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Full Length Research Article

COMBINED ORAL ADMINISTRATION OF ETHINYLESTRADIOL AND LEVONORGESTREL ALTERS THE EXPRESSION OF ANTIOXIDANT AND APOPTOTIC MARKERS IN FEMALE RATS

Wusu A.D.[1*], Bankole H.A.[1], Fatai A.A.[1], kanmodi R.I.[1], Obasieke P.E.[1], Wusu T.D. and Afolabi O.K.[2]

[1]Biochemistry Department, Lagos State University, Lagos, Nigeria.
[2]Biochemistry Department, Ladoke Akintola University of Technology, Ogbomosho, Nigeria.

*Corresponding Author Email Address: adedoja.wusu@gmail.com Phone: +2348026585027

ABSTRACT
The use of ethinylestradiol and levonorgestrel (E/L) for birth control may result in serious adverse effects. Herein, the effect of (E/L) on the expression of genes encoding antioxidant enzymes (superoxide dismutase (SOD) and Catalase (CAT)), and apoptotic regulator genes (B-cell lymphoma 2 (BCL-2), caspase 1 and 3) was evaluated. Thirty-two rats were randomly divided into four equal groups. Group A (Control) received 0.5% DMSO, Group B, C and D received 0.015, 0.030, and 0.060 mg of E/L orally and daily for 21 days respectively. RNA extracted from brain, liver and kidney were purified, and the genes amplified using RT-PCR. Following analysis of the intensity of the amplicon bands on 1.2% agarose, the gene's relative expression compared with the expression β-actin was determined. There was significant downregulation of SOD and CAT genes in the liver, brain and kidney in all groups treated with E/L compared to control. Caspase 1 and 3 expressions were significantly elevated by 3.8 and 3.5 folds, respectively in the brain at the lowest E/L concentration. Expression of BCL-2 was downregulated in the brain and kidney in animals administered E/L at all concentrations. These findings suggest that E/L could modulate the expression of antioxidant and apoptosis marker genes.

Keywords: Ethinylestradiol, Levonorgestrel, Contraceptive, Antioxidant, Apoptosis

INTRODUCTION
Family planning is essential for the health and wellbeing of women of reproductive age (15-49 years) (Adeyemi et al, 2016; Yirgu et al, 2020). A significant benefit of family planning is that it enables couples to make informed decisions on family size and birth spacing; thereby promoting high-quality life for mother, child and family (Madrigal et al, 2019). Moreover, when successful, family planning averts the consequences of unintended pregnancy such as poor antenatal and prenatal care, increased risk of congenital disabilities, and maternal depression. Longer birth interval and decreased unintended pregnancy level have also been crucial to women empowerment (Adebowale et al, 2016; Yusuf et al, 2020). As an integral part of the Sustainable Development Goals, family planning services receive huge global attention, and developing countries are significant targets. This fact reflects the 2012 London Summit on Family Planning aimed to enable 120 million additional women in the world's 69 poorest countries to use and easily access modern contraceptives by 2020 (Ahmed et al, 2019). A significant component of family planning services involves providing effective contraception methods for women who are sexually active but unwilling to get pregnant (Bitzer et al, 2016; Madrigal et al, 2019).

In addition to the traditional rhythm and withdrawal methods, there are several modern contraceptive methods such as contraceptive pills, implants, injectables, patches, vaginal rings, intrauterine devices, condoms, male and female sterilisation, lactational amenorrhea and emergency methods (de Leon et al, 2019; Wiebe et al, 2004). Globally, women of reproductive age rely more on modern contraceptive methods to meet family planning needs (Ajayi et al, 2018; Kantorová et al, 2020).

Oral contraceptives (OCs), fall under modern contraceptives' sub-category, producing short-acting and reversible effects (de Leon et al, 2019). However, the use of OCs is not exclusive to birth control, as females also rely on them to regulate menses and prevent menstrual or endometriosis pain (Cauci et al, 2016). The mechanism of action of OCs in pregnancy control mainly involves preventing ovulation and inducing changes in the female reproductive system that result in the inhibition of fertilisation and implantation (Shukla et al, 2017). OCs may contain only progestin hormone or a combination of progestin and estrogen hormones (Busund et al, 2018).
Ethinylestradiol/levonorgestrel (E/L) is a combined hormonal oral contraceptive pill comprising progestin (levonorgestrel) and estrogen (ethinylestradiol) (Hadji et al, 2020). The progestin and estrogen components are majorly metabolised by Cytochrome P450 3A4 (CYP3A4). Although E/L is well tolerated by most women and presents high contraceptive efficacy when used appropriately, there are several adverse effects (Stocco et al, 2015). Like other hormonal oral contraceptive pills, common adverse effects associated with E/L include bleeding, headache, bloating and fluid retention, nausea, breast tenderness, dysmenorrhea and a decreased libido (Stewart & Black, 2015). Studies have also reported that the use of E/L for birth control may result in serious adverse effects such as thrombotic and thromboembolic disorders, vascular problems, carcinoma of breasts and reproductive organs, hepatic neoplasia, gallbladder disease and ocular lesions (MacGregor, 2017; Shukla et al, 2017; Stocco et al, 2015).

Despite being extensively researched, the precise mechanism for the adverse effects of E/L is scarce (Shukla et al, 2017). The adverse effects of E/L might be mediated by the overproduction of reactive oxygen species (ROS) which impairs the body's antioxidant defence; thereby resulting in oxidative stress. Oxidative stress is implicated in the pathogenesis of numerous diseases, including neurodegenerative and metabolic disorders and respiratory failure and cancer (Liguori et al, 2018). A conglomeration of evidence suggests that severe oxidative stress can trigger apoptotic changes which may be detrimental to cells

(D'Arcy, 2019; Foroughi et al, 2019; Roy et al, 2017; Tiwari et al, 2016). Oxidative stress and apoptosis can combine to generate negative triggers that contribute to the pathophysiology of some chronic diseases, including cancer, diabetes mellitus, autoimmunity, Alzheimer's and Parkinson's (Sharifi-Rad et al, 2020). This study aimed to assess the gene expression of antioxidant (superoxide dismutase and catalase) and apoptotic (BCL-2, caspase 1 and 3) markers following E/L oral administration of E/L to rats.

MATERIALS AND METHODS

Experimental animals

Healthy adult female albino rats weighing between 150-200 g were used in this study. The animals were housed in standard environmental conditions (23–25 °C, 12 h/12 h, light/dark cycle) and fed with a standard rat pellet diet and water *ad libtum*. The animals were allowed to acclimatise to the laboratory conditions for two weeks before the experiment's commencement. The experimental procedures employed in this study conformed to the Institutional Animal Care and Use Committee and were approved by the Animal Ethical Committee of the Faculty of Science, Lagos State University, Nigeria.

Chemicals

Combination 3(0.03mg ethinylestradiol, 0.015mg levonorgestrel purchased from Newton Pharmaceutical Company, Lagos). Dimethyl sulfoxide (DMSO), Heparin, Normal saline, Di-ethyl ether were obtained from Biochemistry Laboratory (Lagos state university, Nigeria). RNA extraction kit were products of Aidlab Biotechnologies Co., Ltd, Beijing, China.

Drug and dose regime

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

Experimental design

Thirty-two rats were randomly divided into four groups of eight animals each. Group A (Control) received DMSO and distilled water. Group B received 0.015 mg/kg body weight of contraceptive. Group C received 0.030mg/kg body weight of contraceptive. Group D received 0.060 mg/kg body weight of contraceptive. The treatment groups were subjected to oral administration of respective daily dose regimes for 21 days, with DMSO used as the vehicle.

Tissue Collection and Analysis

At the end of the 21 days treatment, animals were fasted overnight, and sacrificed under light ether anaesthesia. The liver, kidney and brain were removed from the animals for biochemical analyses. Portions of the tissue were stabilised in RNAlater® (Aidlab Biotechnologies Co., Ltd, Beijing, China) before storage at -80°C for RNA analysis.

Extraction of RNA from organs

Extraction of RNA from animal tissues was carried out using Aidlab EASY spin Plus RNA extraction kit. 20mg of the organ was minced. 350µL of buffer RLT plus was added for lysis of the tissue. Tissues were then homogenised for about 40 seconds using an electronic tissue homogeniser. The homogenised lysate was centrifuged at 13,000rpm for 3 minutes, and the supernatant was transferred into a DNA elimination column. For 60 seconds and at 13,000rpm, the supernatant was centrifuged with flow-through collected in a 2ml collection tube (RNA is present in the flow-through). Centrifugation was done until all samples had been collected as flow through. 350µL of 70% ethanol was added to the flow-through. 700µL of the mixture was then pipetted into an RNA binding column before centrifuging for 30 seconds at 13,000rpm with the flow-through discarded. 700µL of buffer RW1 was added, and it was incubated at room temperature for 1 minute before centrifuging at 12,000rpm for 30 seconds. The flow-through was discarded. 500µL of buffer RW was then added before centrifugation for 30 seconds at 12,000rpm with flow-through discarded. The step was repeated to ensure adequate washing of any contaminant. RNA binding column was placed in the collection tube and centrifuged at 13,000rpm for 2 minutes to obliterate ethanol from the column. The column was then transferred into an RNase free microcentrifuge tube. 50µL RNase free water pre-warmed to about 90°C was added to the middle of the column to increase the RNA yield. After adding RNase free water, the mixture was incubated for 1 minute at room temperature before centrifuging at 12,000rpm for 1 minute to elute RNA. The purity and concentration of the eluted RNA template were quantified using Fisher Scientific nanodrop spectrophotometer. The absorbance ratio of 260:280 nm checked the purity. The eluted RNA template was stored at -80°C.

cDNA synthesis

The Transcriptor First-strand cDNA synthesis kit was used (Roche) according to the manufacturer's instructions. The resultant first-strand cDNA preparation was quantified using the Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies) and stored at -20 ^{o}C. (The step should be done before the RT-PCR).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

According to the manufacturer's instructions, the RT- polymerase chain reaction was carried out using the TransgenEasyScript® one-step RT-PCR Supermix. All reactions were carried out on the ice. The amplicon bands' intensity on 1.2% agarose was analysed using Image J software (Abràmoff et al, 2004). Results were presented as the relative expression of the gene compared to the level of expression of the β-actin gene.

The Sequence of Gene-Specific Primers

	Sequence (5` – 3`)	Gene Bank ID
β-Actin	Forward: GTCAGGTCATCACTATCGGCAAT	NM_031144.3
	Reverse: GAGGTCTTTACGGATGTCAACGT	
Caspase 1	Forward: CACGAGACCTGTGCGATCAT	NM_012762.2
	Reverse: GCGCCACCTTCTTTGTTCAG	
Caspase 3	Forward: GAGCTTGGAACGCGAAGAAA	NM_012922.2
	Reverse: TAACCGGGTGCGGTAGAGTA	
SOD	Forward: GCAGAAGGCAAGCGGTGAAC	NM_017050.1
	Reverse: TAGCAGGACAGCAGATGAGT	
CAT	Forward: GCGAATGGAGAGGCAGTGTAC	NM_012520.2
	Reverse: GAGTGACGTTGTCTTCATTAGCACTG	

Statistical Analysis

Data were expressed as mean ± SEM of four replicates in each group. The statistical differences across groups were determined by one-way Analysis of Variance (ANOVA). Post-Hoc analysis was

done using the Tukey test. *P* values > *0.05* was considered significant.

RESULTS

The expression level of SOD gene in the liver, kidney and brain of rats in control and treatment groups is presented in Fig. 1. The expression pattern of SOD gene shows a significant downregulation across all groups treated with E/L combined oral contraceptive as compared to the control group *(p<0.05)* in all the compartments (liver, kidney and brain). At the highest dose, liver, kidney, and brain had significantly decreased expression level of 72, 69 and 70% respectively compared to the control *(p<0.05).*

Fig 2 depicts the CAT gene expression level in the liver, kidney, and brain of rats after 21 days of administration of different E/L combined oral contraceptive concentrations. CAT gene expression levels were significantly downregulated compared to the control *(p<0.05).* In the liver, the decrease in the expression of the CAT gene was dose-dependent. However, the inhibition of CAT gene expression in the kidney and brain by the E/L OCs amounted to 61% and 57% decrease at the highest concentration.

Fig. 3 shows the gene expression of BCL-2 in the liver, brain and kidney of animals in the control and experimental groups. E/L OCs on the gene expression in the brain and kidney samples of treated rats were dose-dependent. Also, the OCs inhibited the expression of BCL-2 in the kidney. However, its effect was reduced at the highest concentration. The difference in the gene expression level in treated samples was statistically significant compared to the control *(p<0.05).*

The expression of caspase 1 in the liver, brain and kidney of animals in the control and experimental groups is presented in Fig. 4. The results indicated no significant effect of the OC on the expression of Caspase-1 in the liver; however, the OC inhibited the expression of Caspase-1 gene in the brain at higher concentrations. The increase in expression in the kidney was dose-dependent.

Fig. 5 shows the gene expression level of caspase 3 in the liver, brain and kidney of rats in the control and experimental groups. E/L inhibited the expression of Caspase-3 at the lowest concentration (0.015mg/ml) in the brain and kidney and the expression in the liver at a concentration of 0.03mg/ml.

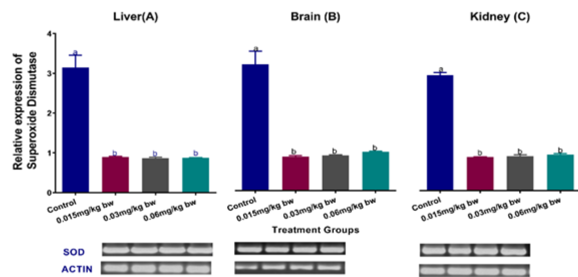

Figure 1: Relative expression of Superoxide Dismutase in the animals' liver, brain, and kidney after exposure to E/L oral contraceptive at different concentrations. Bars with different alphabets are significantly different from each other at p<0.05 compared to control.

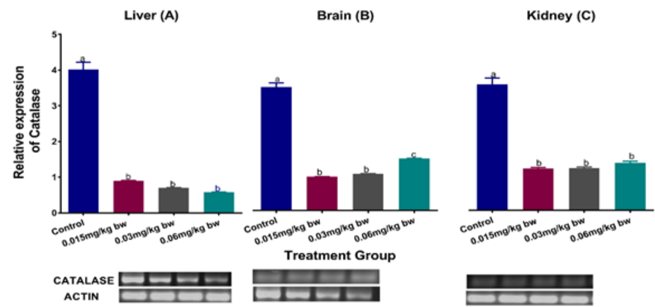

Figure 2: Relative expression of catalase in the animals' liver, brain, and kidney after exposure to E/L oral contraceptive at different concentrations. Bars with different alphabets are significantly different from each other at p<0.05 compared to control.

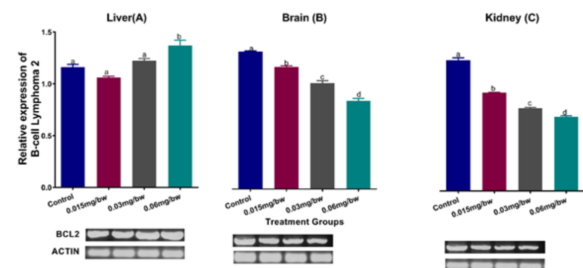

Figure 3: Relative expression of B- cell Lymphoma 2 in the animals' liver, brain, and kidney after exposure to an oral contraceptive. Bars with different alphabets are significantly different from each other at p<0.05 compared to control.

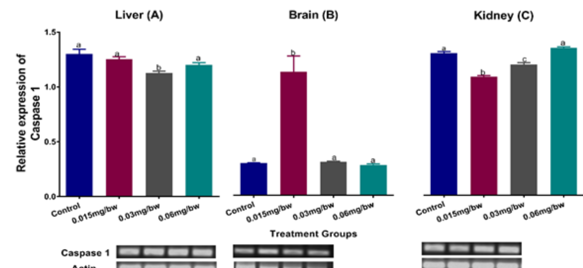

Figure 4: Relative expression of Caspase 1 in the animals' liver, brain, and kidney after exposure to an oral contraceptive. Bars with different alphabets are significantly different from each other at p<0.05 compared to control.

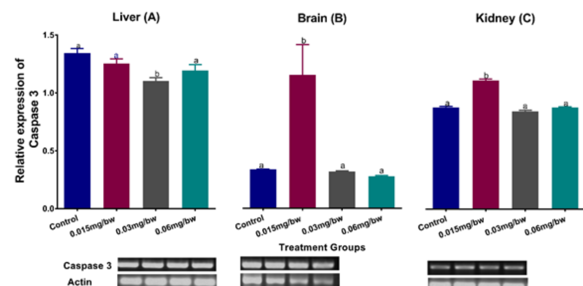

Figure 5: Relative expression of Caspase 3 in the animals' liver, brain, and kidney after exposure to an oral contraceptive. Bars with different alphabets are significantly different from each other at p<0.05 compared to control.

DISCUSSION

Combined oral contraceptives are becoming increasingly popular among women of reproductive ages (de Oliveira et al, 2019; Stocco et al, 2015). Several studies have attributed the use of combined oral contraceptives to the overproduction of free radicals (Cauci et al, 2016; Chen & Kotani, 2012; Finco et al, 2012; Jendryczko et al, 1993). The production and accumulation of free radicals beyond the antioxidant system's scavenging power result in oxidative stress and consequent damage to the primary cellular components including lipids, carbohydrates, proteins and DNA (Liguori et al, 2018; Salim, 2017). Therefore, the antioxidant defence is essential in counteracting free radicals' deleterious effects, thereby preventing subsequent damage to body tissues and organs such as liver, brain, and kidney (Olutope, 2015; Perrone et al, 2018; Poprac et al, 2017). Superoxide dismutase (SOD), catalase (CAT) and peroxidase are antioxidant enzymes that have been identified in organisms (Dar et al, 2019). While SOD catalyses the conversion of superoxide radical anion ($O_2^{\bullet -}$) to hydrogen peroxide (H_20_2), catalase, as well as glutathione peroxidase, are responsible for the subsequent detoxification of H_20_2 to water (H_20) and oxygen (0_2) (Zuo et al, 2019).

The assessment of SOD and CAT activities is a standard method for evaluating antioxidant potential (Alam et al, 2013; Karthishwaran et al, 2018). In this study, rather than assessing SOD and CAT's enzymatic activities, efforts were directed towards investigating the expression of the genes encoding them. This study's findings show that E/L administered to animals significantly decreased CAT and SOD genes expression. Studies have reported changes in the level of antioxidant enzymes in subjects administered E/L contraceptive (Jendryczko et al, 1993; Mushafau et al, 2010). A strong positive correlation may exist between the expression of genes and the level of activity of enzymes that they encode (Aminfar et al, 2019; Arvas et al, 2011). A change in gene expression pattern usually impacts the translation and activity of enzymes (Buccitelli & Selbach, 2020). Although it is unclear why E/L downregulated the expression of CAT and SOD genes in this study, the data suggest that E/L (even at a low concentration) could lead to oxidative stress as a result of low antioxidant enzymes and consequently their activities which are required to match the levels of free radicals which could have been triggered. This explanation is consistent with the finding of Jendryczko et al (1993) which revealed that erythrocyte SOD, CAT, and glutathione peroxidase activities dropped after oral administration of E/L at a low-dose. A reduction in antioxidant defence's physiological activity against free radicals could be a direct consequence of oxidative stress (Palan et al, 2010; Poljsak & Milisav, 2013). Finco et al (2011) also reported that there was an elevation of oxidative stress parameters in the erythrocyte of healthy volunteers following oral administration of ethinylestradiol 50 mcg plus levonorgestrel 125 mcg.

Another critical biochemical process that has been widely studied in the pathogenesis of diseases is apoptosis (Wong, 2011). Apoptosis is thought to be a highly conserved evolutionary process and is responsible for the programmed death of cells, which is critical for normal tissue development and differentiation as well as in the maintenance of cellular homeostasis (Green & Fitzgerald, 2016; Hofmann, 2020; Tzifi et al, 2012). To control cell death and survival, there must be proper regulation and coordination of molecular elements that prevent or enhance the process of apoptosis (Galluzzi et al, 2018). In this study, we investigated the gene expression of well-known regulators of apoptosis, including BCL-2 (B-cell lymphoma 2), caspase 1 and caspase 3.

The human genome encodes over 10 distinct caspases (a class of cysteine proteases) involved in cell death by apoptosis as well as pyroptosis, necroptosis and autophagy (Shalini et al, 2015; Tsapras & Nezis, 2017; Zhang et al, 2015). The activation of caspases from inactive zymogen forms is essential for their involvement in apoptosis (Fan et al, 2017; Hu et al, 2013). The caspase cascade activation leads to the recruitment of several caspases that participate in either the intrinsic or extrinsic pathways of apoptosis. Apoptosis via initiator caspase 8 (extrinsic pathway) or caspase 9 (intrinsic pathway) results in the activation of effector caspase 3/7 (Hu et al, 2013). Apoptosis is executed by caspase 3/7, which themselves are activated by either caspase 8 (extrinsic apoptosis pathway) or caspase 9 (intrinsic apoptosis pathway) (Quintana et al, 2016; Renema et al, 2020). Since caspase 1 activates initiator caspase 8/9, it also plays a role in the activation of caspase 3 (Kinsella & Stallings, 2020).

This study revealed that the modulatory pattern of E/L on the gene expression level of caspase 1 and caspase 3 in the brain was different from that of the liver and kidney. Unlike the liver and kidney, the gene expression of caspase 1 and caspase 3 in the brain appeared lower in the control group as compared to the E/L treated subjects. Su et al (2019) has reported a diminished gene expression of caspase 1 and caspase 3 in the brain of control groups (cognitively normal subjects) as compared to the brain of the experimental subjects (Alzheimer patients). Interestingly, caspase 1 and caspase 3 gene expressions were significantly elevated in the brain at the lowest E/L concentration. Thus, it suggests that a low dose of E/L contraceptive could initiate programmed cell death in the brain. There was no consistency in the modulation of caspase 1 and caspase 3 expression in the liver and kidney. However, their expression was upregulated at the lowest and highest concentration of E/L in the kidney. The observed increase in the gene expression of caspase 1 and caspase 3 may be associated with the anti-apoptotic BCL-2 gene's downregulation, which is consistent with the findings of Khazaei et al (2017); and Vizetto-Duarte et al (2016).

The activation of caspases via proteolytic cleavage is regulated by BCL-2 (B-cell lymphoma) family proteins encoded by the *BCL2* gene (Tzifi et al, 2012). These proteins are generally classified as anti-apoptotic or pro-apoptotic based on the BCL-2 homology (BH) domains present (Stevens & Oltean, 2019). The anti-apoptotic family comprises of BCL-X (possesses multiple BH3 domains), MCL-1 (myeloid leukaemia sequence 1) and BCL-2 (the first identified member of the family) (Warren et al, 2019). The gene expression of BCL-2 (a member of the anti-apoptotic BCL-2 family) was the target of this study. The action of BCL-2 in the inhibition of apoptosis is not only limited to the regulation of caspases as it also inactivates other pro-apoptotic proteins such as BAX (BCL-2 associated X protein) (Tzifi et al, 2012).

Here, we show downregulation of BCL-2 gene expression in the brain and kidney at all concentrations of E/L and in a dose-dependent manner. This could be due to the decreased gene expression of antioxidant enzymes (CAT and SOD). This could result in laxity in defence by antioxidants leading to a surge in free radical levels. The induction of apoptosis by free radicals and a compromised antioxidant defence has been widely reported (Ahn

et al, 2011; Banu et al, 2009; Lavrentiadou et al, 2001; Li et al, 2004; Luanpitpong et al, 2011). Li et al (2004) reported that ROS (Reactive Oxygen Species) suppressed expression of BCL-2 and enhanced the expression of BAX. Contrary to the BCL-2 gene expression pattern in the brain and kidney, higher concentrations (0.03 and 0.06 mg) of E/L produced an upregulation of BCL-2 in the liver.

Conclusion
Combined oral contraceptive (ethinylestradiol and levonorgestrel) altered the expression of genes encoding antioxidant enzymes (CAT and SOD) as well as anti-apoptotic (BCL-2) and pro-apoptotic (caspase 1 and caspase 3) regulators in the brain, liver and kidney of rats. Taken together, the findings in this study suggest that E/L acting via deteriorating antioxidant defence could promote oxidative stress-induced apoptosis.

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