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Effects of Melatonin on Estrous Cycle Changes Induced by Ethanolic Extract of *Cannabis-sativa* in Female Wistar Rats

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Abstract
This study investigated the effects of melatonin and ethanolic extract of Cannabis-sativa (EECS) on oestrus cycle and reproductive hormones in reproduction of female rats. Twenty female 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. Vaginal smear was taken daily to determine the Stages of oestrus cycle using light microscope (x400) between 8am-9am. Gonadotropin releasing hormone (GnRH), Luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol (E), progesterone and prolactin were also determined using standard methods. EECS significantly ($p<0.05$) lengthened the metestrus and diestrus phases of oestrus cycle. However, it significantly ($p<0.05$) shortened the proestrus and estrus phases. CS significantly ($p<0.05$) decreased GnRH, FSH, LH, E, progesterone and prolactin levels respectively. All these effects were ameliorated when combined with melatonin to the level comparable to the control. This study showed the gonadotoxic effects of EECS which could be mediated by endocrine disruption. However, these effects could be ameliorated by melatonin. Since the consumption of CS is increasing globally because of its medical uses leading to its legalization, therefore, consumption of melatonin as supplement is recommended for its users to prevent its gonadotoxic effects.

Keywords: *Cannabis-sativa*, Melatonin, Oestrus cycle, Sexual behaviour, Reproductive hormones

Introduction
According to the International Programme on Chemical Safety (IPCS), endocrine disruptor is exogenous substance that affect the function(s) of the endocrine system (IPCS 2002). Cannabis is obtained from the flowering tops, leaves and resin of the female plant of Cannabis sativa L. (family Cannabidaceae), it is the most commonly abused illicit drugs worldwide [1] with medicinal uses [2]. The active component, Δ9-tetrahydrocannabinol (Δ9-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 [3] and ovotoxic in female [4]. Marijuana is one of the commonly abused substances by women of childbearing age [5]. Statistically, it has been reported that about 3.2% of females are cannabis smokers in Nigeria [6]. It has also been reported that about 64-79% of female are cannabis users nation wide [5] which can lead to pregnancy loss [7], low birth weight [8], prematurity [9], intrauterine growth retardation, presence of congenital abnormalities, prenatal death and delayed the time of commencement of respiration [10]. Cannabinoids have also been reported to have negative effects on the activity of gonadotropin releasing hormone (GnRH)-secreting neurons by direct and indirect mechanisms [11] [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 [15]. It also has direct effect on the ovary [16] leading to the inhibition of ovarian prostaglandin synthesis which is implicated in the mechanism of follicle rupture at ovulation [17].

Melatonin (N-acetyl-5-methoxytryptamine), 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 (•OH), singlet oxygen (1O2), hydrogen peroxide (H2O2), superoxide anion (O2•-), hypochlorous acid (HOCl), peroxynitrite anion (ONOO-), nitric oxide (NO•), 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].

Oestrus cycle is a reproductive cycle of female which consists of proestrus, estrus, metestrus and diestrus [23]. The identification of each phase is based on the proportion among three types of cells observed in the vaginal smear: epithelial cells, cornified cell and leukocytes. Ovulation occurs from the beginning of proestrus to the end of estrus [24]. From the onset of sexual maturity (2 to 3 months) up to the age of 12 months, the mean cycle

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length in the female rat is 4 days [25] and this short cycle makes the rat an ideal animal for investigation of changes occurring during the reproductive cycle [26]. This study investigated the effects of cannabis on oestrus cycle and reproductive hormones together with the ameliorative effects of melatonin in female rats.

Materials and Methods

Animals

Twenty (20) female albino rats (150-160g) were used for this experiment. They were obtained from the Department of Biochemistry, University of Ilorin, Ilorin, Kwara State, Nigeria, 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 [27].

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 300 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 35.2%.

Experimental protocol

After 2 weeks of acclimatization, animals were randomized into four groups (I–1V) of five animals each. Animals in Groups I, II, III and IV were given orally 1mL of distilled water, 2mg/kg body weight (b.wt) of ethanolic extract of *Cannabis-sativa* (EECS), 2mg/kg b.wt of EECS plus 4mg/kg b.wt of melatonin and 4mg/kg BW of melatonin, respectively. The doses were administered once daily for 14 days. The animals were sacrificed under ketamine anesthesia after two weeks of the last treatment.

Drug and assay kits

Melatonin was a product of of Sigma Aldrich Company, Mannheim, Germany. The gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol (E), progesterone and prolactin assay kits were products of Monobind Inc., Lake Forest, California, USA. All other chemicals used were products of Sigma Aldrich Company, Mannheim, Germany.

Determination of stages of oestrus cycles

Vaginal smears were taken daily to determine the Stages of oestrus cycle using light microscope (x400) between 8am-9am. A small amount of a physiological saline solution was inserted into the vagina of the rats with a disposable pipette, removed, placed on a slide and examined under the microscope. The four stages were distinguished by noting characteristic cell types that were visible during each stage. The stages were checked for period of two weeks prior to treatment, two weeks during treatment and two weeks after treatment [28].

The experimental protocol was approved by Ethical Committee of the University of Ilorin, Ilorin, Kwara State, Nigeria (Ref. UERC/ASN/2018/1152), University of Ilorin, Ilorin, Nigeria.

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 [27].

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

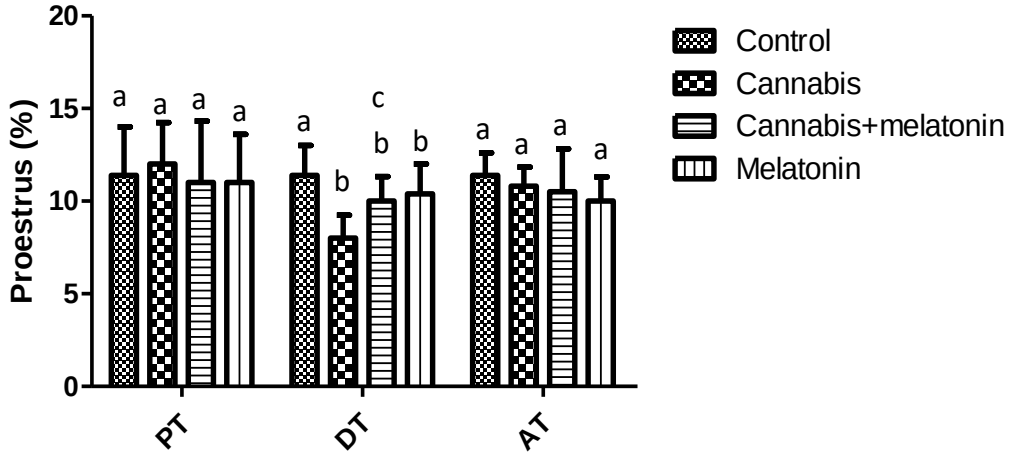

Fig. 4.1: Percentage (%) of proestrus of rats that received EECS with or without melatonin, and melatonin alone. Values are expressed as mean ± S.E.M.

NB: PT – prior to administration; DT – during administration; AT – after administration

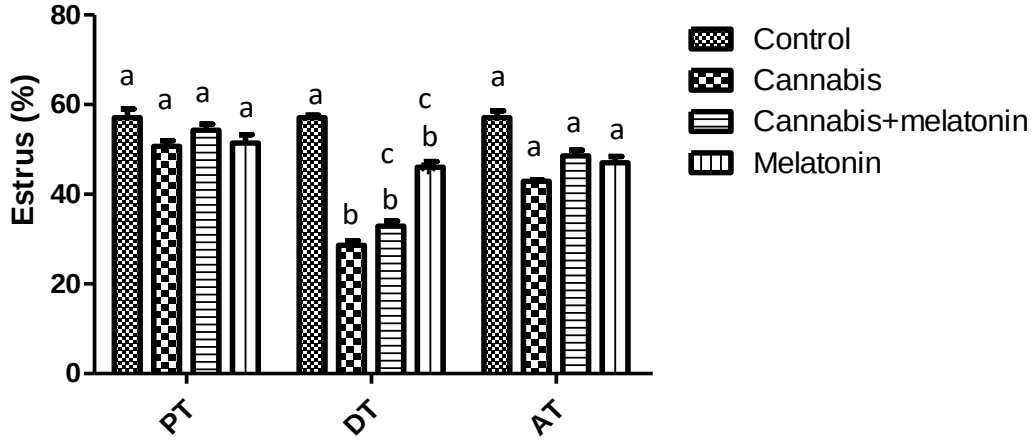

Fig. 4.2: Percentage (%) of oestrus of rats that received EECS with or without melatonin, and melatonin alone. Values are expressed as mean ± S.E.M.

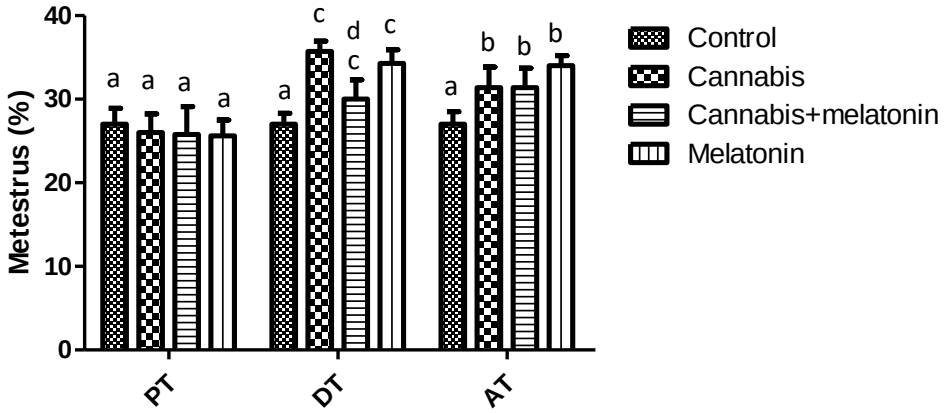

Fig. 4.3: Percentage (%) of metestrus of rats that received EECS with or without melatonin, and melatonin alone. Values are expressed as mean ± S.E.M.

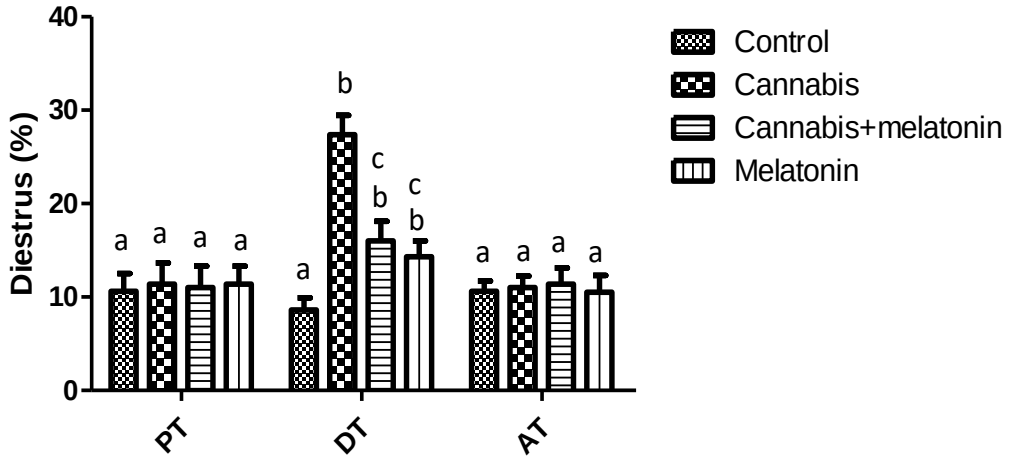

Fig. 4.4: Percentage (%) of diestrus of rats that received EECS with or without melatonin, and melatonin alone. Values are expressed as mean ± S.E.M.

Table 1: Serum reproductive hormones of female Wistar rats following oral administration of distilled water, 2mg/kg body weight (BW) of ethanolic extract of *Cannabis-sativa* (EECS), 2mg/kg BW of EECS and 4mg/kg BW of melatonin, and 4mg/kg BW of melatonin.

	GnRH (pg/ml)	FSH (mlU/ml)	LH (mlU/ml)	Estradiol (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[b]	5.04 ± 0.95[b]	4.17 ± 0.20[b]	38.32 ±11.2[b]	2.78± 0.36[b]	7.82± 0.59[b]
Cannabis+ Melatonin	21.75 ± 0.96[b a]	6.93 ± 1.14[b c]	6.59 ± 1.02[b c]	63.32± 9.42[b c]	7.92± 1.17[b c]	9.18±1.12[b c]
Melatonin	29.43 ± 1.32[b]	7.17 ± 0.79[c]	6.86 ± 1.04[c]	71.68± 6.39[c]	8.41± 1.09[b c]	9.50±0.88[b c]

Values along the column with different superscript are significantly different at 0.05 level of significant.

Discussion

Oestrus cycle is a reproductive cycle of female rats which consists of proestrus, estrus, metestrus and diestrus [23]. Ovulation occurs from the beginning of proestrus to the end of oestrus [24]. From the onset of sexual maturity (2 to 3 months) up to the age of 12 months, the mean cycle length in the female rat is 4 days and this short cycle makes the rat an ideal animal for investigation of changes occurring during the reproductive cycle [25]. The identification of each phase is based on the proportion among three types of cells observed in the vaginal smear: epithelial cells, cornified cell and leukocytes. In this study, there was a significant lengthening of the metestrus and dioestrus of cannabis-treated group which was shortened when administered with melatonin. This could be due to low level of LH of cannabis-treated group which was raised when administered with melatonin. This study is consistent with the report that Δ^9-THC suppressed the proestrus rise in the plasma LH in rats [29]. This study showed that melatonin may be used to suppress the inhibitory effect of cannabis on the plasma LH in rats. An irreversible of metestrus and a reversible of diestrus were observed for all the treated groups after treatment. The reason for this irreversibility of metestrus could be due to residual effect of the treatment. There was also a significant shortening of proestrus of cannabis-treated group which was lengthened when administered with melatonin. This may be due to decrease in estradiol level of cannabis-treated group which was elevated when combined with melatonin. Proestrus is characterized by rise in estradiol produced by the developing follicles [30]. There was a significant shortening of estrus for cannabis-treated group compared with the other groups which was lengthened when administered with melatonin. This may be due to decrease in LH and estradiol levels of cannabis-treated group which was elevated when combined with melatonin. This was consistent with the finding of Senger [30]. The estrus phase was reversed to normal level after two weeks of treatment for all the treated groups. Normal female reproductive functions depend on the secretion of LH and

FSH by the pituitary gland under the influence of hypothalamic gonadotropin-releasing hormone (GnRH). In females, LH stimulates the theca cells of the ovaries to secrete progesterone while FSH induces the granulosa cells of the growing follicles to produce E. Therefore, the decrease in the levels of GnRH, LH, FSH, oestrogen and progesterone observed in the cannabis-treated group may be ascribed to an inhibitory effect on the hypothalamic-pituitary axis which was ameliorated when combined with melatonin. The lowering of serum prolactin levels observed in the cannabis-treated group could be due to the antioestrogenic nature of 'THC' [31].

Conclusion

This study concluded that the gonadotoxic effects of EECS could be mediated by endocrine disruption. However, these effects could be ameliorated by melatonin. Since the consumption of CS is increasing globally because of its medical uses leading to its legalization, therefore, consumption of melatonin as supplement is recommended for its users to prevent its gonadotoxic effects.

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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