stress activates inflammatory pathways, elevating cytokines such as tumor necrosis factor alpha (TNF-α), and interleukin 1 alpha (IL-1α), which promote vascular remodeling and atherogenesis (Solleiro-Villavicencio & Rivas-Arancibia, 2018; Zahan et al., 2020).

Although epidemiological studies have associated OC use with increased risk of thromboembolic events, hypertension, and other cardiovascular complications (Cameron et al., 2023; Vinogradova et al., 2015; Yonis et al., 2025; Zakiyah et al., 2025), the underlying molecular mechanisms remain poorly understood. The aim of this study was to investigate how E/L modulates the expression of antioxidant gene *PON2* and pro-inflammatory genes *IL-1α* and *TNF-α* in rat tissues. By examining expressions of these genes in tissues relevant to inflammation and oxidative stress regulation, this research seeks to provide mechanistic insights into how E/L may influence pathways implicated in CVD development.

Materials and Methods

Experimental animals

150-200g healthy female albino rats were kept at room temperature, observed under a 12 h/12 h light/dark cycle, and given animal feed and water ad libitum. Before the experiment, the animals were given two weeks to adjust to their new surroundings. The experimental procedures were approved by the Animal Ethical Committee of the Faculty of Science, Lagos State University (LASU), Nigeria.

Chemicals

Normal saline, diethyl ether, dimethyl sulfoxide (DMSO), and heparin were acquired from the laboratory of the Department of Biochemistry, LASU, Nigeria. RNA extraction kits were products of Aidlab Biotechnologies Co., Ltd, Beijing, China.

Drug and dose regime

Combination-3® (0.03mg Ethinylestradiol, 0.015mg levonorgestrel) was acquired from the Newton Pharmacy Limited, Lagos, Nigeria.

Experimental design

Thirty-two female albino rats were randomly assigned to one of four groups, each with eight animals. Group A (control) received DMSO (0.1%) and distilled water, while Groups B, C, and D were given graded oral E/L dosages of 0.015, 0.03, and 0.060 mg/kg body weight, respectively, for 21 days with DMSO as the carrier.

Tissue Collection and Analysis

After the 21-day treatment, the animals were fasted overnight before being sacrificed under light ether anesthesia, and their blood collected into heparinized vials through cardiac puncture. The animals' liver, kidney, and brain samples were harvested for gene expression analysis, stabilized in RNAlater®, and preserved at -80 °C.

RNA Extraction

RNA was extracted from animal tissues using the Aidlab EASY spin Plus RNA extraction kit. 20mg of the organ sample was chopped into bits and homogenized using an electric tissue homogenizer. A Fisher Scientific nanodrop spectrophotometer was used to determine the concentration and purity of the eluted RNA template (using a 260:280 nm absorbance ratio), which was then kept at -80°C.

cDNA synthesis

CDA synthesis was carried out using the Transcriptor First-strand cDNA synthesis kit (Roche). The nascent-strand cDNA preparation was subsequently quantified using the Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies) and preserved at -20 ºC.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The TransgenEasyScript® one-step RT-PCR Supermix was used to perform the RT-PCR protocol. The amplicon bands on 1.2 percent agarose were examined using Image J software (Abràmoff et al., 2004), and presented as the gene's relative expression compared to the reference β-actin gene. The sequences of gene-specific primers are presented in Table 1.

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Table 1. *The sequence of gene-specific primers*

	Sequence (5` – 3`)	Gene Bank ID
β-Actin	Forward: GTCAGGTCATCACTATCGGCAAT Reverse: GAGGTCTTTACGGATGTCAACGT	NM_031144.3
IL1 α	Forward: GGGCCTCAAGGGGAAGAATC Reverse: ATGTCCCGACCATTGCTGTT	NM_031512.2
TNFα	Forward: ATCCGAGATGTGGAACTGGC Reverse: AAATGGCAAATCGGCTGACG	M_012675.3
PON2	Forward: ATTCTTTAGCGTGGGCCTCAA Reverse: CGTTGGCTGAGTCGAATCCT	NM_001013082.1

Statistical Analysis

Data were expressed as the mean ± SEM of four replicates and subjected to one-way analysis of variance (ANOVA) to determine differences between groups. Post-hoc analysis was carried out using the Tukey test. All statistical analyses were conducted using GraphPad Prism software (version 10). P values of less than 0.05 were considered statistically significant.

Results

Our results revealed distinct tissue-specific expression patterns prior to E/L administration. Overall, the brain showed the lowest basal expression levels among the three genes examined. *PON2* showed high basal expression in the liver and kidney, while *TNF-α* and *IL-1α* displayed the greatest expression in the liver. These inherent differences likely reflect variations in the oxidative and inflammatory capacities of each tissue.

Our assessment of *PON2* gene expression revealed significant downregulation in the liver and kidney of E/L-treated animals compared to controls, with the most pronounced decrease observed in the kidney at the highest dose (Fig. 1). Conversely, brain *PON2* expression increased progressively with increasing E/L concentrations. Our evaluation of *TNF-α* expression showed significant upregulation in all examined organs at all administered doses (Fig. 2). The liver exhibited a clear dose-dependent increase, while brain and kidney *TNF-α* levels peaked at intermediate doses relative to controls. Furthermore, our results revealed that *IL-1α* expression demonstrated tissue-specific responses to E/L treatment (Fig. 3). Both the brain and kidney showed increased *IL-1α* expression with increasing E/L doses, whereas the liver experienced significant dose-dependent downregulation compared to controls. These findings collectively highlight differential, organ-specific modulation of genes involved in oxidative stress and inflammation following E/L exposure.

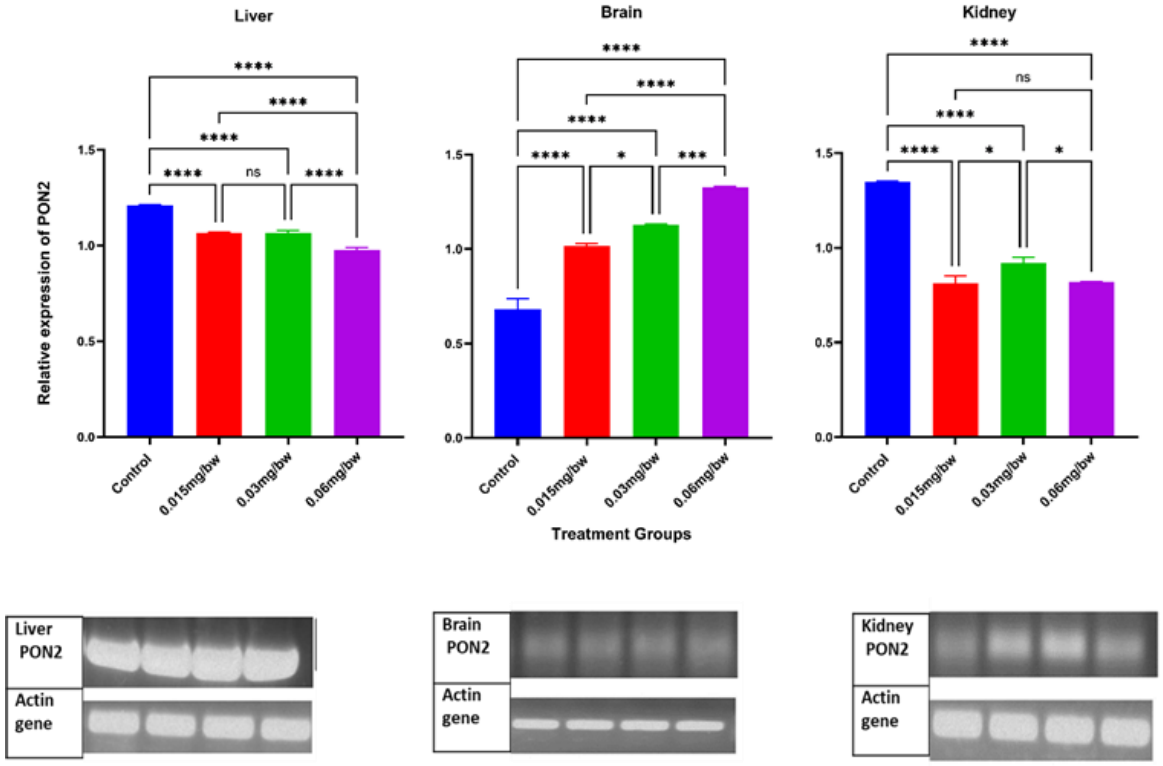

Figure 1. *PON2 expression in the organs of animals treated with graded doses of E/L oral contraceptive. * P <0.05, ** P <0.01, *** P <0.001.*

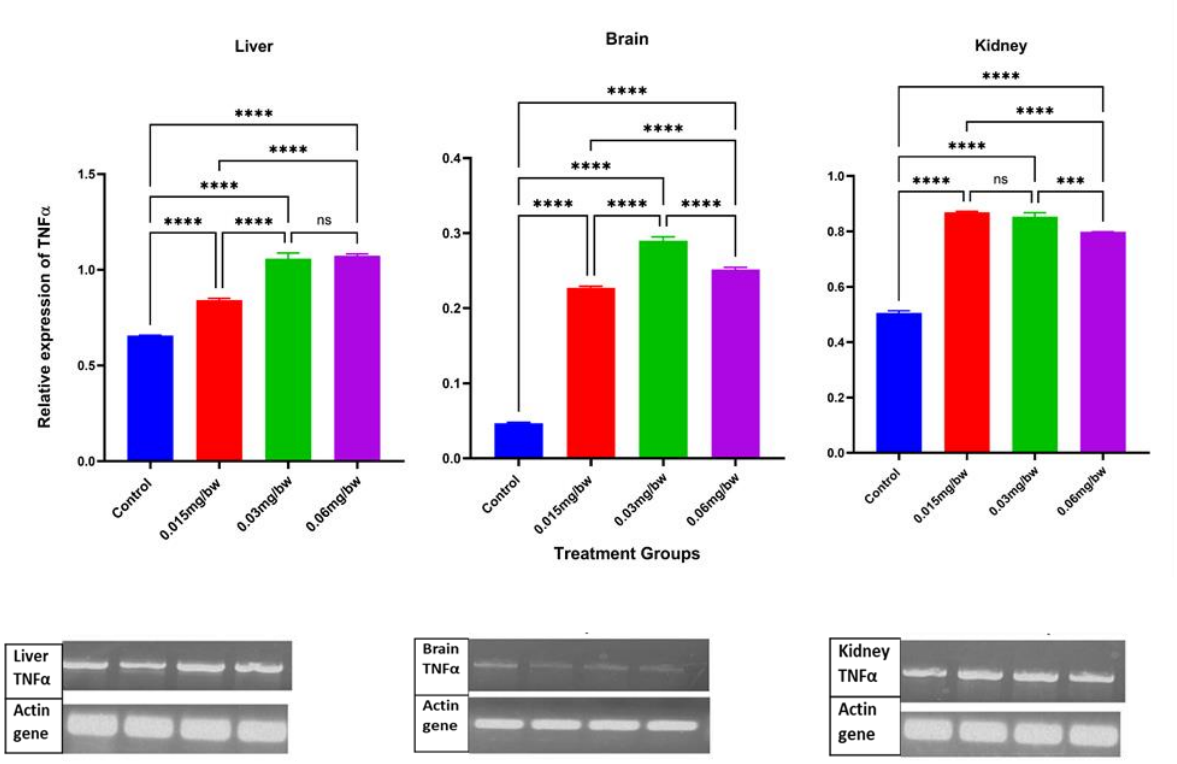

Figure 2. *TNF-α expression in the organs of animals treated with graded doses of E/L oral contraceptive. * P <0.05, ** P <0.01, *** P <0.001. P <0.001.*

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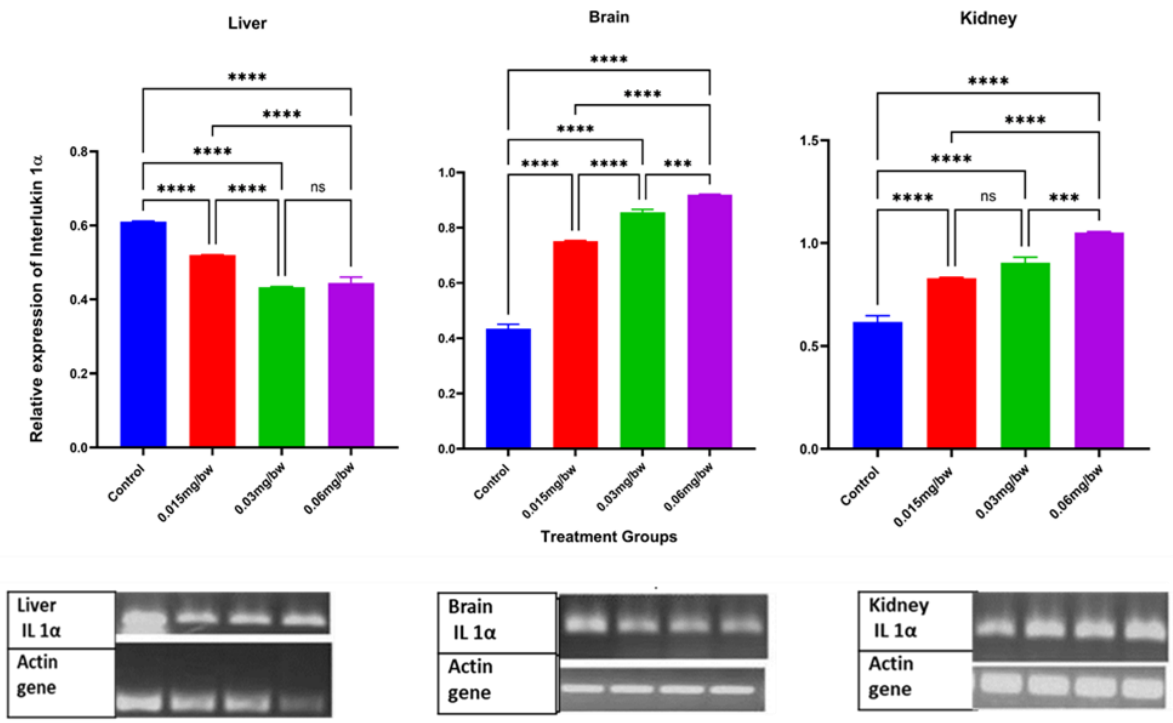

Figure 3. *IL-1α expression in the organs of animals treated with graded doses of E/L oral contraceptive. * P <0.05, ** P <0.01, *** P <0.001.*

Discussion

This study employed a murine model to evaluate the effects of E/L on the expression of oxidative stress– and inflammation-related genes (*PON2, TNF-α, and IL-1α)* across multiple organs.

The differential expression of *PON2* across organs may reflect tissue-specific responses to hormonal exposure. The brain, which is highly susceptible to oxidative stress, may upregulate *PON2* as a compensatory antioxidant mechanism (Cobley et al., 2018; Ghahremani et al., 2018), whereas the liver and kidney—major sites of metabolism and excretion—may exhibit suppressed expression due to differing redox demands. Previous studies have shown that estrogenic compounds can exert organ-specific effects on antioxidant gene regulation, possibly mediated by differential receptor distribution and local redox states (Ansell et al., 2004; Hayashi et al., 1997; Strehlow et al., 2003). Although basal *PON2* expression in the brain was lower than in the liver and kidney, E/L exposure elicited a pronounced upregulation of *PON2* in the brain (Fig.1), indicating a tissue-specific inducible antioxidant response to oxidative stress.

The elevated *PON2* level following E/L administration is consistent with previous findings showing that *PON2* is inducible under oxidative stress. Almutairi et al. (2017) reported significant *PON2* upregulation in rat brain tissues after cisplatin exposure, while Ghahremani et al. (2018) demonstrated that quercetin-induced *PON2* expression serves as a neuroprotective response against chlorpyrifos-mediated oxidative stress. Although cisplatin and quercetin differ from E/L in their pharmacological actions, these studies converge on a shared mechanism involving oxidative stress–driven *PON2* activation. These findings underscore PON2's role as a key antioxidant enzyme upregulated in response to chemically induced oxidative insults. The observed increase in brain *PON2* expression in this study may therefore represent a compensatory neuroprotective response to E/L-induced oxidative imbalance. Further investigation into the molecular mechanisms underlying these responses is warranted.

TNF-α and *IL-1α* are prominent pro-inflammatory cytokines that play pivotal roles in immune activation and tissue homeostasis. In this study, all E/L doses elicited significant upregulation of *TNF-α* across hepatic, renal, and brain tissues (Fig. 2), whereas *IL-1α* upregulation was restricted to the brain and kidney (Fig. 3). This induction may represent an adaptive response to oxidative stress triggered by E/L exposure, possibly aimed at counteracting free radical accumulation resulting from antioxidant depletion. However, sustained cytokine elevation may perpetuate inflammation and promote chronic disease development, including CVD (Ferrucci & Fabbri, 2018; Wong et al., 2012). Clinical studies have similarly shown increased serum TNF-α in individuals with CVD (Tuomisto et al., 2006) and elevated plasma TNF-

α in women receiving E/L-containing oral contraceptives for polycystic ovary syndrome (Yousuf et al., 2017). In addition, Raez (1997) demonstrated that levonorgestrel and 17β-estradiol enhance IL-6 production by osteoblasts, providing further evidence of estrogenic modulation of inflammatory gene expression.

While gene expression analysis provides valuable insights into transcriptional changes, it does not necessarily reflect corresponding protein levels or functional activity. The absence of protein-level validation is a limitation of this study. Future work should incorporate these methods to confirm whether the observed mRNA changes translate into altered protein expression and activity. Importantly, while our findings suggest that E/L exposure may influence molecular pathways linked to CVD risk, we did not measure direct cardiovascular endpoints such as blood pressure, serum oxidative markers, or histopathological changes. Another limitation of this study is the relatively small sample size (n=8 per group). Although this sample size aligns with similar studies in rodent models (Mota-Martorell et al., 2020; Thiriet et al., 2008), it may reduce the statistical power to detect subtle but biologically meaningful differences. Therefore, our conclusions regarding CVD risk must be interpreted with caution, as our results indicate potential molecular alterations associated with CVD pathogenesis rather than definitive evidence of increased disease risk.

Collectively, this study provides preliminary evidence that E/L administration modulates the expression of key genes involved in oxidative stress and inflammation in a tissue-specific manner. These findings underscore the need for further research to elucidate the functional consequences of these changes and their relevance to cardiovascular health.

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