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Full length Research Article

Carvedilol and Clomiphene Combination Therapy Alleviates Inflammation and Redox Imbalance in Experimental PCOS: Role of Nrf2/HMOX-1 and Nfkb Signaling

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Summary: Polycystic ovary syndrome (PCOS) is characterized by hyperandrogenemia, irregular menstrual cycle, and small cysts on the ovaries. This condition can be morphological or biochemical (elevated testosterone). Elevated testosterone (hyperandrogenemia) is the hallmark of PCOS, which can inhibit follicular development, anovulation, or cause irregular menstrual changes. Unfortunately, there is no cure for PCOS, and available treatment options are restricted to mitigating its symptoms. This study was, however, designed to investigate the synergistic effect of clomiphene (CLO) and carvedilol (CAL) on PCOS-induced female infertility. Thirty female Wistar rats were randomized into 5 groups (n= 6/group) control, PCOS, PCOS+ CLO, PCOS+CAL, and PCOS+ CLO+CAL. The administration was once daily via the oral route and lasted for 15 days. Clomiphene and carvedilol synergistically ameliorated PCOS-induced elevated serum gonadotropin-releasing hormone, luteinizing hormone (LH), testosterone and prolactin, and decreased follicle stimulating hormone (FSH), estrogen and progesterone. This was accompanied by the downregulation of PCOS-induced overexpression of ovarian LH, androgen, and FSH receptors. It was also accompanied by a decrease in inflammatory markers such as ovarian interleukin 1 beta and Nuclear factor kappa B (NF-κB) and apoptosis markers such as ovarian caspase 3 and an increase in ovarian Nuclear factor erythroid-2-related factor 2 (Nrf2), Heme Oxygenase 1 (HO 1 or HMO-1), catalase and glutathione reductase. This study shows that carvedilol and clomiphene combination therapy alleviates inflammation and redox imbalance in experimental PCOS.

Keywords*: Hormonal imbalance; Apoptosis; Folliculogenesis; Infertility, Polycystic Ovarian Syndrome*

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INTRODUCTION

Polycystic ovarian syndrome (PCOS) is a multifaceted and varied disorder that affects women in their reproductive years, with a global prevalence of 5-10% (Joham *et al*., 2022). It is a significant contributor to infertility and is characterized by the appearance of numerous small cysts on the ovaries, menstrual irregularities, and hyperandrogenism (Chaudhuri, 2023). The precise pathophysiology of PCOS is still unclear, but it is thought to result from a complex interplay of genetic, environmental, and lifestyle factors (Dabadghao, 2019). Unfortunately, there is no cure for PCOS, and available treatment options are restricted to mitigating its symptoms (Al Khalifa, 2021). Based on current knowledge, the mechanistic basis of PCOS, though unclear, is closely related to oxidative stress, inflammation, and endocrine dysfunction (Yeon *et al*., 2010; Akintoye *et al*., 2023).

Nuclear factor erythroid-2-related factor 2 (Nrf2)/Heme Oxygenase 1 (HO 1) signaling protects the body system from oxidative stress. Nrf2 is considered a major defense mechanism from oxidative stress by stimulating the removal of reactive oxygen species (ROS) via the activation of genes encoding the phase II detoxifying and antioxidant enzymes such as HMOX-1 (Xiong *et al*., 2015). Also, Nuclear factor kappa B (NF-κB) promotes inflammation through the transcriptional induction of pro-inflammatory cytokines and chemokines (Sun *et al*., 2013). These pro-inflammatory cytokines can induce inflammation directly or stimulate the differentiation of inflammatory T cells (Liu *et al*., 2017).

Pharmacological interventions, such as oral contraceptive pills and anti-androgen administration, have

been utilized to manage the symptoms of PCOS (Chouhan, 2022). Fertility treatments such as ovulation induction with clomiphene citrate (CLO) have also been employed. While these treatments can be efficacious in addressing specific symptoms, PCOS remains an incurable condition (Alesi *et al*., 2022). As such, there is an urgent need for ongoing research to identify more effective treatments that target the underlying pathophysiological mechanisms of this disorder (Xu *et al*., 2022). It was predicted that combining drugs with different mechanisms of action could be more advantageous in PCOS management rather than a monotherapy treatment. Hence, this study was designed to explore the synergistic effect of carvedilol (CAR) and CLO in PCOS animals.

CLO is a selective estrogen receptor modulator that promotes ovulation and ameliorates infertility in women with PCOS (Cunha and Póvoa, 2021). Although CLO is the first line pharmacological therapy for ovulation induction in women with PCOS, it still possess some side effects such as multiple pregnancies. Also, CLO may not be effective for treating oxidative stress (Wahab, 2019) which is part of PCOS pathogenesis. CAL is a beta-blocker with ability to prevent hyperandrogenism, a hallmark of PCOS (Aikoye *et al*., 2019). It has also been shown to possess properties as a modulator of redox and inflammatory signaling pathways and an enhancer of insulin sensitivity; these factors are all key drivers in the pathophysiology of PCOS (Bakr *et al*., 2022). Despite the potential complementarity of their mechanisms of action, the combination of carvedilol and clomiphene has yet to be extensively investigated as a possible treatment option for PCOS. Therefore, co-administration of these medications may provide a multifaceted approach to addressing the complex nature of PCOS. Hence, this study investigated the synergistic effect of carvedilol and clomiphene in ameliorating PCOS-induced endocrine dysfunction in an experimental rat model. Furthermore, the study explored the role of Nrf2/HMOX-1 and NF-κB signaling as the possible mechanism of action.

MATERIALS AND METHODS

Drugs and Chemicals: The drugs utilized in this study were acquired from reputable sources. Letrozole, manufactured by Novartis Pharmaceuticals in India, was utilized for the study. Carvedilol was procured from Ciron Drugs and Pharmaceuticals, also located in India. Clomiphene citrate tablets were obtained from Doppel Farmaceutici Sri Italy. Furthermore, ELISA kits, such as Gonadotropin-releasing hormone (GnRH), Follicle Stimulating Hormone (FSH), Luteinizing hormone (LH), Testosterone, Estrogen, Progesterone, Prolactin, and Catalase, were purchased from Bio-Inteco in the United Kingdom

Animals: For this study, thirty virgin female Wistar rats weighing 190-210g were kept at 25 ± 2°C and maintained on a 12-hour light-dark cycle. Rat chow and water were made available ad libitum. During the study, strict adherence was held to the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals to ensure the welfare and ethical treatment of the animals.

Experimental method: At the onset of the study, the animals were divided into five groups consisting of six animals each. Group 1 (control) received a daily oral dose of 1 mg/kg of distilled water throughout the study. The remaining animals were induced with polycystic ovary syndrome (PCOS) by administering a daily oral dose of 1 mg/kg of letrozole dissolved in distilled water for 21 days (Marouf *et al*., 2022; Akintoye *et al*., 2023). After PCOS induction, the animals were randomly divided into four treatment groups as follows: Group 2 (PCOS untreated) received oral doses of 1 mg/kg of distilled water, Group 3 received clomiphene citrate in distilled water at 1 mg/kg orally (PCOS treated with CLO), Group 4 received carvedilol 10 mg/kg in distilled water by oral gavage (PCOS treated with CAL), and Group 5 received 1 mg/kg clomiphene citrate and 10 mg/kg carvedilol by oral gavage (PCOS treated with CLO and CAL). All treatment groups received their respective treatments for 15 days, and overnight fasted animals were euthanized via intraperitoneal administration of 4 mg/kg xylazine and 40 mg/kg of ketamine. The dosages of CLO and CAL are similar with previously reported doses (Akintoye *et al*., 2023). The ovaries were also harvested, trimmed off of surrounding tissues, and kept in Bouin solution for histological processing.

Estrous phase determination: PCOS induction was confirmed using estrus cycle. The estrous phase of estrus cycle was determined through vaginal smears collected daily for 21 days between 7 am and 9 am to minimize variations due to circadian rhythms (Akintoye *et al*., 2023). To collect the smears, approximately 40 µl of normal saline was injected into the vagina of the rats using a micropipette and then aspirated in. Afterward, about 15 µl of the fluid aspirated from the vagina was placed onto a clean, air-dried slide and analyzed under a light microscope with 40x objective lenses. The estrous phase was determined based on the proportion of three types of cells: leukocytes (little round ones), epithelial cells (round and nucleated), and cornified cells (irregular anucleated ones).

Biochemical analysis: Blood samples were collected from the experimental rats through cardiac puncture 24 h after the last treatment dose. The blood samples were stored in plain bottles and separated by centrifugation at 3000 rpm/min for 15 minutes and was used for hormonal assays. The left ovaries were also harvested, homogenized in cold phosphate buffer solution and stored for biochemical analysis (Interleukin 1 B (IL-1B) and catalase).

Ovarian tissue gene expression: Right ovaries were harvested from the female rats and dissected into two longitudinal halves. The manufacturer's instructions were followed to isolate total RNA using TRIzol Reagent (ThermoFisher Scientific). DNAse I treatment (ThermoFisher Scientific) was applied to remove extracted RNA. DNA-free RNA was instantly transcribed into cDNA using the ProtoScript® First Strand cDNA Synthesis Kit (NEB). Polymerase Chain Reaction (PCR) amplification was performed using OneTaq® 2X Master Mix (NEB) with the following forward and reverse primer sets used: Nuclear factor-erythroid factor 2-related factor 2, HO-1, Glutathione reductase (GSR), Follicle-stimulating hormone receptor (FSHR), Luteinizing hormone receptor (LHR), Androgen receptor (AR), NF-κB, and Caspase 3.

Forward and Reverse Primers

NRF2:

Forward primer	GTCAGCTACTCCCAGGTTGC
Reverse primer	CAGGGCAAGCGACTGAAATG

HO-1:

Forward primer	CGACAGCATGTCCCAGGATT
Reverse primer	AGGAGGCCATCACCAGCTTA

GSR

Forward primer	CGGAAACTCGCCCATAGACT
Reverse primer	TGGACGGCTTCATCTTCAGT

FSHr

Forward primer	CATTCTTGGGCACGGGATCT
Reverse primer	GGTGAGCACAAACCTCAGTTC

LHr

Forward primer	TCACAGCTGCAGTCCCGAG
Reverse primer	GGGAGATAGGTGAGAGATAGTCTGG

AR

Forward primer	AATGTACAGCCAGTGCGTGA
Reverse primer	GCCCATCCACTGGAATAATGC

NFKB

Forward primer	TTCAACATGGCAGACGACGA
Reverse primer	AGGTATGGGCCATCTGTTGAC

Casp 3

Forward primer	GGAGCTTGGAACGCGAAGAA
Reverse primer	GAGTCCATCGACTTGCTTCCAT

The mean ± SEM (n = 6) values of the gene/Actin ratio from the 1.5% agarose in TAE buffer gel image were plotted using bar graphs. The computation of these values was done through Image-J. The representative snapshots of the pooled sample were also included.

Statistical analysis: To perform statistical analysis, Graph-pad Prism version 9 was used. A nonparametric (one-way ANOVA) test was conducted, with statistical significance set at ($p < 0.05$).

RESULTS

Effect of PCOS induction on oestrus cycle: The vaginal smears of rats with polycystic ovarian cysts showed a dioestrus aphase with a predominant leukocyte cell type upon microscopic inspection.

Effect of clomiphene and carvedilol on reproductive hormones: As shown in Table 1, PCOS significantly decreased serum GnRH, LH, FSH, estradiol, and progesterone and increased serum prolactin compared with the control. These observed alterations were reversed by CLO, CAR, and CLO+CAR treatment, although animals treated with CLO+CAR exhibited better ameliorative effects. Similarly, CLO and CAR prevented PCOS-induced significant increase in AR, LH, and FSH gene expression compared with the control when used singly or combined (Figure 1).

Table 1: Effect of Carvedilol and Clomiphene on Reproductive Hormones

	Control	PCOS	PCOS+CLO	PCOS+CAR	PCOS+CLO+CAR
GnRH(ng/ml)	4.40 ±0.4	5.03±0.23a	4.02.4±0.28b	4.12±0.26b	4.30±0.75b
FSH(mIU/ml)	0.46±0.034	0.17±0.011a	0.455±0.03b	0.36±0.039abc	0.32±0.01abc
LH (mIU/ml)	2.14±0.19	5.91±0.57a	3.47±0.35ab	3.13±0.22ab	2.33±0.38bcd
Prolactin (ng/ml)	2.68±0.11	4.2±0.21a	3.09±0.26ab	3.76±0.97abc	2.58±0.01bcd
Testosterone (ng/ml)	0.24±0.01	0.52±0.03a	0.26±0.01b	0.27±0.02b	0.25±0.01b
Estradiol (ng/ml)	3.25±0.83	1.15±0.93a	2.77±0.17ab	3.01±1.01ab	3.34±0.38bcd
sProgesterone (ng/ml)	8.04±0.11	4.65±2.01a	8.12±0.21b	8.18±0.19b	8.01±0.24b

The presented data represents the means±SEM, where n = 6. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple post hoc test. The statistical analysis showed that there were significant differences ($p < 0.05$) between the control group and the groups with PCOS, PCOS+CLO, PCOS+CAR, and PCOS+CLO+CAR. PCOS refers to Polycystic Ovarian Syndrome, CLO refers to Clomiphine, CAR refers to carvedilol, GnRH refers to Gonadotropin Releasing Hormone, FSH refers to Follicle Stimulating Hormone, and LH refers to Luteinizing Hormone. The symbols a, b, c, and d indicate the levels of statistical significance compared to each group.

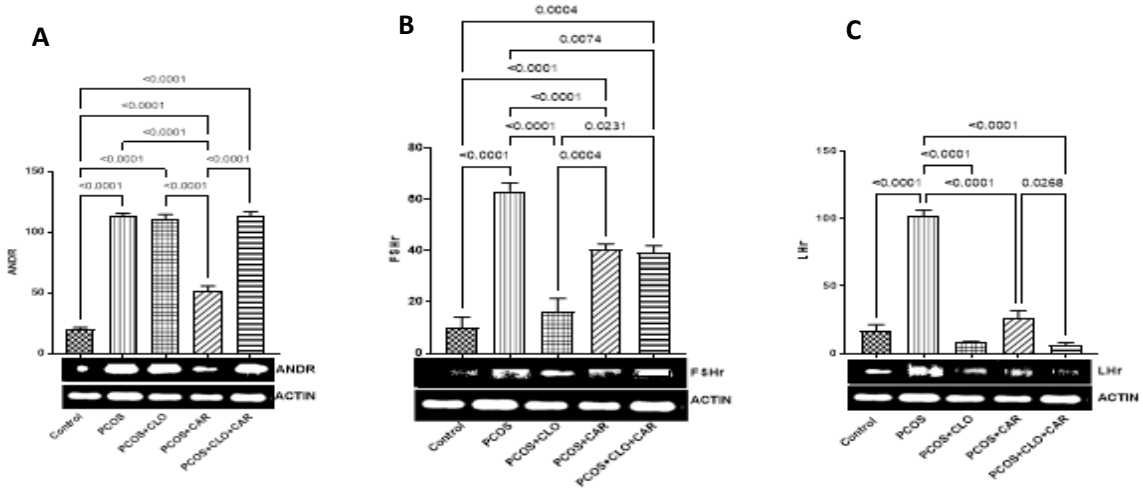

Figure 1:
Effect of Carvedilol and Clomiphene on Reproductive hormone receptors: The values for the quantified bands of the specified genes from each sample in the five groups were expressed as means±SEM, where n = 6. The data was analysed using one-way analysis of variance (ANOVA), which was then followed by Tukey's multiple post hoc test. The gel image presented is representative of the pooled samples. Each bar graph shown represents the control-normalized relative expression of the Androgen receptor (AR), Follicle stimulating hormone receptor (FSHr), and Lueutinizing hormone receptor (LHr) genes in relation to b-actin. The groups analyzed in this study include CONTROL, PCOS (polycystic ovarian syndrome), CLO (clomiphene), CAR (Carvedilol), PCOS+CLO+CAR).

Effect of Carvedilol and Clomiphene on Inflammatory markers: There was a significant increase in ovarian IL-1β and NF-κB gene expression in animals in the PCOS untreated group compared with the control. This observed difference was ameliorated by clomiphene, carvedilol, and clomiphene + carvedilol (Figure 2), although animals in the clomiphene + carvedilol treatment group exhibited a better ameliorative effect.

Effect of Carvedilol and Clomiphene on oxidative stress markers: PCOS significantly led to decreased ovarian catalase activities and downregulation of GSR, HMOX-1, and Nrf2 mRNA compared with the control (Figure 3). These observed differences were reversed in all the PCOS-treated groups, although clomiphene + carvedilol treatment was more effective in reversing the redox imbalance.

Effect of Carvedilol and Clomiphene on apoptotic markers: As shown in Figure 4, the expression of caspase 3 mRNA was upregulated in animals in the PCOS group. However, the observed upregulation was ameliorated by treatment with clomiphene and clomiphene + carvedilol.

Effect of Carvedilol and Clomiphene on histology of the ovary: The animals in the control group showed preserved ovarian outer cortex and an inner medulla. The cortical region is composed of follicles at varying degrees of maturity (Figure 5). The Graafian follicle is lined by cuboidal epithelium and contains a matured oocyte with a defined zona pellucida, zonal granulosa, antrum, and defined theca follicular cells. The medulla contained blood vessels and lymphatics. The corpus luteum appeared unremarkable. Unlike the animals in the control group, those in the PCOS showed a distorted ovarian outer cortex and an inner medulla. Furthermore, the animals treated with clomiphene and clomiphene with carvedilol showed preserved ovarian outer cortex and inner medulla and contained features consistent with the control. Although the animals treated with carvedilol also showed preserved ovarian outer cortex and inner medulla, their medulla contained severely congested blood vessels.

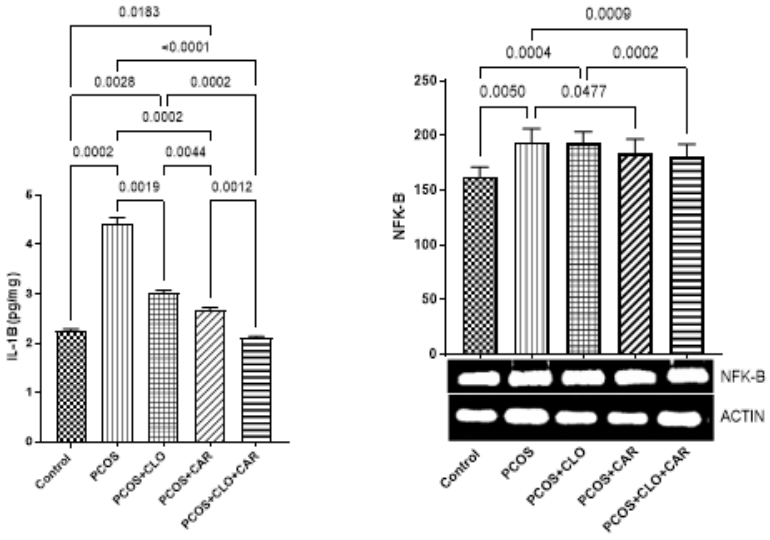

Figure 2:
Effect of Carvedilol and Clomiphene on Inflammatory markers: Serum concentration of IL-1B expressed as mean ± SEM, n=6. The values for the quantified bands of the specified genes from each sample in the five groups were expressed as means±SEM, where n = 6. The data was analysed using one-way analysis of variance (ANOVA), which was then followed by Tukey's multiple post hoc test. The gel image presented is representative of the pooled samples. Each bar graph shown represents the control-normalized relative expression of the NFKb gene in relation to b-actin. The groups analyzed in this study include CONTROL, PCOS (polycystic ovarian syndrome), CLO (clomiphene), CAR (Carvedilol), PCOS+CLO+CAR)

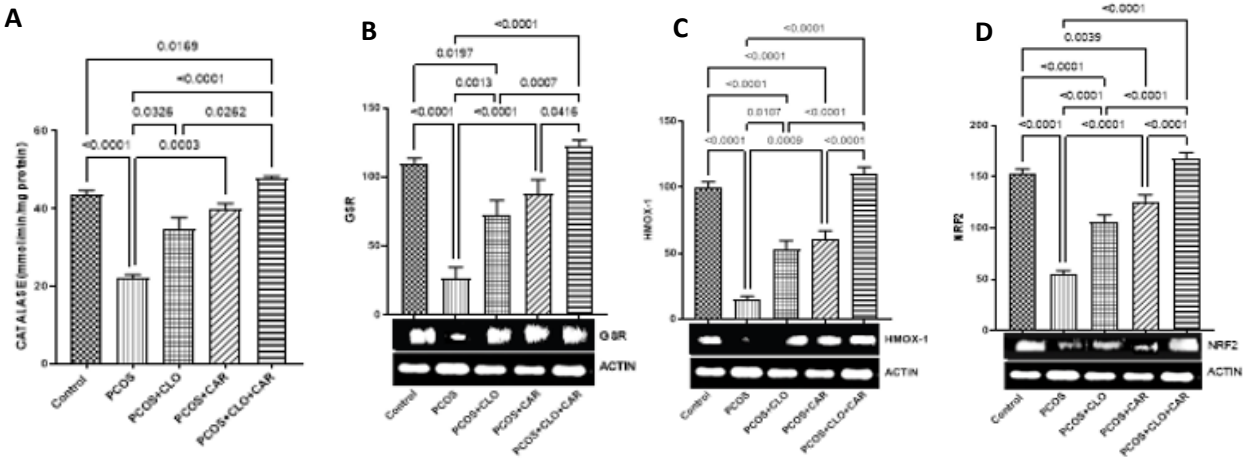

Figure 3:
Carvedilol and Clomiphene synergistically improved the antioxidant status of PCOS rats: Serum concentration of Catalase expressed as mean ± SEM, n=6. The values for the quantified bands of the specified genes from each sample in the five groups were expressed as means±SEM, where n = 6. The data was analysed using one-way analysis of variance (ANOVA), which was then followed by Tukey's multiple post hoc test. The gel image presented is representative of the pooled samples. Each bar graph shown represents the control-normalized relative expression of the GSR, HMOX-1, NRF2, gene in relation to b-actin. The groups analyzed in this study include CONTROL, PCOS (polycystic ovarian syndrome), CLO (clomiphene), CAR (Carvedilol), PCOS+CLO+CAR)

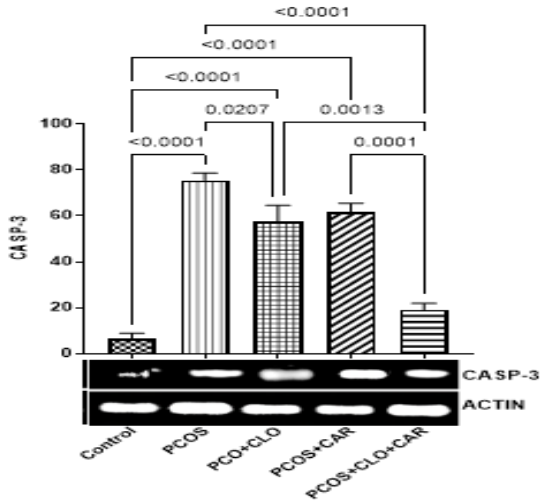

Figure 4:
Carvedilol and Clomiphene demonstrates synergistic anti-apoptotic properties: The values for the quantified bands of the specified genes from each sample in the five groups were expressed as means±SEM, where n = 6. The data was analysed using one-way analysis of variance (ANOVA), which was then followed by Tukey's multiple post hoc test. The gel image presented is representative of the pooled samples. Each bar graph shown represents the control-normalized relative expression of the Caspase 3 gene in relation to b-actin. The groups analyzed in this study include CONTROL, PCOS (polycystic ovarian syndrome), CLO (clomiphene), CAR (Carvedilol), PCOS+CLO+CAR

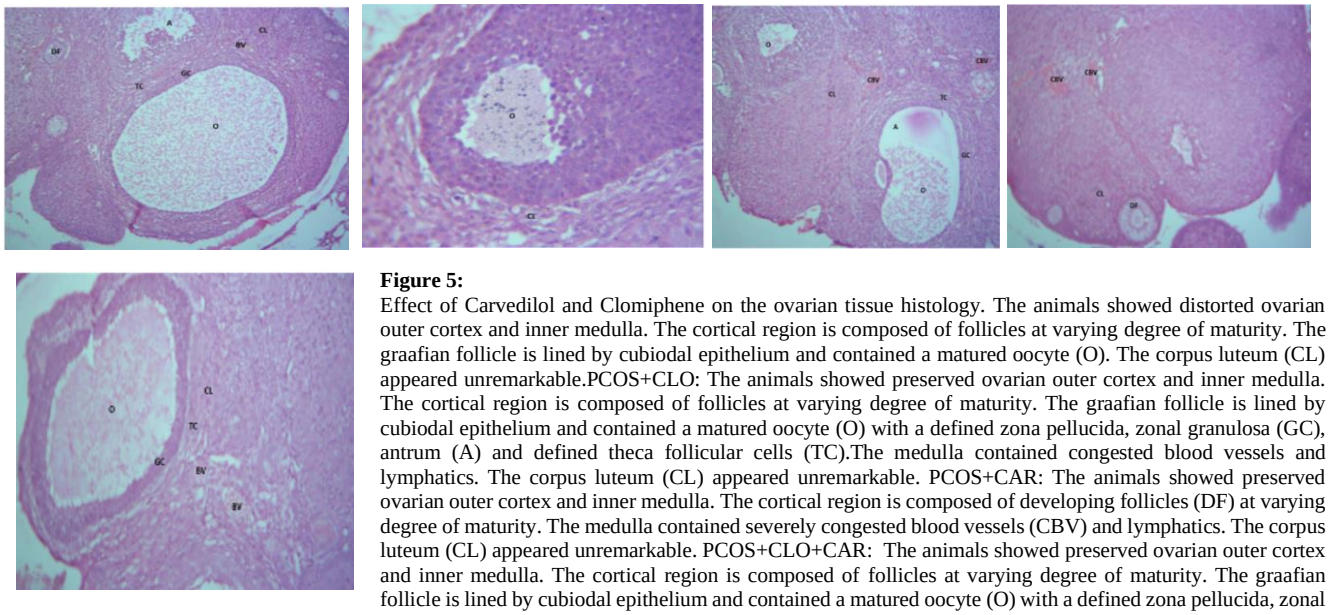

Figure 5:
Effect of Carvedilol and Clomiphene on the ovarian tissue histology. The animals showed distorted ovarian outer cortex and inner medulla. The cortical region is composed of follicles at varying degree of maturity. The graafian follicle is lined by cubiodal epithelium and contained a matured oocyte (O). The corpus luteum (CL) appeared unremarkable.PCOS+CLO: The animals showed preserved ovarian outer cortex and inner medulla. The cortical region is composed of follicles at varying degree of maturity. The graafian follicle is lined by cubiodal epithelium and contained a matured oocyte (O) with a defined zona pellucida, zonal granulosa (GC), antrum (A) and defined theca follicular cells (TC).The medulla contained congested blood vessels and lymphatics. The corpus luteum (CL) appeared unremarkable. PCOS+CAR: The animals showed preserved ovarian outer cortex and inner medulla. The cortical region is composed of developing follicles (DF) at varying degree of maturity. The medulla contained severely congested blood vessels (CBV) and lymphatics. The corpus luteum (CL) appeared unremarkable. PCOS+CLO+CAR: The animals showed preserved ovarian outer cortex and inner medulla. The cortical region is composed of follicles at varying degree of maturity. The graafian follicle is lined by cubiodal epithelium and contained a matured oocyte (O) with a defined zona pellucida, zonal granulosa (GC), antrum (A) and defined theca follicular cells (TC). The medulla contained mildy congested blood vessels and lymphatics. The corpus luteum (CL) appeared unremarkable

DISCUSSION

This study reported for the first time that clomiphene and carvedilol synergistically decrease oxidative stress, inflammatory, and apoptotic biomarkers in Letrozole-induced PCOS in female rats. Interestingly, these ameliorative effects were consistent with the maintenance of hormonal balance, expression of hormone receptors, and significant improvement in the histology of the ovary.

The findings from this study that PCOS disrupts endocrine functions by increasing GnRH, LH, testosterone, and prolactin production and decreasing FSH, estrogen, and progesterone release is similar to previous studies (Dennett and Simon, 2015; Oyebanji *et al*., 2018; Mikhael *et al*., 2019). An increase in testosterone (hyperandrogenism) is the hallmark of PCOS (Ashraf *et al*., 2019). An increase in testosterone disrupts folliculogenesis by stimulating the production of primordial follicles and elevating the number of small antral follicles (Nisenblat and Norman, 2009). Ideally, GnRH is released from the hypothalamus and acts on the pituitary gland to secrete gonadotropins (LH and FSH). While LH acts majorly on the ovarian theca cells to stimulate the production of androgens, FSH acts on the ovarian granulosa cells to act on the androgens produced from the theca cells and converts it into estrogens (majorly estradiol) which is the major female sex hormone that stimulates the development of follicles (Nisenblat and Norman, 2009). Unfortunately, PCOS is characterized by an increase in GnRH which favors the production of LH and not FSH (Fauser *et al*., 1991; Dennett and Simon, 2015), leading to the classical increase in LH/FSH ratio and, consequently increase in circulating testosterone. Due to the increase in LH secretion, the growth of the follicles in the theca cells will be arrested mostly in the preantral and antral stages leading to theca cells hyperplasia and the formation of cyst-like structure on the ovary (Abbott *et al*., 2005; Dennett and Simon, 2015). Also, the increase in testosterone has been linked with hyperinsulinemia, suggesting multiple pathways for PCOS-induced hyperandrogenism. PCOS has been shown to increase insulin production from the pancreas, which can mimic LH's tropic action on the theca cells (Wu *et al*., 2014).

Furthermore, the observed decrease in progesterone indicates a distorted menstrual cycle known as an

anovulatory cycle. The hypothalamic-pituitary-ovarian (HPO) axis promotes ovulation by selecting a dominant follicle and priming the endometrium for implantation (Cha *et al*., 2012). Once the ovary is released during ovulation, the empty follicle sac will start secreting progesterone to inhibit the hypothalamic and pituitary glands, thereby reducing estrogen production and increasing progesterone. Furthermore, the observed hyperprolactinemia from this study disagreed with the study of Yang *et al*., 2020. This could result from decreased dopamine secretion (Chaudhari *et al*., 2018) negatively correlated with prolactin secretion (Gragnoli *et al*., 2016). It is tempting to conclude that the associated infertility with PCOS results from PCOS-induced hyperprolactinemia since hyperprolactinemia has been reported to cause infertility (Odetayo and Olayaki, 2022). This study agrees with previous studies that clomiphene and carvedilol can individually ameliorate PCOS-induced hormonal imbalance (Rezyamfar *et al*., 2016; Takasaki *et al*., 2018). However, findings from this study also showed that the combination of both drugs is also effective in treating PCOS-induced hormonal imbalance.

In addition, the findings from this study that PCOS caused overexpression of AR, FSHR, and LHR supports our claim that PCOS is an endocrine disruptor and clomiphene and carvedilol synergistically ameliorated PCOS-induced hormonal imbalance. Overexpression of AR and LHR indicates an increase in testosterone and LH synthesis, while that of FSHR could result from decreased FSH synthesis. FSH-induced decrease in FSHR mRNA has previously been reported (Themmen *et al*., 1991; Griswold *et al*., 2001). In fact, an increase in FSHR expression has been reported in hypophysectomized rats (Maguire *et al*., 1997). Griswold *et al*., 2001 observed a 50% decrease in FSHR mRNA levels after 5 hours of FSH stimulation. This decrease in FSHR mRNA could be due to the ability of FSH to decrease the expression of upstream stimulatory factor 1 (USF1) and increase the accumulation of inhibitor of DNA binding/differentiation (ID)-2 repressor protein to distort transcription of FSHR (Viswanathan *et al*., 2009).

Also, this study reported that PCOS induced inflammatory response evidenced by the increase in IL-1β and NF-κB, which agreed with a previous study that chronic inflammation is one of the pathogenesis of PCOS (Rudnicka *et al*., 2021). NF-κB is activated via two main pathways, canonical and noncanonical (Liu *et al*., 2017). The canonical pathway involves responding to different stimuli, while the noncanonical response is to a specific group of stimuli. NF-κB is a major regulator of inflammatory responses. Its overexpression is a hallmark of chronic inflammatory diseases (Liu *et al*., 2017; Akhigbe *et al*., 2021) since it stimulates the release of pro-inflammatory markers such as IL-1β (Afolabi *et al*., 2022a). Since overexpression of NF-κB has been implicated in various inflammatory diseases, targeting NF-κB signaling could open a new therapeutic window in treating PCOS complications. The findings from this study that clomiphene and carvedilol synergistically ameliorate PCOS-induced overexpression of NF-κB is novel and suggests their effectiveness in treating inflammatory diseases.

Oxidative stress has been implicated in PCOS-induced ovarian damage (Mohammadi, 2019). It is due to a PCOS-led imbalance between pro-oxidants and antioxidants, resulting in ovarian damage. Furthermore, PCOS is associated with excessive adipokines generation (Lin *et al*., 2021), resulting in cytokine storm which aggravates oxidative damage leading to structural and functional cellular impairment and cell death (Akhigbe *et al*., 2021; Adeyemi *et al*., 2022). Oxidative stress also leads to the generation of reactive oxygen species (ROS), which can destroy the ovarian follicles because of the high content of polyunsaturated fatty acids in the cells (Afolabi *et al*., 2022b). The findings from this study that clomiphene and carvedilol upregulate the gene expression of Nrf2, HO-1, and GSR and serum catalase suggests their antioxidant capacity in PCOS-induced oxidative stress. Nrf2 is a cytoprotective factor that regulates the expression of proteins responsible for protecting the cell against oxidative stress, inflammation, and apoptosis (Loboda *et al*., 2016). Nrf2 regulates the expression of different cytoprotective genes, such as the ones that regulate the synthesis of glutathione and HO-1, a potent antioxidant. Apart from being an antioxidant, HO-1 also regulates different important biological activities such as inflammation, apoptosis, cell proliferation, and angiogenesis (Loboda *et al*., 2016). Putting these pieces of information together, it is tempting to conclude that combining clomiphene and carvedilol synergistically reverses PCOS-induced female infertility by acting as antioxidants via Nrf2/HO-1-dependent mechanism.

The observed PCOS-induced oxidative stress accompanied by an increase in the inflammatory response in the ovaries explains the observed PCOS- induced increase in caspase 3 gene expression. Since it has been shown that oxidative stress can stimulate inflammatory response and vice versa, PCOS could possibly lead to overwhelming oxido-inflammatory damage to the ovary. PCOS possibly induces the generation of free radicals, which stimulate caspase 3 by stimulating the intrinsic apoptotic pathway (Aitken and Koppers, 2010; Hamed *et al*., 20222). Also, the pro-apoptotic pathway may be activated by NF-κB by enhancing the phosphorylation of c-Jun and caspase 3 activations (Li *et al*., 2017). Findings from this study showed that clomiphene and carvedilol reduced apoptosis in animals with PCOS, which could result from their anti-inflammatory and antioxidant capacity since both inflammation and oxidative stress form a vicious cycle with apoptosis (Akhigbe *et al*., 2021). Also, clomiphene and carvedilol could activate peroxisome proliferator-activated receptor-γ (PPARγ), which might inhibit p65 NF-κB (involved in inflammatory response), and upregulates IkB to inhibit NF-κB, thereby halting inflammatory response (Edwards and O'Flahert, 2008; Zayed *et al*., 2018; Hassanein *et al*., 2020). PPARγ activation can also stimulate Nrf2 expression, stimulating the release of HO-1, thereby conferring cellular protection (Edwards and O'Flahert, 2008; Zayed *et al*., 2018; Hassanein *et al*., 2020).

Also, this study showed that PCOS distorts the histology of the ovary, evidenced by a distorted ovarian outer cortex and an inner medulla. This finding is in tandem with the study of Wang *et al*., 2012, who also reported morphologic differences and organelle structure changes in the ovaries of animals with PCOS.

In conclusion, this study is an extension of existing data on the pathophysiological processes of PCOS. The present study also explored the therapeutic activities of clomiphene and carvedilol in PCOS-induced ovarian dysfunction. This

study demonstrated for the first time that clomiphene and carvedilol protect the ovary by ameliorating the effect of PCOS-induced oxido-inflammatory damage and apoptosis via the stimulation of Nrf2/HMOX-1 and downregulation of NfkB signaling. This suggests that combining clomiphene and carvedilol is a novel therapeutic candidate to be explored in managing PCOS. However, clinical studies are strongly recommended to validate the protective potentials of clomiphene and carvedilol in PCOS patients.

Declarations

Ethical Approval

The animals were carefully handled as stated by the National Institute of Health (NIH), and ARRIVE guidelines for reporting experimental findings were strictly followed. The experimental research protocol was designed according to the National Research Council's guidelines for the Care and Use of Laboratory Animals, and ethical approval was obtained from the Institutional Ethical Review Committee.

Competing Interest

The authors have no conflicts of interest to declare

Authors' contributions

AAJ, AOO, OAF and OLA conceived and designed the study: AAJ, AOO, OAI, OST, OAF, BAM, and OLA carried out the experiments. AAJ, AOO, OAI, OST, OAF, BAM, and OLA contributed reagents and analytical kits. AAJ and OAF analyzed and interpreted data. OAF drafted the manuscript. AAJ, AOO, OAI, OST, OAF, BAM and OLA read and approved the final manuscript

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