Histological and Immunohistochemical Alterations of the Amygdala of Female Wistar Rats Administered Oral Ethinyl Estradiol and Levonogestrel Combination

Ekanem TB,[1] *Nwakanma AA,[2] Eluwa MA,[1] Ekong MB[3]

ABSTRACT

Combined oral contraceptive pills containing two synthetic hormones, Ethinyl estradiol and Progestin were evaluated in this study. Mood and brain structure changes in individuals on combined oral contraceptives are inconsistent, warranting the histological and immunohistochemical studies of the Amygdala of female Wistar rats administered oral Ethinyl estradiol and Levonogestrel combination. Thirty-two female Wistar rats weighing between 180g and 200g were equally divided into four groups (1-4). Control and groups administered 0.002mg/kg Levonogestrel plus 0.00043mg/kg Ethinyl estradiol for 21, 42 and 63 days. Twenty-four hours after the last administrations, the rats were anaesthetized and the brains perfusion-fixed transcardially with 10% buffered Formalin. The whole brain was excised and fixed in 10% buffered Formalin for 48 h. The Amygdala was dissected, processed for paraffin sectioning, stained with Haematoxylin and Eosin and immunolabelled with anti-glial fibrillary acidic protein. Histological results of the Amygdala showed hypertrophied nuclei in the test groups, especially in the 63 days Levonogestrel plus Ethinyl estradiol group. These test groups also showed GFAP positive cells, whose expression was increased in the 21 and 42 days Levonogestrel plus Ethinyl estradiol groups, except in the 63 days Levonogestrel plus Ethinyl estradiol group which decreased compared with the control. In conclusion combined oral Ethinyl estradiol plus Levonogestrel induced cellular alteration and reactive Astrocytes in the Amygdala, except in the 63 days Levonogestrel plus Ethinyl estradiol group which was decreased implying stress due to chronic exposure.

Keywords: Oral, Contraceptive, Amygdala, Histology, Immunohistochemistry, Wistar

INTRODUCTION

Hormonal pill use is the most common contraceptive method in the world.[1-3] They come as single or combined oral contraceptives containing two synthetic hormones, Ethinyl estradiol and Progestin, which act by altering the natural fluctuation of gonadal hormones and consequently suppresses ovulation.[4,5] Contraceptives are taken primarily to prevent pregnancy; however, their use is known to affect brain activity. Neuroimaging during contraceptive use revealed that oral contraceptives affect emotional reactivity and fear learning systems such as the Amygdala, Hippocampus and ventromedial Prefrontal cortex.[4-7] The Amygdala is well known for its role in modulating memory for emotionally arousing stimuli, and studies have shown that the Amygdala's response to emotional stimuli can be influenced by sex hormones.[8-11]

Department of Anatomical Sciences,[1] University of Calabar, Calabar, Nigeria
Department of Anatomy,[2] Chukwuemeka Odumegwu Ojukwu University, Uli, Nigeria
Department of Anatomy,[3] University of Uyo, Uyo, Nigeria

*Corresponding author: akudoekeoma@yahoo.com

Longitudinal and cross-sectional studies assessing contraception related mood changes yielded inconsistent results. Nevertheless, the use of hormonal contraceptives was found to be associated with either increase, decrease or no overall change in negative effect.[1,2,4,6,10] It is unclear whether the inconsistency of findings reflect actual differences between women in their mood-related responses to hormonal contraceptives or whether they are due to differences in study design or pill types.[12] Goldstein *et al*. reported that menstrual phases influence Amygdala activity, while increased Amygdala response to emotional stimuli has also been reported.[13-15] This pattern of reactivity across the menstrual cycle suggests an attenuating effect of Estrogen on the Amygdala and agonistic effects of Progesterone in naturally cycling women.

Structural and functional investigations revealed baseline changes to the brain in women using hormonal contraceptives. In a structural brain analysis, hormonal contraceptive use significantly

increased grey matter volume in the Pre- and Postcentral gyri, Parahippocampal gyrus, Fusiform gyrus and the Superior, Middle and Inferior temporal gyri.[13] White mater differences have also been observed in the Fornix with higher mean diffusivity scores in oral contraceptive users.[16] Potential associations of mood changes and brain structural changes induced by hormonal contraceptives are still unclear. The Amygdala, a brain structure necessary in this regard has been associated with many neuropsychiatric diseases, and many studies revealed its effects on depression.[17,18] This study, therefore, investigated the Amygdala micro structural changes of Wistar rats administered a combined oral contraceptive.

MATERIALS AND METHODS

Thirty-two female Wistar rats weighing between 180g and 200g were obtained from Chris Farm in Awka, Anambra State, Nigeria. Ethical approval was obtained from the University of Calabar Ethical Committee, and the animals were handled according to the guidelines for animal care of the United States' National Institute of Health. The animals were allowed access to rat chow (Vital Feeds Limited, Nigeria) and water *ad libitum* throughout the experimental period and maintained in the animal house of the Faculty of Basic Medical Sciences Chukwuemeka Odumegwu Ojukwu University, Uli. The animals were acclimatized for two weeks under standard laboratory conditions before and throughout the experiment.

The combined oral contraceptive pill used was purchased from a Sheffield Pharmacy, World Bank, Owerri, Nigeria. Each beige coloured tablet contained 0.15 mg Levonorgestrel and 0.030 mg Ethinyl estradiol. Each tablet was ground to powder using a glass mortar and dissolved in 175 mL of water. Therapeutic dosages were calculated per bodyweight of the animal. The animals were equally divided into four groups: 1-4. Group 1 animals were the control and did not receive any drug treatment, groups 2-4 received 0.002 mg/kg Levonorgestrel plus 0.00043 mg/kg Ethinyl estradiol (LEE), orally, for 21, 42 and 63 days. The animals were sacrificed 24 h after Ketamine anaesthesia (40 mg/kg i.p). The brains were perfusion fixed with 10% buffered Formalin and post-fixed for 48 h. Each animals' Amygdala was dissected and processed for Paraffin embedding. Sections were cut at 10 µm and routinely processed for Haematoxylin and Eosin (H & E) staining, as well as immunolabelled with anti-glial fibrillary acidic protein (GFAP).

For the immunolabelling, serial paraffin sections on slides were brought to water and antigen retrieval was performed in Citrate buffer (pH 6.0) in a microwave oven for 5 minutes. This was followed by protein blocking with 3% Hydrogen peroxide for 10 minutes. Sections were thereafter pre-incubated in 2% normal Goat serum for 30 minutes and incubated in Mouse monoclonal anti-GFAP (Novocastra, Leica Biosystems, NCL-L-GFAP-GA5, 1:100) for an hour. This was followed by 30 min incubation in Goat anti-mouse secondary antibody (1:100). Detection of the reaction was utilizing the Avidin-biotin complex, with Diaminobenzidine as the chromagen. Sections were then counterstained with Harris haematoxylin, dehydrated, cleared and cover-slipped with DPX. Processed slides were viewed under the light microscope and photomicrographs obtained using a computer-assisted digital microscope's camera.

RESULTS

Histology of the central Amygdaloid cells of the control group revealed normal histological features - there were numerous cell types of different sizes. The central amygdaloid cells of the 21 days LEE group showed slight hypertrophy compared with the control. The central Amygdaloid cells of the 42 days LEE group showed slight hypertrophy compared with the control. The central Amygdaloid cells of the 63 days LEE group showed hypertrophy compared with the control (Figure 1).

Immunolabelling

The central Amygdaloid cells of the control group showed GFAP positive cells, with expression in the soma and processes.

The central Amygdaloid cells of the 21 days LEE group showed GFAP positive cells whose expression appear similar to the control, but with less of the expression seen in the soma. The central Amygdaloid cells of the 42 days LEE group showed increased expression of GFAP positive cells, with less of the expression seen in the soma. The central Amygdaloid cells of the 63 days LEE group showed less expression GFAP positive cells, with less of the expression seen in the soma (Figure 2).

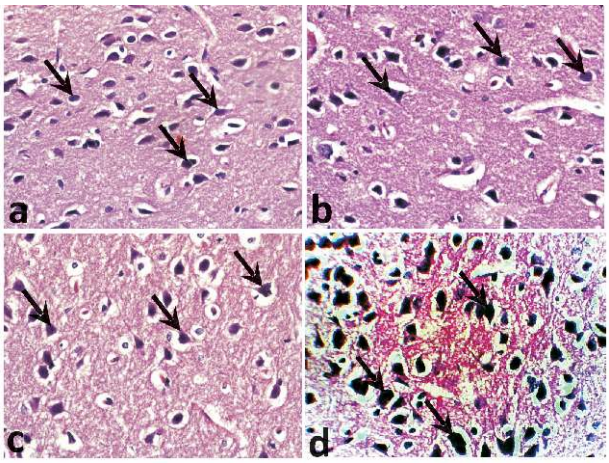

Figure 1: Sections of the central Amygdaloid cells

a. The control group revealed normal cells of different sizes (arrows).
b. The 21 days LEE group showed slight hypertrophied cells (arrows).
c. The 42 days LEE group showed slight hypertrophied cells (arrows).
d. The 63 days LEE group showed hypertrophied cells (arrows).

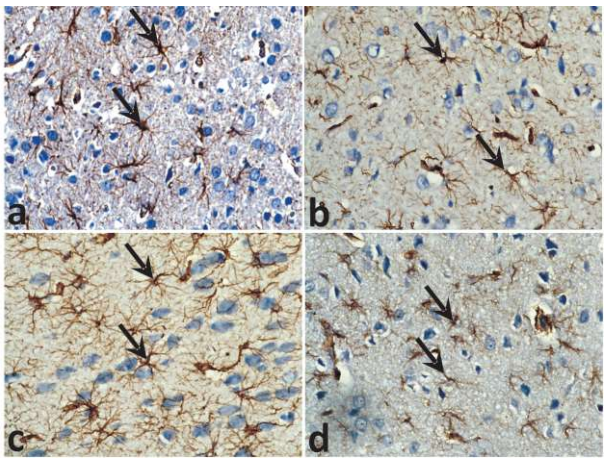

Figure 2: The sections of the central Amygdaloid cells

a. The control group showing GFAP positive cells (arrows), with expression in the soma and processes.
b. The 21 days LEE group showing GFAP positive cells (arrows) whose expression appear similar to the control, but with less of the expression seen in the soma.
c. The 42 days LEE group showing increased expression of GFAP positive cells (arrows), with less of the expression seen in the soma.

DISCUSSION

This study investigated the histological and immunohistochemical effects on the Amygdala of female Wistar rats administered oral Ethinyl estradiol and Levonorgestrel combination. The histology of the Amygdala of the test groups, and especially the 63 days Levonorgestrel plus Ethinyl estradiol group showed hypertrophied cells. Hypertrophy is a cellular alteration resulting in increased cellular size, which may be physiological or pathological. Physiological hypertrophy arises when the tissue tries to cope with insults, as in this case with Levonorgestrel plus Ethinyl estradiol, which may be reversible.[19] In pathological

conditions, cells are traumatized to a point of degeneration, where hypertrophy is one of the processes, and in this instance may be irreversible.[19,20]

Combined oral contraceptive is reported to significantly increase grey matter volume in the Cerebrum, which may have also occurred in the Amygdala of the present study.[13] Several studies have shown that the Amygdala's response to emotional stimuli can be influenced by sex hormones.[10,11] Oral contraceptives reduce Amygdala's reactivity to emotional stimuli and changes to the brain of women using hormonal contraceptives have been reported.[13,15]

The test groups also showed GFAP positive cells, whose expression was increased in the 21 and 42 days Levonorgestrel plus Ethinyl estradiol groups, except in the 63 days Levonorgestrel plus Ethinyl estradiol group which decreased. GFAP is a structural cytoskeletal protein, which also serves as a marker for Astrocytes.[21] Increased GFAP expression as seen in the present study may indicate Astrogliosis which occurs in reactive astrocytes.[22] The presence of reactive Astrocytes is an indication of the early stage of neuronal cell damage, which may have been the case in the present study.[23]

Decreased GFAP expression as observed in the 63 days Levonorgestrel plus Ethinyl estradiol group may indicate Astrocytic stress and degeneration changes.[24] This is because oral contraceptives have been implicated in stress, which is one of the aetiologies of depression known to affect brain areas involved in emotion, including the Amygdala.[25,26] Decreased GFAP expressions have also been reported in both stress and depressive conditions.[27,28] This may also explain the reduction of reactive Astrocytes in the 63 days treated group. The present result is at variance with a previous report by Nwakanma *et al.*, which may be due to the brain area involved.[29]

The Amygdala is a part of the limbic system necessary for emotion and behaviour control. It functions in regulating anxiety, aggression, fear conditioning, emotional behaviour and social cognition.[19] This function may be impaired with the structural alteration observed in the present study, and especially in the 63 days Levonorgestrel plus Ethinyl estradiol group.

CONCLUSION

The present study showed that oral intake of Levonorgestrel plus Ethinyl estradiol leads to cellular alteration in microstructure and GFAP expressions in the Amygdala, which was duration dependent. Hence, prolonged intake of the combined Levonorgestrel plus Ethinyl estradiol may be deleterious to the Amygdala.

REFERENCES

1. Champaloux SW, Tepper NK, Mosour M, Curtis KM, Whiteman MK, Marchbanks PA, *et al*. Use of combined hormonal contraceptives among women with migraines and risk of ischemic stroke. *Am J Obstet Gynecol*. 2017;216:489.e1-489.e7.
2. Lee JJML, Tan TC, Ang SB. Female sexual dysfunction with combined oral contraceptive use. *Singapore Med J*. 2017;58:285-8.
3. Busund M, Bugge NS, Braaten T, Waaseth M, Rylander C, Lund E. Progestin-only and combined oral contraceptives and receptor-defined premenopausal breast cancer risk: the Norwegian woman and cancer study. *Int J Cancer* 2018;142:2293-302.
4. Toffoletto S, Lanzenberger R, Gingnell M, Sundstrom-Poromaa I, Comasco E. Emotional and cognitive functional imaging of estrogen and progesterone effects in the female human brain: a systematic review. *Psychoneuroendocrinology* 2014;50:28-52.
5. Cooper DB, Mahdy H. Oral contraceptive pills. StatPearls Publishing: *Treasure Island (FL)* 2019.
6. Montoya ER, Bos PA. How oral contraceptives impact social-emotional behaviour and brain function. *Trends Cogn Sci*. 2017;21:125-36.
7. Lewis CA, Kimmig AS, Zsido RG, Jank A, Derntl B, Sacher J. Effects of hormonal contraceptives on mood: a focus on emotion recognition and reactivity, reward processing and stress response. *Curr Psychiatry Rep*. 2019;21:115.