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Impact of Hormonal Contraceptives on Splenic Architecture and Cytokine Response in Neisseria gonorrhoeae-Infected mice

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ABSTRACT

Neisseria gonorrhoeae is a Gram-negative bacterium causing gonorrhea, a sexually transmitted infection. To study the effect of combined oral contraceptives(COC) on the immune response of mice, we selected the key lymphoid organ, spleen which is known to integrate innate and adaptive immunity and is sensitive to hormonal and environmental influences. Female BALB/ c mice were divided into 4 groups named as COC Treated(T), Infected with *N. gonorrhoeae* (I), COC Treated Infected(TI), and Control where mice were kept without any treatment (C). The spleen's histological examination showed that although infection alters the splenic structure, leading to amyloidosis, hemosiderin buildup, and a loss of red and white pulp differentiation, pretreatment of the group with COC only led to decreased cellularity and structural disarray of splenic compartments.

MDA levels in the TI group were stable, indicating a compensatory antioxidant response. SOD activity was significantly decreased in the T group, but remained insignificantly altered in the I & TI group. Catalase activity dropped insignificantly in all groups, indicating a disabled H_2O_2 detoxification system. while GSH levels remain maintained. These results indicate that infection and COC perturb the splenic redox balance, but in combination may activate adaptive antioxidant systems. Limited inflammatory response in spleen during *Neisseria gonorrhoeae* infection was observed. TNF- α levels declined, suggesting host adaption. COC treatment and bacterial infection maintained high IL- 1β levels, while TI suppressed inflammatory action and delayed pathogen clearance. An, in general, upregulation of IFN-γ in TI group suggests inactivation of cell mediated immunity. The minimum IL- 10 induction in all groups indicates inadequate anti-inflammatory regulation.

Keywords: *Neisseria gonorrhoeae, Infection, Inflammatory response, COC, cytokines, oxidative stress.*

1. INTRODUCTION

Ethinylestradiol–levonorgestrel (EEL) is a widely used oral contraceptive used to prevent pregnancy and manage gynecological disorders, but long-term use has been linked to adverse metabolic effects, including cardiovascular disease risk factors (Michael & Olatunji, 2018, Olaniyi et al 2021). EEL administration can cause insulin resistance, impaired glucose homeostasis, with elevated triglyceride levels,renal glucose and lipid metabolism and depressed anti-oxidants (Olaniyi and Olatunji,2019) leading to a pro-inflammatory metabolic state. As a central location for systemic immune surveillance, the spleen is an essential secondary lymphoid organ that controls both innate and adaptive immune responses. Through its diverse population of immune cells, which includes lymphocytes, dendritic cells, and macrophages, it plays a crucial role in removing blood-borne pathogens and coordinating the production of cytokines(Zhao et al., 2020).

The etiologic agent of gonorrhea, a sexually transmitted infection (STI) that continues to be a serious global public health concern, is *Neisseria gonorrhoeae.* It has been found to be resistant to the majority of currently available antibiotics by the World Health Organization (WHO) through surveillance, underscoring the immediate threat of widespread, incurable gonorrhea infections. Although it can also colonize the ocular, nasopharyngeal, and anal mucosa. *N. gonorrhoeae* primarily colonizes the genital mucosa. *N. gonorrhoeae* expresses a variety of factors that enable replication and survival in these environmental niches, as well as factors that modulate and elude the host immune system, because it colonizes the genital, rectal, and oral mucosa. Since *N. gonorrhoeae* does not express strong exotoxins, pathology is mostly the result of damage brought on by the activation of innate immune responses at the sites of colonization (Quillin and Seifert, 2018).

Even though *Neisseria gonorrhoeae mainly* infects mucosal surfaces, the spleen is frequently involved in defense mechanisms outside of the local infection site due to systemic immune activation. Hormone-induced immunosuppression may impede systemic pathogen clearance and contribute to the persistence or spread of disease in gonococcal infection. Thus, examining the antioxidant levels, cytokine expression, and spleen histopathology in BALB/c mice treated with COC offers a useful framework for comprehending the systemic effects of hormonal contraceptives during bacterial infections.

2. MATERIALS AND METHODS

Animal Care

Adult female BALB/c mice (4-6 weeks reproductively mature) were procured and housed in the Central Animal House, Panjab University, Chandigarh. The Institutional Animal Ethics Committee's approved guidelines were followed in all animal procedures. (PU/45/99/CPCSEA/ IEAC/2023/840). The mice were acclimatized for seven days before commencing experiment and housed in standard polypropylene cages at a regulated room temperature. They were fed standard mouse pellets (Ashirwad Industries, Punjab, India) and provided water ad libitum with a 12-hour light/dark cycle.

Bacterial strain and growth conditions

Neisseria gonorrhoeae (strain WHO–M STD-223) was taken from the Department of Microbiology, PGIMER in Chandigarh, India and cultured at 37°C in 7% CO_2 on Difco™ GC Medium Base GC agar enriched with BD BBL Hb bovine freeze-dried hemoglobin based as per the instructions on the label.All of the media used for bacterial culture was obtained from Difco (Becton Dickinson, Sparks, Md).

Experimental design

Adult female BALB/c mice (15-20g) were randomly divided into four groups(n=12 each)

Control (C): No bacterial infection nor COC treatment was given.

COC Treated (T):A commercially available COC (0.03 mg of ethyl estradiol + 0.15 mg of levonorgestrel) per tablet. Micewere given human equivalent dose (0.74 µg COC / 20g body weight) for 20 days.

Infected with *N. gonorrhoeae***(I):** Mice were intravaginally inoculated with a dose of *N. gonorrhoeae* at 1.2×10^8 CFU/mL following Abutaleb et al. (2022). The dosage was created using the description of the McFarland technique (Faro et al., 2016). Each mouse's vagina was rinsed with 50 mM HEPES (pH = 7.4) before inoculation. Twenty microliters of the bacterial suspension were given to each mouse. On Days -2 through +1, mice were also administered vancomycin (0.04 mg, intraperitoneally, twice daily) and streptomycin sulfate (0.24 mg, intraperitoneally, twice daily) to prevent the overgrowth of commensal flora under estradiol influence.Throughout the experiment, water was replaced every other day while trimethoprim sulfate (0.04 g/100 mL) was administered. Streptomycin sulfate (2.4 mg/L) was added to the trimethoprim water from day +2 until the end of the study.

Treated infected (TI): Mice pretreated with COC for twenty days were infected with *N. gonorrhoeae* on day 21.

Mice in all groups were euthanized, and their spleens were extracted on days 3, 5, 7, and 10 after treatment or infection periods to study histopathology, antioxidant levels, as well as pro- and anti-inflammatory cytokine immunoassays.

Histopathology: The spleen tissues were removed, thoroughly rinsed with physiological saline, fixed in Aqueous Bouin's solution, and processed for paraffin embedding following standard histological methods. Serial sections were cut, dewaxed, rehydrated, and stained with hematoxylin-eosin to allow for microscopic analysis of histopathological alterations (Bancroft et al., 1996).

Assessment of Oxidative Stress Markers: In order to assess oxidative stress, we examined important indicators in each experimental group. Lipid peroxidation was determined by the Thiobarbituric Acid Reactive Substances (TBARS) assay (Wills, 1966) for MDA concentrations, and the Lück (1965), Kono (1978), and Ellman's (1959) methods were used for measuring catalase, SOD levels, and GSH activity, respectively. The total protein content in spleen homogenateswas determined using the Lowry method (1951)with standard graph plotted using bovine serum albumin (BSA).

Cytokine Analysis: Tissue samples were aseptically taken from 3 mice per group at the times indicated (days 3, 5, 7, and 10) in order to analyze the cytokines .The samples were homogenized in 0.3 mL of PBS, and the supernatant was obtained by centrifuging the mixture for 20 minutes at 4 °C and 5000 rpm. The cytokine concentrations (TNF-α, IL-1β, IFN-γ, and IL-10) were then measured with commercial ELISA kits (Bioassay Technology Laboratory China).

Statistical analysis All quantitative data are expressed as the Mean ± standard error of the mean (SEM). Statistical evaluations were conducted using IBM SPSS Statistics 21. One-way Analysis of Variance (ANOVA) and the Dunnett post-hoc test were employed to assess the differences in cytokine levels and antioxidant enzyme activities across the four experimental groups. A p-value of less than 0.05 ($p < 0.05$) was considered a statistically significant result.

3. RESULTS

Histopathological analysis

The spleen parenchyma of the control mice showed a clear white and red pulp area when investigated under a microscope. (Fig. 1a). Treated Group (T) showed slight hyperemia of the red pulp area. Extensive vacuolation, decreased cellularity, and increased macrophages were also observed in the red pulp area (Fig. 1b). In the Infected Group (I) the red pulp was severely hyperemic, and the lymphocyte arrangement in the white pulp was loose with an increase in macrophages and a few multinuclear giant cells. Extensive hemosiderin accumulation (brown granules) due to red pulp hemorrhage was noted on day 3, which decreased by day 5. Severe congestion and enlarged red pulp were observed, with necrotic areas more prominent on 5 days post-infection (Fig. 1c). In the fourth group i.e., TI group, spleen showed decreased cellularity and structural disarray of splenic compartments, loose lymphocyte arrangement in the white pulp, increase in macrophages, and reduced vacuolization in the red pulp area (Fig. 1d).

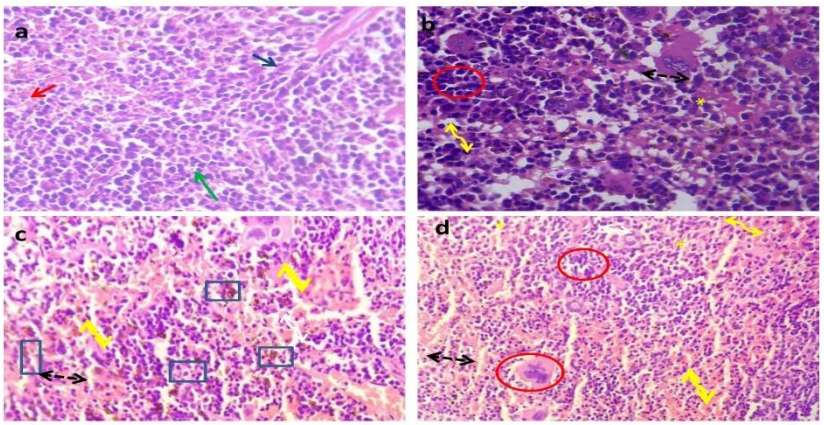

FIGURE 1(a-d). Histopathology of mice spleen(H & E staining, 40X) . (a) Spleen from control group showing normal architecture with intact white pulp (red arrow), well-organized red pulp (green arrow), and normal trabeculae (blue arrow). (b) Spleen from COC treated group showing marked pathological changes including hyperemia of red pulp (double arrow -doted) ,extensive vacuolation in the red pulp (asterik), decreased pals number,size and cellularity ((double arrow) ,the macrophages (red circle)(c)Spleen from infected group showing hemosiderin accumulation (in square) , decreased cellularity ((double arrow) and hyperemia of red pulp (double arrow -doted black)(d)Spleen from combined COC + infection group showing decreased cellularity with structural disorganisation of splenic compartments (double arrow), the macrophages (red circle), hyperemia of red pulp (double arrow -doted black)

Oxidative Stress Markers

The Malondialdehyde (MDA) levels**,** indicating lipid peroxidation and oxidative stress, were measured in the spleen of mice across the four groups. MDA levels in the control spleen ranged approximately between 8 and 9 nanomoles/10 mg of protein throughout the experiment period. COC treatment (T) consistently resulted in higher spleen MDA levels compared to the Control group across all observed time points, indicating that the spleen experiences a mild degree of oxidative stress on treatment with oral contraceptives alone. Bacterial infection (I) showed an initial increase in MDA levels at Day 3 compared to control which surprisingly, from Day 5 onwards declined, indicating that bacterial infection did not cause sustained oxidative stress in the spleen under these conditions. For the Treatment followed by Infection (TI) group, MDA levels initially declined when compared with the infected group but later they became similar to that as for the I group suggesting that the oxidative stress did not escalate despite prior COC exposure(Fig. 2a).

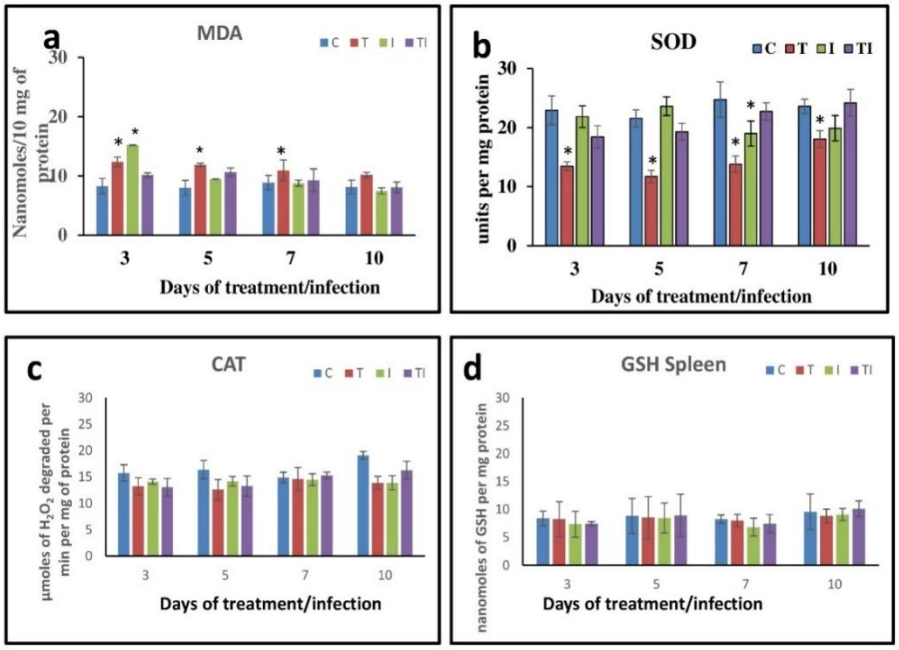

FIGURE 2 (a-d). Bar graph showing the biochemical changes in the spleen of Control (C), COC Treated (T), Infected (I), and COC Treated + Infected (TI) mice (a) MDA (b) SOD (c) catalase (d) Reduced GSH on days 3, 5, 7, and 10 of their respective treatments/infection

Superoxide dismutase (SOD) activity was significantly reduced in the T group throughout the experiment while in the I group the change was, in general, insignificant as compared to controls. The TI group demonstrated comparatively little decline as compared to infected group indicating a partial recovery of antioxidant defense in the combined condition (Fig. 2b). Catalase (CAT) activity did not show significant variations among the groups, with the TI group remaining comparable to both T and I throughout the study period (Fig. 2c). Similarly, splenic GSH levels remained largely unchanged, with no significant differences between the TI, T, and I groups, suggesting that glutathione homeostasis was unaffected by either treatment or their combination (Fig. 2d).

Cytokines response

TNF-α levels apparently remained near to baseline in all the groups when compared with control with statistically insignificant rise or fall throughout the observation period but TI group noticed large decrease on 10 day indicating decreased inflammatory response probably for survival of bacteria (Fig. 3a). IL-1β level was significantly increased in the COC treated group at all the time points. An, in general, increase was also observed in the I group but in the TI group the IL-1β level declined and became similar to controls at all the time points suggesting that infection in the presence of COCs (TI group) appears to inhibit prolonged IL-1β production, possibly through hormonal regulation of cytokine gene expression (Fig. 3b). The IFN-γ concentration in the Infected (I) group was significantly higher than the Control (C) group on day 5 and 7, subsequently declining by day 10. However, the TI group exhibited significant rise at 3 days with decline on day 5 onwards as compared to I group (Fig. 3c). suggesting that the COC exposure might be influencing the basal Th -1 cytokine production. The level of IL-10 in the spleen for both Treated (T) infected (I) and infected group post treatment TI showed insignificant increase to control(C) (Fig. 3d). indicating augmentation of anti inflammatory pathways to suppress inflammatory pathway to inhibit inflammation and pathogen clearance.

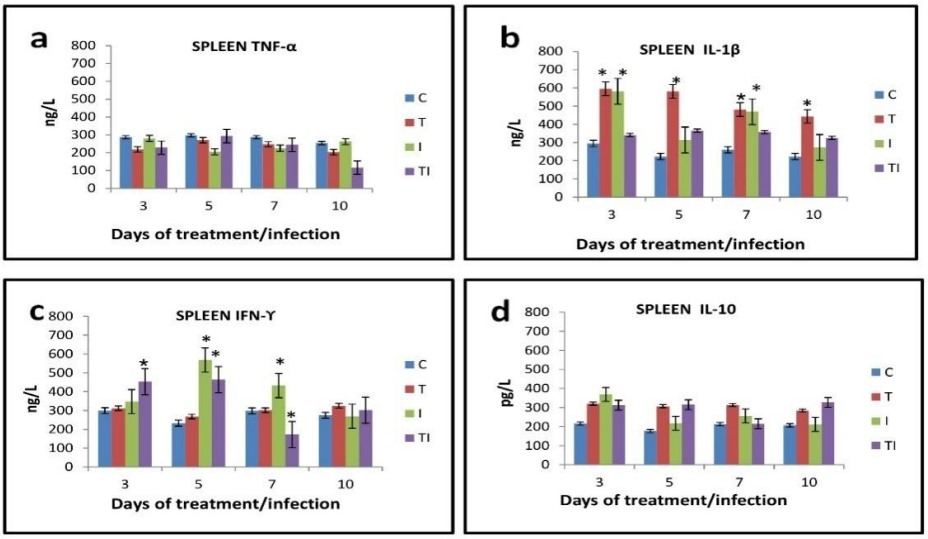

FIGURE 3 (a-d). Bar graph showing the cytokines changes in liver of Control (C), COC Treated (T), Infected (I), and COC Treated + Infected (TI) (a) TNF-α (b) IL-1β (c) IFN-γ (d) IL-10 concentration levels on days 3, 5, 7, and 10 of their respective treatments/infections.

4. DISCUSSION

The spleen, as a specialized lymphoid organ, is located within the circulatory system and is positioned to monitor and respond to hormonal and environmental factors including infections with antifungal and antibacterial immunological reactivity, which integrates the innate and adaptive immune systems in a systematic manner. The spleen is believed to be a sensitive sentinel organ for identifying immunomodulatory effects of environmental factors due to its crucial role in the inflammatory response such as chemical pollutants or pharmaceuticals that impact normal hormone-regulation of immune responsiveness(Elmore, 2006; Gear and Belcher, 2017). Apart from removing the older erythrocytes , blood-borne microorganisms and cellular debris from the circulation by involving macrophages, it recycles iron from the erythrocytes (Elmore, 2006). Histological analysis of the spleen in control mice revealed the classic architecture characterized by a clear distinction between red and white pulp, the presence of PALS (periarteriolar lymphoid sheaths), venous sinuses, and well-organized lymphoid follicles as per previous descriptions of normal murine spleen histology (Mebius& Kraal, 2005; Steiniger, 2015). Estradiol causes alterations in the spleen, including increased monocyte and phagocyte proliferation, enhanced functional activity, and increased cell number and size in splenic cord macrophages (Weng et al., 2020; Pandey et al., 2022). Zhao et al. (2020)studied and found that bisphenol S exposure caused significant spleen morphological changes, including damage to white pulp, loss of red and white pulp distinction, reduced cellularity in the periarteriolar lymphoid sheath, and splenomegaly. Similar findings were made by Shaibi,,et al. (2022), who found that exposure to bisphenol A (BPA) resulted in spleen damage, including changes in spleen size and histopathological changes. Hussen et al. (2024) found that after 35 days of estrogen exposure, the spleens of adult female BALB/c mice showed hyperplastic changes, blood vessel congestion, and varied degrees of amyloidosis. Maranghi et al. (2013) found lymphocytic infiltration in the spleen in mice exposed to persistent organic pollutants, supporting previous studies identifying the murine immune system as a sensitive target of 17β-estradiol. According to Gear and Belcher (2017), female mice exposed to BPA or EE showed decreased cellularity, decreased PALS size and number, and mild to moderate increases in apoptotic lymphocytes. The main changes in the red pulp observed was hemosiderin accumulation and increased cellularity brought on by enhanced extramedullary hematopoiesis (EMH). Increased erythrocyte lysis and iron recycling are reflected in the presence of this pigment, which is a consequence of hemoglobin metabolism (Mebius, & Kraal ,2005).In mice, where the spleen is haematopoietically active throughout life, exposure to EE caused EMH with an abundance of erythroid precursors. This is probably a compensatory reaction to the inhibition of bone marrow hematopoiesis caused by estrogen. The peripheral erythrocyte count and hemoglobin levels are decreased as a result of this suppression (Landshman& Bleiberg, 1979).

Amyloidosis, a prevalent sign in infected individuals, is detected in the red pulp of the spleen, altering normal spleen architecture and causing atrophy in lymphoid tissues(Ge et al., 2007).Spleen in mice also displayed hyperemia, macrophage

infiltration, vacuolation, and haemosiderin accumulation, similar to malaria and leishmaniasis (Shaibi et al., 2022). Hemosiderin deposits and vacuolated macrophages indicate ongoing hemolysis and erythrophagocytosis (Engwerda et al., 2005; Chang et al., 2021). By day 10, the recovery group's splenic architecture had partially returned, but there was still no clear distinction between the red and white pulp. This implies that splenic remodeling is delayed even though systemic recovery may start. This is consistent with previous research that found that the structural and functional reconstitution of spleen tissue frequently lags behind pathogen clearance (Elchaninov et al.,2022). Persistent amyloid deposits or fibrosis, which impede stromal cell function and germinal center reformation, may also be the cause of incomplete recovery (Del et al., 2012). Additionally, acute infection may cause irreversible damage to follicular dendritic cells, which are crucial for B cell maturation and antigen presentation (Allen et al., 2007). The post-treatment TI infection group showed mild disruptions in splenic architecture, resulting in a decrease in the cellularity and structural disarray of the splenic compartments, suggesting a protective role for COC.

Our study sought to evaluate the dynamics of oxidative stress markers—MDA, SOD, CAT, and GSH—in the spleen following COC treatment (T), infection (I), and combined treatment plus infection (TI). The results reveal distinct temporal patterns of oxidative stress and antioxidant responses across these groups. The study found elevated MDA levels in the T group and I group, indicating increased lipid peroxidation due to COC treatment and infection. This suggests oxidative disruption may last longer than the acute infection response. The TI group's elevated MDA suggests an adaptive or protective reaction, reducing initial oxidative damage through compensatory antioxidant reactions(Cemek et al., 2006). SOD acts as a first line of defense against ROS by converting superoxide radicals into hydrogen peroxide. The primary defense against superoxide radicals was compromised, as evidenced by the significantly lower SOD activity in the I group on Day 7 and in the T group at all time points. The TI group, on the other hand, showed comparatively constant SOD activity; similar to those of the I group, despite slight variations (Sies& Jones, 2020)Catalase activity decreased in T, I, and TI groups over 10-days, potentially compromising the spleen's ability to neutralize H_2O_2, potentially worsening oxidative damage and hindering healing (Poljsak&Milisav, 2013)As a vital intracellular antioxidant, GSH is stable in the TI group, suggesting resilience in non-enzymatic antioxidant capacity and may help buffer ROS even when enzymatic defenses fail to improve their oxidative stability occurs (Pizzino et al., 2017).Osman et al. (2017) in his study, demonstrated a significant decrease in SOD and CAT activities, and nonsignificant decrease in GSH, and a concurrently significant increase in MDA levels as compared to the control. Oral contraceptive pills can increase lipid oxidation due to free oxidative radical generation, a decline in antioxidant defense systems, or both. This can be due to increased allele frequency of GPx and SOD enzymes, bone marrow erythroblast maturation, or oxidation of enzyme proteins. Combination of oral contraceptive pills can disrupt oxidative status (Jendryczko et al., 1992; Fallah et al., 2011).The catabolism of exogenous hormones through P450 cytochrome activities may lead to increased ROS production and depletion of reduced glutathione(Finco et al., 2012; Zal et al., 2012).

Research indicates that (E/L) treatment diminishes CAT and SOD gene expression in animals, thereby affecting enzyme translation and activity. E/L contraceptives may induce oxidative stress by reducing antioxidant enzyme levels, thereby modifying gene expression (Arvas et al., 2011; Aminfar et al., 2019; Buccitelli& Selbach, 2020).E/L in low dose can decrease erythrocyte SOD, CAT, and glutathione peroxidase activities (Jendryczko et al., 1993), affecting antioxidant defense against free radicals. When ethinylestradiol 50 mcg and levonorgestrel 125 mcg were administered orally to healthy volunteers, Finco et al. (2011) observed an increase in oxidative stress parameters.According to Shukla et al. (2012), pregnant BALB/c mice infected with *Styphimurium* encountered severe tissue damage due to an increased bacterial load and liver enzymes, leading to substantial lipid peroxidation and reduced antioxidant activity when orally challenged with Salmonella enterica. Earlier findings of Abdulkareem et al.(2022)found that Swiss albino mice had higher levels of MDA at the conclusion of a 3-week *P. berghei* infection and the fact that Oral contraceptive treatment (COC) inhibits parasite growth and reduces parasite-induced lipid peroxidation, hyperlactatemia, and hepatic lipid accumulations, suggesting potential benefits for malaria and metabolic disorders.

The immune response to infection and hormonal treatment was evaluated using cytokine profiling in the spleen. TNF-α, IL-1β, IFN-γ, and IL-10 are cytokines that play a crucial role in immune regulation and inflammation .Their varied expression across treatment groups is a complex reflection of host-pathogen and hormone-immune interactions .Over the course of the observation period, TNF-α levels in the Control, T, and I groups did not exhibit any statistically significant variations which points to a splenic inflammatory state that is more regulatory while responsive activity probably concentrated in primary immune tissues like the liver or genital tract, even though TNF-α is a major initiator of systemic inflammation (Aggarwal, 2003). The TI group's TNF-α levels had significantly dropped by Day 10 suggesting that the host may have adapted to bacterial presence to reduce tissue damage from ongoing inflammation. A decrease in TNF-α, potentially through altered host cytokine responses, could also represent a bacterial tactic to evade immune clearance (Rittirsch et al., 2008).In order to avoid a septic-like state after the initial pro-inflammatory surge peaked around day 3 , a negative feedback regulation localized inflammatory response at the infection site, the genital tract with less systemic spillover to the spleen. At every time point, the T group's levels of IL-1β, a strong pro-inflammatory cytokine were markedly higher, suggesting that COC treatment alone was responsible for the long-term inflammatory activation. This supports findings that synthetic hormones like COCs can modulate immune responses by upregulating inflammatory gene expression even in the absence of infection(Soria-Jasso et al., 2019; Dai et al., 2024). The study found that IL-1β levels in the I group increased during infection

between days 3 and 7, indicating an inflammatory response.(Delano and Ward, 2016). However, the TI group showed stable IL-1β level, suggesting concurrent COC treatment may reduce the typical IL-1β response, potentially delaying pathogen removal (Yu et al., 2007). IFN-γ, a key Th1 cytokine, showed a transient increase in the I group, peaking on Days 5 and 7, before declining by Day 10. In the TI group, IFN-γ levels increased up to Day 5 but declined by Day 7, suggesting a shorter Th1 activation window. These findings align a classical cell-mediated immune response to infection and indicate that hormonal contraceptives may suppress Th1-type responses, potentially compromising the host's ability to clear certain infections (Schroder et al., 2004; Wira et al., 2015).According to Moore et al. (2001), IL-10 is essential for preventing immunopathology by reducing the host immune response to infections. All groups experienced a negligible rise in IL-10, an anti-inflammatory cytokine that controls overreactive immune responses, when compared to the control. This slight increase might be the result of a mild attempt by the host to maintain tissue integrity and balance pro-inflammatory signals, particularly in the face of ongoing immunological stress. The lack of a strong IL-10 response, however, indicates that either pro-inflammatory mediators successfully counterbalanced the anti-inflammatory arm or the spleen did not strongly activate it(Bhol et al.,2024).The study reveals that COC treatment modulates host immune responses during infection, potentially altering immune activation and pathogen clearance.

5. CONCLUSION

The spleen plays crucial role for immune surveillance as is sensitive to hormonal and infectious stressors. Infection and COC treatment disrupt splenic architecture and redox balance, but COC co-administration may partially protect by mitigating structural damage and preserving antioxidant capacity and maintains a stable cytokine profile, emphasizing the immunological complexity of hormonal contraceptive exposure. Further investigation is needed to understand long-term effects of COCs on redox homeostasis.

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7. CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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