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Ameliorative Effect of Melatonin on Reproductive Hormones in Ethanol Extracts of *Cannabis sativa*-Treated Female Wistar Rats

[1] A. Oluwasola, [2]O.E. Ayoola., [3] A. F. Odetayo., [1]Garba Saa'du., [4]L.A. Olayaki*
1. Department of [1]Human Physiology, Faculty of Health Sciences, Al-Hikmah University, P.M.B. 1601, Ilorin, Nigeria.
2. Department of [2]Public Health, Faculty of Basic Medical Sciences, Kwara State University, Malete P.M.B. 1530, Ilorin, Nigeria.
3. Department of [3]Physiology, College of Medicine, Federal University of Health Sciences, Ila-Orangun, Nigeria.
4. Department of [4]Physiology, Faculty of Basic Medical Sciences, University of Ilorin, P.M.B. 1515, Ilorin, Nigeria.

Abstract
This study investigated the effects of melatonin and ethanol extracts of Cannabis-sativa on reproductive hormones in female rats. Twenty female rats were assigned into four groups of five animals each, such that the rats in groups 1, 2, 3and 4 received orally 1mL distilled water, 2mg/kg of ethanol extract of Cannabis sativa (EECS), 2mg/kg of EECS+4mg/kg of melatonin and 4mg/kg of melatonin, respectively for 14 days. Gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH), luteinizing hormone (LH), oestradiol (E), progesterone and prolactin were quantified according to the instruction provided by assay kit manufacturers, using microplate immunoenzymometric (EMA/ELISA) assays. EECS significantly ($p<0.05$) decreased GnRH, FSH, LH, E, progesterone and prolactin levels respectively when compared with the control. However, co-administration of melatonin with EECS significantly ($p<0.05$) increased all the aforementioned reproductive hormones to the level comparable with the control. Histological study of hypothalamus and pituitary gland revealed that EECS-treated rats showed scanty and shrink nissl bodies and severe degeneration of acidophils and basophils when compared with the control. These pathological changes were ameliorated by melatonin. This study concluded that the effects of CS on reproductive hormones could be mediated by damage to the endocrine system. However, these effects of EECS on the reproductive hormones could be ameliorated by melatonin. The study recommended that consumption of melatonin could be used as supplement to prevent the side effects of EECS on the reproductive hormones.
Keywords: *Cannabis-sativa*; Melatonin; Endocrine system; Reproductive hormones

Introduction
Cannabis is an annual herbaceous flowering plant obtained from the flowering tops, leaves and resin from the female plant of *Cannabis sativa* L. (family *Cannabidaceae*) [1]. It is the most commonly abused illicit drugs worldwide [2] with medicinal uses [3]. The active component, Δ^9-tetrahydrocannabinol (Δ^9-THC), has been used for treating migraine headache, glaucoma, nausea, and anorexia. However, its detrimental effects on reproductive system have been reported. For instance, it has been shown to be spermatotoxic in male [4] and ovotoxic in female [5] [6]. Marijuana is one of the commonly abused substances by women of childbearing age [7]. It has been reported that about 64-79% of female are cannabis users nation wide [7] which can lead to pregnancy loss [8], low birth weight [9], prematurity [10], intrauterine growth retardation, presence of congenital abnormalities, prenatal death and delayed the time of commencement of respiration [11]. Cannabinoids have also been reported to have negative effects on the activity of gonadotropin releasing hormone (GnRH)-secreting neurons by direct and indirect mechanisms [12]. It also has direct effect on the pituitary gland through its receptors [13]. Moreover, it has been shown to have direct oestrogenic effect on the uterus [14] leading to the binding of 3β-estradiol to oestrogen receptors [14]. It also has direct effect on the ovary [15] thus, inhibiting ovarian prostaglandin synthesis which is involved in follicular rupturing during ovulation [16].
Melatonin (N-acetyl-5-methoxytryptamine) is obtained from serotonin and was first discovered and isolated in the pineal gland of cows [17]. It is expressed in the darkness because its highest level always coincides with the dark phase of light/dark cycle [18]. It is secreted in the pineal gland and other extra-pineal sources like retina, gut, skin, bone marrow, lymphocytes, and ovaries [19]. Its ability to scavenge free radicals like hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), hypochlorous acid (HOCl), peroxynitrite anion ($ONOO^-$), nitric oxide ($NO^{\bullet}$), and others in many conditions [20] directly by free radical scavenging actions [21]. Its role in reproduction has been contradictory, as both detrimental and beneficial effects have been reported [22].
This study investigated the effects of melatonin and EECS on reproductive hormones in female rats.

Corresponding Author's E-mail: droluwasola@alhikmah.edu.ng

Materials and Methods

Animals

Twenty (20) female albino rats (135-163g) were used for this experiment. They were obtained from the Department of Biochemistry, University of Ilorin, Ilorin, Kwara State, Nigeria, were housed at room temperature with unrestricted access to diet and water and maintained on a daily light/dark cycle. Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed.

Extraction of *Cannabis sativa* leaves

Extraction of *Cannabis sativa* (*CS*), which was kindly donated by National Drug Law Enforcement Agency (NDLEA), Nigeria, for research purpose only, was done with Soxhlet apparatus by soaking 800 grams of CS in 98% ethanol for 48 hours. It was filtered and the filtrate was poured into a round bottom conical flask which was fixed with a rotary evaporator. It was then evaporated and cooled. The dried yield of the extract was 62%.

Experimental protocol

After 2 weeks of acclimatization, twenty female rats (135-163g) rats were assigned into
four groups of five animals each, such that the rats in groups I, II, III and IV received orally 1mL distilled water, 2mg/kg body weight (BW) of EECS, 2mg/kg BW of EECS plus 4mg/kg BW of melatonin and 4mg/kg BW of melatonin, respectively for 14 days.

Preparation of serum

The female rats were sacrificed under ketamine anesthesia and blood was collected from the heart puncture into sample bottles. The blood was left for 30 min to clot and thereafter centrifuged at 625×*g* for 10 min using a Uniscope Laboratory Centrifuge (Model SM800B, Surgifield Medicals, Essex, England). The serum was collected into plain bottles with the aid of a Pasteur pipette. Sera were stored in a freezer maintained at -5 ℃ and used within 12 hours of preparation.

Quantification of reproductive hormones

The serum hormone concentrations of GnRH, FSH, LH, E, progesterone and prolactin were quantified according to the instruction provided by assay kit manufacturers, using microplate immunoenzymometric (EMA/ELISA) assays. The serum hormone concentrations were then interpolated from their respective calibration curves. The analyzer was calibrated and validated for use with rat sera. All the assay kits used were products of Monobind Inc., Lake Forest, California, USA. All other chemicals used were products of Sigma Aldrich Company, Mannheim, Germany.

Histological Studies

The hypothalamus and pituitary gland were removed from the animals, blotted in blotting paper, cut into small pieces, fixed in 10% buffered formalin, dehydrated through ascending grades of ethanol (70%, 90% and 95% v/v), cleaned in xylene and embedded in paraffin wax (melting point, 56 ℃) (Krause, 2001). Tissue sections (5 to 6 µm) were prepared according to the procedure described by Drury and Wallington (Drury and Wallington, 1980) and stained with hematoxylin and eosin (H&E). The processed histological slides were read with a light microscope (Olympus, Model CX21FSI, China). Photomicrographs of the ovaries were captured at a ×400 magnification with Presto Image Folio Package Software.

Statistical analysis

Results were expressed as the mean ± standard error of mean. Data were analyzed using a one-way analysis of variance, followed by the LSD post-hoc test to determine significant differences in all the parameters with Students Package for Social Science, version 20.0 (SPSS Inc., Chicago, USA). Differences with values of $P<0.05$ were considered statistically significant.

Results

EECS significantly ($p<0.05$) decreased all the reproductive hormonal levels (FSH, LH, Oestrogen, Progesterone, Prolactin and GnRH) of EECS-treated groups when compared with the control. However, co-administration of melatonin with EECS significantly ($p<0.01$) increased all the aforementioned reproductive hormones to the level comparable with the control (table 1).

Table 1: Effect of different concentration of EECS on serum reproductive hormones of female wistar rats

	GnRH (pg/ml)	FSH (mlU/ml)	LH (mlU/ml)	Oestradiol (pg/ml)	Progesterone (ng/ml)	Prolactin (ng/ml)
Control	32.24±3.93[a]	7.83±1.52[a]	7.00±1.97[a]	75.0±11.07[a]	13.00± 0.63[a]	16.82±2.39[a]
Cannabis	11.38±0.77[c]	5.04±0.95[c]	4.17±0.20[c]	38.32 ±11.2[c]	2.78±0.36[c]	7.82±0.59[c]
Cannabis+Melatonin	21.75±0.96 [c]	6.93±1.14[c]	6.59±1.02[c]	63.32±9.42[c]	7.92±1.17[c]	9.18±1.12[c]
Melatonin	29.43±1.32[c b]	7.17±0.79[c b]	6.86±1.04[c b]	71.68±6.39[c b]	8.41±1.09[cb]	9.50±0.88[c b]

Values were expressed as mean±SEM, [a]p<0.05 vs treated groups; [c]p<0.05 vs control, [b]p<0.01 vs 2mg/kg BW and 4mg/kg BW respectively

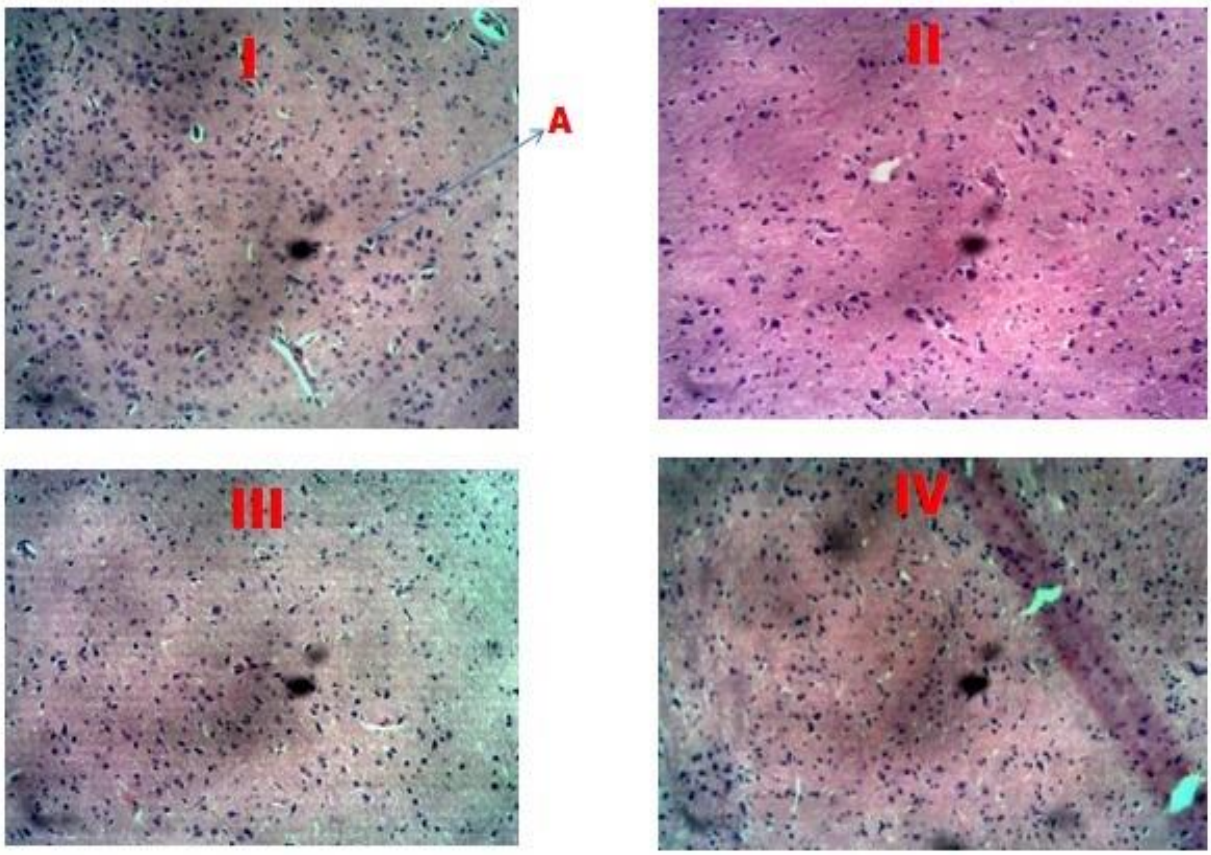


Fig. 4.1: Histological examination of the hypothalamus (Nissl staining, ×400). Photomicrographs of the cross section of hypothalamus of female rat orally administered distilled water,

Control (I) showing numerous nissl bodies, EECS, 2mg/kg body weight (B.W.) (II) showing scanty and shrink nissl bodies, cannabis, 2mg/kg B.W.+melatonin, 4mg/kg B.W (III) showing moderate nissl bodies, and melatonin, 4mg/kg B.W. (IV) showing numerous nissl bodies for 14 days. A -Nissl body

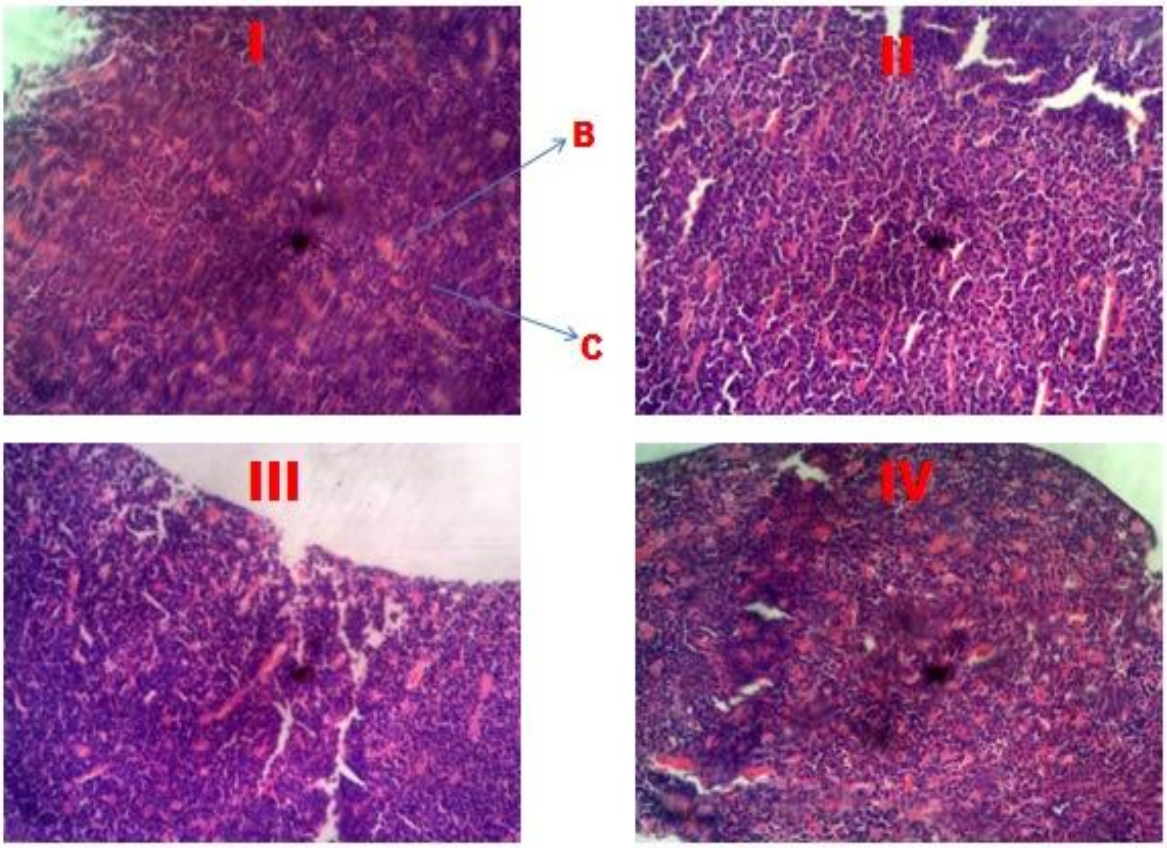


Fig. 2: Histological examination of the pituitary gland (hematoxylin and eosin staining, ×400). Photomicrographs of the cross section of pituitary gland of female rat orally administered distilled water,

Control (I) showing intact structural architecture of acidopils and basophils, EECS, 2mg/kg body weight (B.W.) (II) showing severe degeneration of acidopils and basophils, cannabis, 2mg/kg B.W.+melatonin, 4mg/kg B.W (III) showing mild degeneration of acidopils and basophils, and melatonin, 4mg/kg B.W. (IV) showing intact structural architecture of acidopils and basophils for 14 days. B-acidophil, C-basophil

Discussion

Reproductive hormones such as estrogen and progesterone play an important role in pregnancy and childbirth. These hormones increase steadily over the course of pregnancy and drop suddenly following delivery, typically returning to pre-pregnancy levels within two weeks [23]. Given their important role in the gestational process, many experts have speculated that these reproductive hormones and other biological factors, such as stress hormones, immune and inflammatory markers, and genetic and epigenetic factors, play a role in the development of postpartum depression [24]

Although research implicating the role of reproductive hormones and postpartum depression is still evolving, there is some evidence for the role of stress hormones, such as cortisol, β-endorphin, and corticotropin-releasing hormone (CRH). These hormones are implicated in depression more broadly and may be activated during pregnancy. For example, evidence supports associations between unusually high elevations of corticotropin-releasing hormone (CRH) in mid-to-late pregnancy and postpartum depressive symptoms during the initial months following delivery [24]. Study has shown that the entire endocannabinoid system is active at the ovarian level and cannabinoid receptors 1 and 2 (CB_1R and CB_2R) and anadamide (AEA), have been identified in ovarian tissue [25]. Immunostaining showed expression of CB_1R and CB_2R in the medulla and cortex of the ovary. In the cortex, the receptors are expressed in the granulosa cells of primordial, primary, secondary and tertiary follicles and in the theca cells of secondary and tertiary follicles. Both receptors have also been observed in the corpus luteum and corpus albicans. In vertebrates, many authors have reported inhibitory effects exerted by endocannabinoids on the reproductive physiology in both sexes [26]. In this study, decrease in the level of GnRH, FSH, LH, estradiol and

progesterone levels in the EECS-treated rats could be due to the inhibitory effect of *Cannabis sativa* on the GnRH neurons in the hypothalamus [25]. These effects were reduced by melatonin when co-administered with EECS which could be due to the circadian role of melatonin on reproductive hormones [27]. Some studies have reported increase in prolactin level following the administration of *Cannabis sativa* to both male and female rats [28]. However, the decrease in the prolactin level which was observed in the this study following the administration EECS could be due to the stimulatory activity of dopaminergic neurons in the hypothalamus by the *CS* [29]. These effects were reduced by melatonin when co-administered with EECS which could be due it ability to stimulate nocturnal prolactin release [30]. More so, the hypothalamus and pituitary gland of EECS-treated rats showed scanty nissl bodies (which are proteins that are essential for the production of GnRH hormones in the hypothalamus) and severe degeneration of acidophils and basophils (cells secreting prolactin and gonadotropes in the pituitary gland). These effects could have probably led to the decrease in the reproductive hormones observed in this study. These pathological changes were ameliorated by melatonin.

5. Conclusion

This study concluded that effect of CS on reproductive hormones could be mediated by damage to the endocrine system. However, this effect of EECS on the reproductive hormones could be ameliorated by melatonin. The study recommended that, consumption of melatonin could be used as supplement to prevent the side effects of EECS on the female reproductive hormones.

6. Conflict of Interests

The authors declare no conflict of interests. The authors alone are responsible for the writing and content of the papers.

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