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Research Article

Ameliorative effect of combined melatonin and vitamin C on *Cannabis sativa*-induced reproductive hormonal toxicity

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Keywords:
Cannabis sativa; *Endocrine disruption; Hyperprolactinemia; Hypothalamic-pituitary-gonadal axis; Melatonin; Vitamin C.*

ABSTRACT

Background: Decline in fertility seen in *Cannabis sativa* (CS) consumers has been related to its influence on reproductive hormones but the mechanism(s) involved is not fully understood. Moreover, the possible beneficial or detrimental effect of melatonin and vitamin C on cannabis-associated effects on reproductive hormones is yet to be investigated and necessitated this study. **Methods**: Fifty-five (55) male albino rats (250-300g) were randomly divided in a blinded fashion into 5 oral treatment groups as follow: Group I (control, n=5) received 10% ethanol (1 ml/kg) for 30 days. Groups 2, 3, and 4 consisted of 15 rats each that were subdivided to receive CS (2 mg/kg) only, CS (2 mg/kg)+melatonin (4 mg/kg), and CS (2 mg/kg)+vitamin C (1.25 g/kg) respectively for 20-, 30-, or 40 days (n=5 rats each). Group V (n=5) received CS (2 mg/kg) + melatonin (4 mg/kg)+vitamin C (1.25 g/kg) for 30 days. **Results**: The CS reduced gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), and testosterone but increased estradiol and prolactin. In addition, co-administration of *CS* with melatonin and vitamin C abolished the effect of cannabis on these parameters when combined but not when administered separately (except for prolactin and GnRH). **Conclusion**: Cannabis causes downregulation of hypothalamic-pituitary-gonadal axis, endocrine disruption, and hyperprolactinemia. These effects (except hyperprolactinemia) could be reversed by melatonin and vitamin C only when combined but not when administered separately.

INTRODUCTION

Cannabis sativa (marijuana), the most widely used illicit drug in the world, is a "chemical factory", producing more than 500 different chemical compounds (Rosales-Corral *et al*., 2015). Various mental health problems like impaired memory and attention, reduced motor skills, anxiety, and addiction have been observed in some of its users (Degenhardt *et al.*, 2010; Hall and Solowij, 1998). Its legalization has been increasing globally due to its documented beneficial effects in the management of many diseases like cancer (Velasco *et al*., 2016), inflammation (Juknat *et al*., 2016), pain (Lynch, 2016), and epilepsy (Tzadok *et al*., 2016).

Although it is well known that chronic marijuana use transiently decreases male fertility in animal models and humans (Murphy *et al.*, 1994), the mechanism involved remain unclear. Some studies have implicated reduction in some male reproductive parameters such as testosterone secretion, sperm production, sperm motility, sperm viability, luteinizing and follicle stimulating hormone as possible culprits (Nahas *et al*., 2002; Schuel and Burkman, 2005; Alagbonsi *et al.*, 2016).

Vitamin C (ascorbate) acts as a potent water-soluble anti-oxidant in biological fluids by scavenging physiologically relevant reactive oxygen species and reactive nitrogen species (Halliwell, 1996). Melatonin (N-acetyl-5-methoxy-tryptamine) is the main pineal hormone synthesized from tryptophan, predominantly at night (Arendt, 1995). Melatonin is critical for the regulation of circadian and seasonal changes in various aspects of physiology and neuroendocrine function (Pevet *et al.*, 2002). Being a potent naturally occurring anti-oxidant (Zhang and Zhang, 2014), its beneficial effect on male fertility would be expected as there are numerous links between oxidative stress and male factor infertility (El-Tohamy, 2012). However, decline

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in progression of spermatogenesis, testosterone, human chorionic gonadotropin binding sites, and luteinizing hormones (LH) at the end of juvenile period have been reported in immature male rats treated with melatonin (Olivares *et al.*, 1989). Even in female mammals, it has been evaluated as an oral contraceptive due to its ability to inhibit pre-ovulatory LH surge (McElhinny *et al.*, 1996).

Because melatonin is produced endogenously and also occurs naturally in some foods (Reiter *et al.*, 2015), it is being sold as a dietary supplement in the U.S. under the dietary supplement Health and Education Act of 1994 without pre-market approval from the Food and Drug Administration. Vitamin C has also been grossly abused by users because of its wide perception as an anti-oxidant. Moreover, legislation and decriminalization of cannabis possession is increasing worldwide. This will lead to an increasing use of cannabis and consequently an increase in infertility. There is therefore the need to be proactive in solving the reproductive health problems that may arise from increasing use of cannabis due to its decriminalization.

We have recently shown that the cannabis-induced gonadotoxicity is partly mediated by oxidative stress. Furthermore, melatonin and vitamin C deteriorated cannabis-induced gonadotoxicity when administered separately but ameliorated it when combined in rats (Alagbonsi *et al.*, 2016). However, the mechanism involved in the regulation of reproductive hormones by cannabis is not fully understood. Moreover, the possible beneficial or detrimental effect of melatonin and vitamin C on cannabis-associated effects on reproductive hormones is yet to be investigated and is of interest to us.

MATERIALS AND METHODS

Animals

Fifty five male albino rats (250-300g) obtained from the Department of Biochemistry, University of Ilorin, Kwara State, Nigeria, were housed at room temperature with free access to food and tap water *ad libitum* and maintained on a daily light/dark cycle. In addition to experimental protocol approval by our institutional ethics committee, "principles of laboratory animal care (NIH publication No. 85-23, revised 1985)" were followed.

Extraction of Cannabis Sativa leaves

Cannabis sativa leaves (CS), which was kindly donated by National Drug Law Enforcement Agency (NDLEA), Nigeria, for research purpose only, was done with 98% ethanol in Soxhlet apparatus for 4-8 hours as previously described (Dixit *et al.*, 1974; Mandal and Das, 2010; Alagbonsi *et al*., 2016). Its percentage yield was 21.2%.

Experimental protocol

After 2 weeks acclimatization to their new environment with standard laboratory diet and water given *ad libitum*, the animals were randomly divided in a blinded fashion into 5 oral treatment groups as follow: Group I (control, n=5) received 10% ethanol (1 ml/kg) for 30 days. Groups 2, 3, and 4 consisted of 15 rats each that were subdivided to receive CS (2 mg/kg) only, CS (2 mg/kg)+melatonin (4 mg/kg), and CS (2 mg/kg)+vitamin C (1.25 g/kg) respectively for 20-, 30-, or 40 days (n=5 rats each). Group V (n=5) received CS (2 mg/kg)+melatonin (4 mg/kg)+vitamin C (1.25 g/kg) for 30 days.

After determining the lethal dose that killed 50% of the treated animals (Yassa *et al.*, 2010), the 2 mg/kg dose of CS was arrived at as the tenth of its LD_{50}. Ethanol was used to dissolve CS extract, while normal saline was used to dissolve melatonin and vitamin C (Biopharma Nigeria Ltd.). Animals were sacrificed a day after the last treatment under ketamine anesthesia and plasma was collected from each sample and preserved at -20 °C.

Estimation of reproductive hormones

Enzyme-linked immunosorbent assays of Testosterone (Monobind Inc., Lake Forest, CA, USA. Product Code: 3725-300), Estadiol (Monobind Inc., Lake Forest, CA, USA. Product Code: 4925-300), Progesterone (Monobind Inc., Lake Forest, CA, USA. Product Code: 4825-300), Luteinizing Hormone (Monobind Inc., Lake Forest, CA, USA. Product Code: 625-300), Follicle Stimulating Hormone (Monobind Inc., Lake Forest, CA, USA. Product Code: 425-300), Prolactin (Monobind Inc., Lake Forest, CA, USA. Product Code: 725-300), Gonadotropin Releasing Hormone (Elabscience, catalog No: E-EL-R0451c) were done spectrophotometrically (Spectramax plus, Molecular devices, Sunnyvale, CA, USA) following the kits' manufacturer procedures.

Data processing

Data were analyzed with one-way ANOVA using SPSS version 16.0 (IBM Corporation, Armonk, NY, USA), followed by a post-hoc Least Significance Difference (LSD) test for multiple comparisons. Data were presented as the Mean ± SEM. p values ≤ 0.05 were considered statistically significant.

RESULTS

Effects of cannabis with(out) melatonin and/or vitamin C on testosterone in rats

Cannabis administration to rats caused reductions in testosterone concentrations at 20 days ($p<0.01$), 30 days ($p<0.001$), and 40 days ($p<0.01$) when compared

to control. Administration of cannabis+melatonin caused more reductions in testosterone concentrations than that caused by cannabis alone at 20 days (p<0.001), 30 days (p<0.001), and 40 days (p<0.001) when compared to control. The testosterone

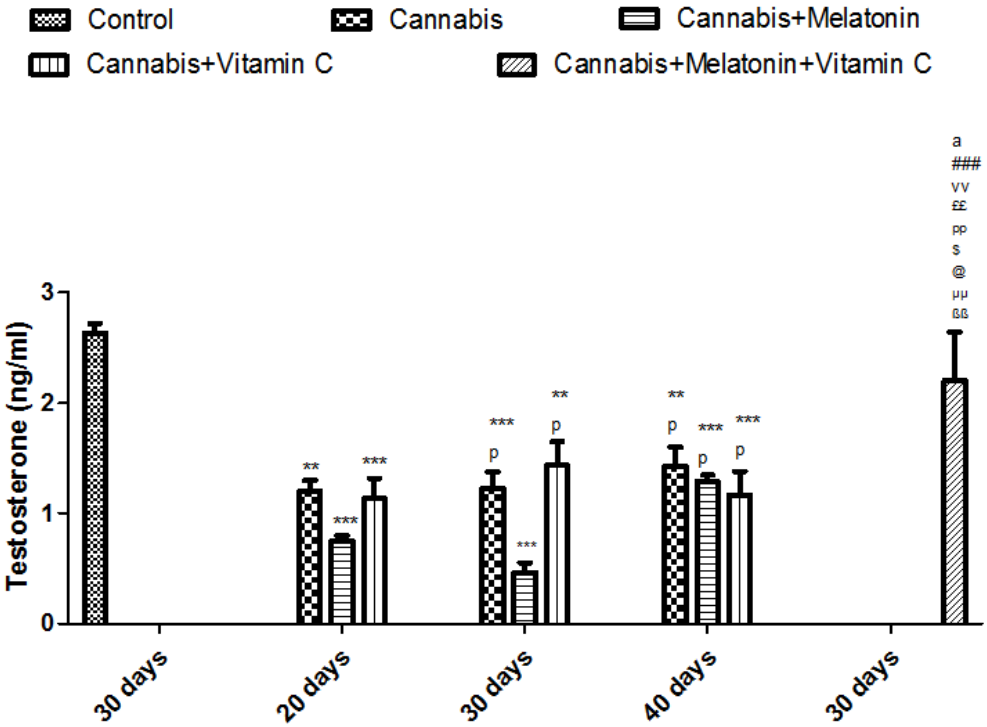

Fig.1: Testosterone concentration in rats given cannabis with(out) melatonin and/or vitamin C. Values are expressed as Mean±S.E.M (n=5). $^{**}p$<0.01, $^{***}p$<0.001 vs. control; ^{a}p<0.05 vs. cannabis 20 days; $^{###}p$<0.001 vs. cannabis+melatonin 30 days; ^{vv}p<0.01 vs. cannabis+vitamin C 20 days; $^{££}p$<0.01 vs. cannabis 30 days; ^{p}p<0.05, ^{pp}p<0.01 vs. cannabis+melatonin 30 days, $^{\$}p$<0.05 vs. cannabis+vitamin C 30 days; $^{@}p$<0.05 vs. cannabis 40 days; ^{µµ}p<0.01 vs. cannabis+melatonin 40 days; ^{ßß}p<0.01 vs. cannabis+vitamin C 40 days.

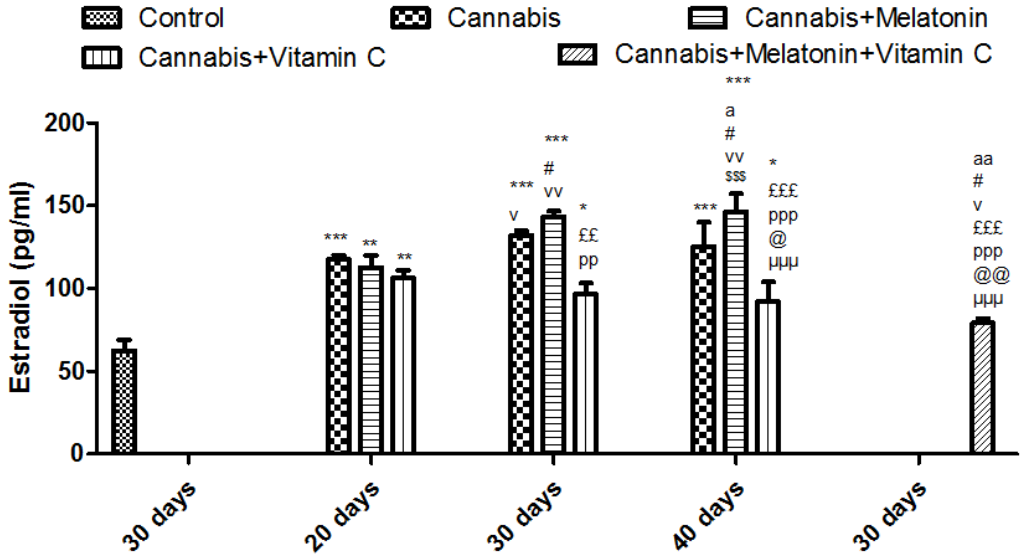

Fig.2: Estradiol concentration in rats given cannabis with(out) melatonin and/or vitamin C. Values are expressed as Mean±S.E.M (n=5). $^{*}p$<0.05, $^{**}p$<0.01, $^{***}p$<0.001 vs. control; ^{a}p<0.05, ^{aa}p<0.01 vs. cannabis 20 days; $^{\#}p$<0.05 vs. cannabis+melatonin 20 days; ^{v}p<0.05, ^{vv}p<0.01 vs. cannabis+vitamin C 20 days; $^{££}p$<0.01, $^{£££}p$<0.001 vs. cannabis 30 days; ^{pp}p<0.01, ^{ppp}p<0.001 vs. cannabis+melatonin 30 days, $^{\$\$\$}p$<0.001 vs. cannabis+vitamin C 30 days; $^{@}p$<0.05, $^{@@}p$<0.01 vs. cannabis 40days; ^{µµµ}p<0.001 vs. cannabis+melatonin 40 days.

concentrations in rats that received cannabis+vitamin C were lower at 20 days (p<0.001), 30 days (p<0.01), and 40 days (p<0.001) than in the control but comparable to the levels in rats that received cannabis only for similar durations. Lastly, the testosterone concentration in rats that received cannabis+melatonin+vitamin C is comparable to the control level (p>0.05), but significantly higher than the concentration in all the other treatment subgroups (p<0.05, p<0.01, p<0.001) (Figure 1). This shows that the reduced testosterone in rats receiving cannabis could be prevented by melatonin and vitamin C only when combined but not when separated.

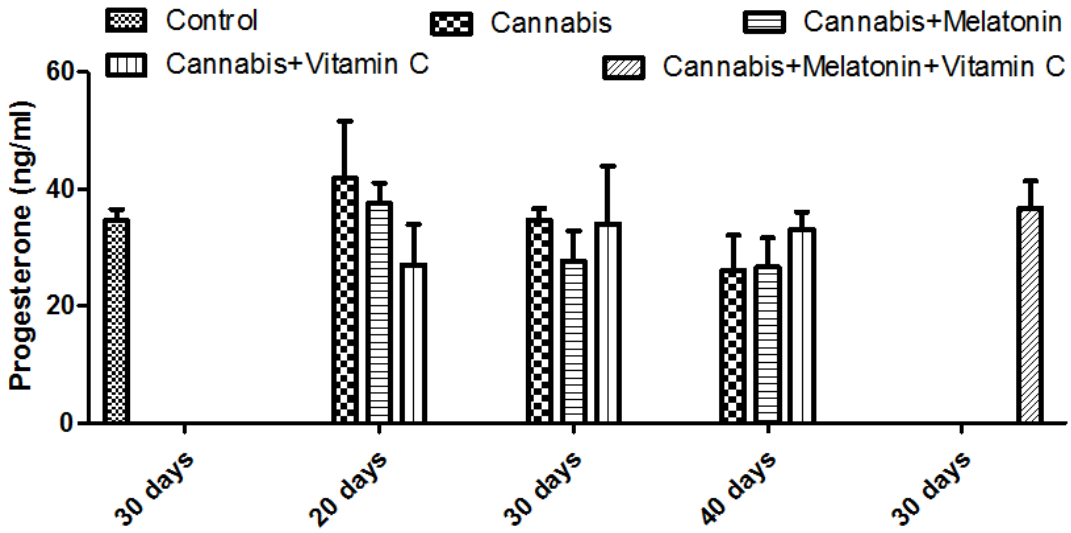

Fig. 3: Progesterone concentration in rats given cannabis with(out) melatonin and/or vitamin C. Values are expressed as Mean±S.E.M (n=5).

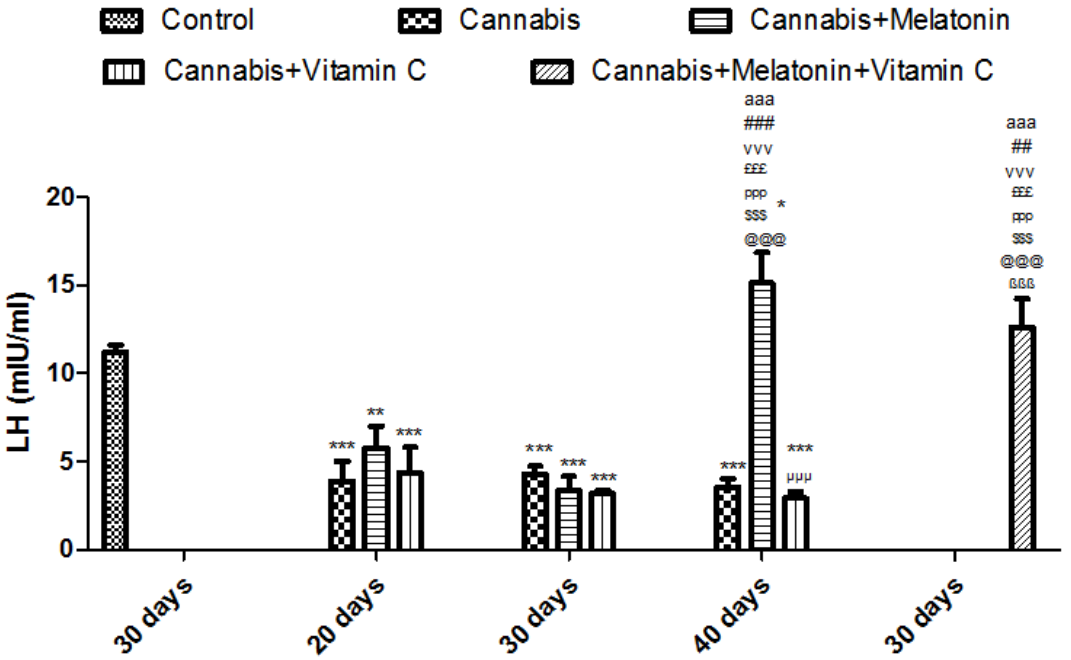

Fig. 4: Luteinizing Hormone (LH) concentration in rats given cannabis with(out) melatonin and/or vitamin C. Values are expressed as Mean±S.E.M (n=5). $^{*}p$<0.05, $^{**}p$<0.01, $^{***}p$<0.001 vs. control; ^{aaa}p<0.001 vs. cannabis 20 days; $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. cannabis+melatonin 20 days; ^{vvv}p<0.001 vs. cannabis+vitamin C 20 days; $^{£££}p$<0.001 vs. cannabis 30 days; ^{ppp}p<0.001 vs. cannabis+melatonin 30 days, $^{\$\$\$}p$<0.001 vs. cannabis+vitamin C 30 days; $^{@@@}p$<0.001 vs. cannabis 40 days; ^{µµµ}p<0.001 vs. cannabis+melatonin 40 days; ^{ßßß}p<0.001 vs. cannabis+vitamin C 40 days.

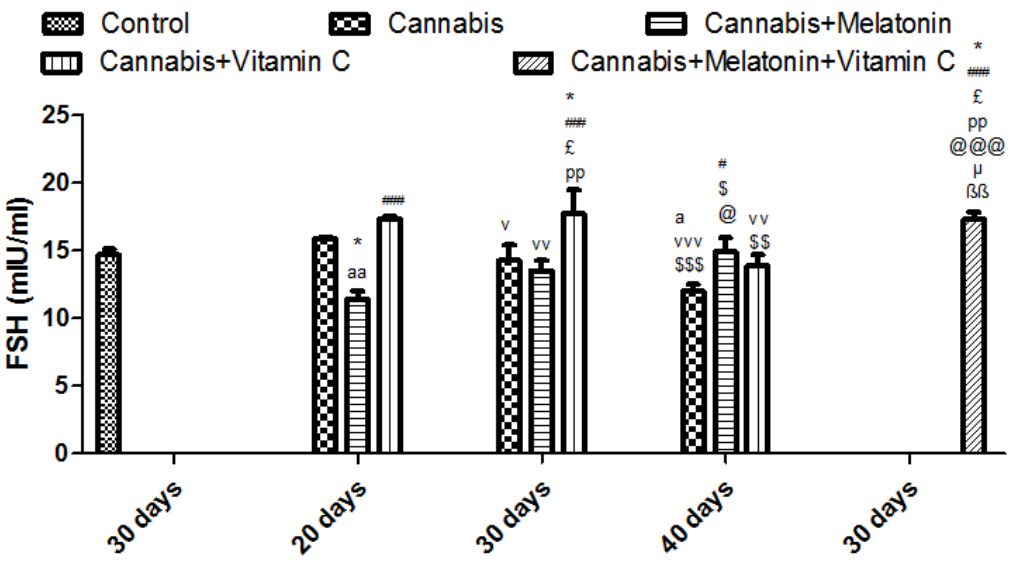

Fig. 5: Follicle Stimulating Hormone (FSH) concentration in rats given cannabis with(out) melatonin and/or vitamin C. Values are expressed as Mean±S.E.M (n=5). $^{*}p<0.05$ vs. control; $^{a}p<0.05$, $^{aa}p<0.01$ vs. cannabis 20 days; $^{\#\#\#}p<0.001$ vs. cannabis+melatonin 30 days; $^{v}p<0.05$, $^{vv}p<0.01$, $^{vvv}p<0.001$ vs. cannabis+vitamin C 20 days; $^{£}p<0.05$ vs. cannabis 30 days; $^{pp}p<0.01$ vs. cannabis+melatonin 30 days, $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ vs. cannabis+vitamin C 30 days; $^{@}p<0.05$, $^{@@@}p<0.001$ vs. cannabis 40 days; $^{µ}p<0.05$ vs. cannabis+melatonin 40 days; $^{ßß}p<0.01$ vs. cannabis+vitamin C 40 days.

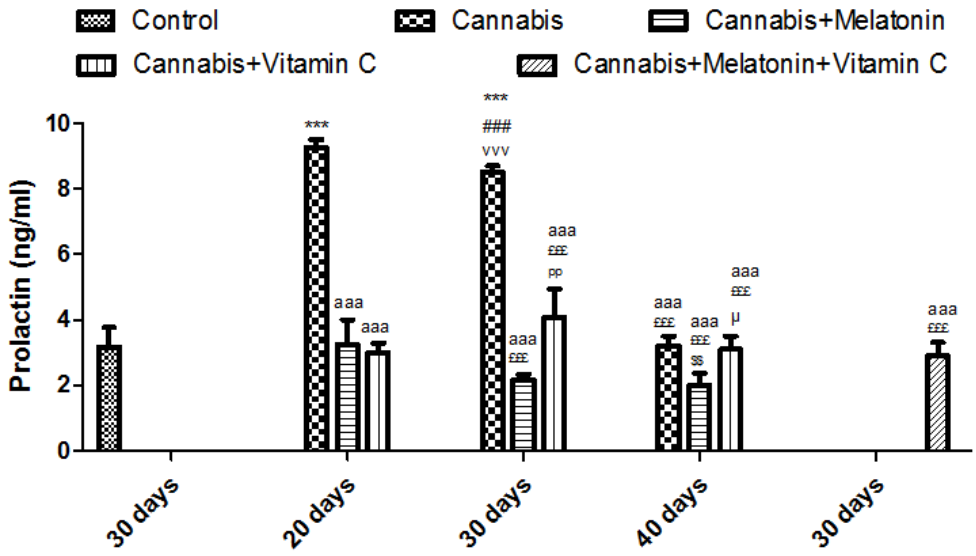

Fig. 6: Prolactin concentration in rats given cannabis with(out) melatonin and/or vitamin C. Values are expressed as Mean±S.E.M (n=5). $^{***}p<0.001$ vs. control; $^{aaa}p<0.001$ vs. cannabis 20 days; $^{\#\#\#}p<0.001$ vs. cannabis+melatonin 20 days; $^{vvv}p<0.001$ vs. cannabis+vitamin C 20 days; $^{£££}p<0.001$ vs. cannabis 30 days; $^{pp}p<0.01$ vs. cannabis+melatonin 30 days, $^{\$\$}p<0.01$ vs. cannabis+vitamin C 30 days; $^{µ}p<0.05$ vs. cannabis+melatonin 40 days.

Effects of cannabis with(out) melatonin and/or vitamin C on estradiol in rats

Cannabis administration to rats caused increase in estradiol concentrations at 20 days ($p<0.001$), 30 days ($p<0.001$), and 40 days ($p<0.001$) when compared to control. Rats that received cannabis+melatonin also had increased estradiol concentrations at 20 days ($p<0.01$), 30 days ($p<0.001$) and 40 days ($p<0.001$) when compared to control. The testosterone level in rats that received cannabis+melatonin for 30 days and 40 days are higher, albeit insignificant ($p>0.05$), than in rats that received cannabis only for same duration.

Similarly, estradiol concentrations in rats that received cannabis+vitamin C for 20 days (p<0.01), 30 days (p<0.05), and 40 days (p<0.05) were higher than in control. It is however noteworthy that the extent of increases in estradiol level observed in cannabis+vitamin C subgroups are smaller compared to cannabis subgroups (p>0.05, p<0.01, p<0.05) and cannabis+melatonin subgroups (p>0.05, p<0.01, p<0.001) at 20 days, 30 days, and 40 days respectively. Lastly, rats that received cannabis+melatonin+vitamin C had estradiol concentration that is comparable to the control level (p>0.05), and below the level in cannabis 20 days (p<0.01), cannabis+melatonin 20 days (p<0.05), cannabis+vitamin C 20 days (p<0.05), cannabis 30 days (p<0.001), cannabis+melatonin 30 days (p<0.001), cannabis 40 days (p<0.01), and cannabis+melatonin 40 days (p<0.001) subgroups (Figure 2). This shows that the increased estradiol in rats receiving cannabis could be prevented by melatonin and vitamin C only when combined but not when separated.

Effects of cannabis with(out) melatonin and/or vitamin C on progesterone in rats

Cannabis administration to rats with(out) supplementation with melatonin and/or vitamin C caused no significant changes in progesterone concentration when compared to control (p>0.05) (Figure 3). This suggests that progesterone is unaffected by cannabis administration in rats.

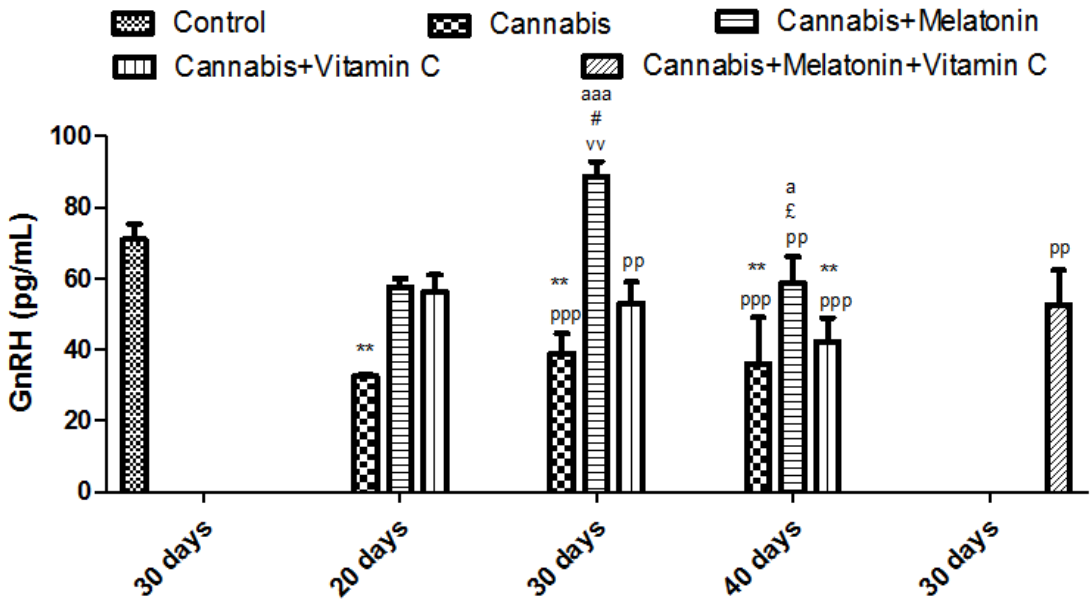

Fig.7: Gonadotropin Releasing Hormone (GnRH) concentration in rats given cannabis with(out) melatonin and/or vitamin C. Values are expressed as Mean±S.E.M (n=5). $^{**}p$<0.01 vs. control; ^{a}p<0.05, ^{aaa}p<0.001 vs. cannabis 20 days; $^{\#}p$<0.05 vs. cannabis+melatonin 20 days; ^{vv}p<0.01 vs. cannabis+vitamin C 20 days; $^{£}p$<0.05 vs. cannabis 30 days; ^{pp}p<0.01, ^{ppp}p<0.001 vs. cannabis+melatonin 30 days.

Effects of cannabis with(out) melatonin and/or vitamin C on luteinizing hormone in rats

Cannabis administration to rats caused decrease in luteinizing hormone levels (LH) at 20 days (p<0.001), 30 days (p<0.001), and 40 days (p<0.001) when compared to control. Rats that received cannabis+melatonin also had reduced LH at 20 days (p<0.01) and 30 days (p<0.001) when compared to control, but increased LH at 40 days when compared to control (p<0.05), cannabis 20 days (p<0.001), cannabis+melatonin 20 days (p<0.001), cannabis+vitamin C 20 days (p<0.001), cannabis 30 days (p<0.001), cannabis+melatonin 30 days (p<0.001), cannabis+vitamin C 30 days (p<0.001), cannabis 40 days (p<0.001), and cannabis+vitamin C 40 days (p<0.001) subgroups. In addition, rats that received cannabis+vitamin C also had reduced LH at 20 days (p<0.001), 30 days (p<0.001), and 40 days (p<0.001) when compared to control. Lastly, LH in rats that received cannabis+melatonin+vitamin C was comparable to the control level (p>0.05), but was significantly higher than the level in cannabis 20 days (p<0.001), cannabis+melatonin 20 days (p<0.01), cannabis+vitamin C 20 days (p<0.001), cannabis 30 days (p<0.001), cannabis+melatonin 30 days (p<0.001), cannabis+vitamin C 30 days (p<0.001), cannabis 40 days (p<0.001), and cannabis+vitamin C 40 days (p<0.001) subgroups (Figure 4). This shows

that the reduced LH in rats receiving cannabis could be prevented by melatonin and vitamin C only when combined but not when separated.

Effects of cannabis with(out) melatonin and/or vitamin C on follicle stimulating hormone in rats.

The follicle stimulating hormone concentrations (FSH) in rats that received cannabis for 20 days, 30 days, and 40 days were not significantly different from that of the control ($p>0.05$). Treatment of rats with cannabis+melatonin for 20 days ($p<0.05$) but not for 30 days ($p>0.05$) and 40 days ($p>0.05$) significantly reduced FSH when compared to control. Rats that received cannabis+vitamin C for 20 days and 30 days had higher FSH when compared to control ($p>0.05$, $p<0.05$), cannabis+melatonin 20 days ($p<0.001$, $p<0.001$), cannabis 30 days ($p<0.05$, $p<0.05$), cannabis+melatonin 30 days ($p<0.01$, $p<0.01$), cannabis 40 days ($p<0.001$, $p<0.001$), cannabis+melatonin 40 days ($p>0.05$, $p<0.05$), and cannabis+vitamin C 40 days ($p<0.01$, $p<0.01$) subgroups respectively. Lastly, rats that received cannabis+melatonin+vitamin C had FSH that is significantly higher than the control ($p<0.05$), cannabis+melatonin 20 days ($p<0.001$), cannabis 30 days ($p<0.05$), cannabis+melatonin 30 days ($p<0.01$), cannabis 40 days ($p<0.001$), cannabis+melatonin 40 days ($p<0.05$), and cannabis+vitamin C 40 days ($p<0.01$) subgroups (Figure 5). This suggests that cannabis treatment did not affect FSH, while supplementation with melatonin and vitamin C caused some changes in FSH of rats receiving cannabis.

Effects of cannabis with(out) melatonin and/or vitamin C on prolactin in rats

Cannabis administration to rats caused increased prolactin concentration at 20 days and 30 days but not at 40 days when compared to control ($p<0.001$), cannabis+melatonin 20 days ($p<0.001$), cannabis+vitamin C 20 days ($p<0.001$), cannabis+melatonin 30 days ($p<0.001$), cannabis+vitamin C 30 days ($p<0.001$), cannabis 40 days ($p<0.001$), cannabis+melatonin 40 days ($p<0.001$), cannabis+vitamin C 40 days ($p<0.001$), and cannabis+melatonin+vitamin C 30 days ($p<0.001$). Supplementation of cannabis-treated rats with melatonin and/or vitamin C abolished the effects of cannabis by reducing the prolactin concentration to levels that are comparable to the control ($p>0.05$) (Figure 6). This suggests that cannabis increased prolactin level, which was prevented by melatonin and/or vitamin C in rats receiving cannabis.

Effects of cannabis with(out) melatonin and/or vitamin C on gonadotropin releasing hormone in rats

Cannabis administration to rats caused reductions in gonadotropin releasing hormone concentration (GnRH) at 20 days ($p<0.01$), 30 days ($p<0.01$), and 40 days ($p<0.01$) when compared to control. Rats that received cannabis with melatonin and/or vitamin C had GnRH that are comparable to the control level ($p>0.05$). Moreover, rats that received cannabis+melatonin for 30 days had significantly higher GnRH than the cannabis 20 days ($p<0.001$), cannabis+melatonin 20 days ($p<0.05$), cannabis+vitamin C 20 days ($p<0.01$), cannabis 30 days ($p<0.001$), cannabis+vitamin C 30 days ($p<0.01$), cannabis 40 days ($p<0.001$), cannabis+melatonin 40 days ($p<0.01$), cannabis+vitamin C 40 days ($p<0.001$), and cannabis+melatonin+vitamin C ($p<0.01$) subgroups (Figure 7). This suggests that cannabis reduced GnRH, which was prevented by melatonin and/or vitamin C in rats receiving cannabis.

DISCUSSION

A high intratesticular level of testosterone has been established to be a major prerequisite for sperm production and functions including count, motility and morphology (Snyder, 2000; Gray *et al*., 2005). The involvement of oxidative stress in cannabis-induced gonadotoxicity has recently been shown in rats (Alagbonsi *et al*., 2016). In the present study, administration of cannabis to rats caused reduction in testosterone, which was sustained with addition of either melatonin or vitamin C. The reduction of testosterone by cannabis with(out) melatonin or vitamin C in this study, in addition to the reduction of paired-testis-body-weight ratio previously reported by us (Alagbonsi *et al*., 2016), convincingly show that hypogonadism is implicated in cannabis-induced infertility. It further shows that neither melatonin nor vitamin C can reverse the cannabis-induced gonadotoxicity when administered separately.

It is desirable to know what was responsible for the hypogonadism observed with administration of cannabis with(out) melatonin or vitamin C in this study. The testicular secretion of testosterone in males has been widely known to be solely controlled by the pituitary luteinizing hormone (LH) (Coquelin and Desjardins, 1982). By implication, it connotes that steady pulsatile secretion of LH is required for high testicular Leydig cell testosterone secretion needed for sperm production and functions. In the present study, administration of cannabis to rats caused reduction in LH, which was sustained with separate addition of either melatonin or vitamin C. The similarity of this trend to the pattern observed for testosterone showed that the hypogonadism in rats that received cannabis only, or in rats that received either melatonin or vitamin C in addition to cannabis, is related to hypogonadotropism.

It is also of interest to know if the origin of the hypogonadotropic hypogonadism observed with cannabis treatment in this study is from the hypothalamus and the pituitary gland. Because the secretion of gonadotropins by pituitary gland is under the direct influence of hypothalamic GnRH, distinguishing patients with hypogonadotropic hypogonadism of pituitary origin from those with primary hypothalamic disease is achieved by GnRH testing. People with pituitary disease would not respond to GnRH, whereas those with hypothalamic disorders would secrete LH and FSH normally after administration of GnRH (Gingrich, 2010). In our present study, melatonin or vitamin treatment reversed the cannabis-induced reduction in GnRH to levels that are similar to the control (except for rats that received cannabis and vitamin C for 40 days) but did not concomitantly reverse the cannabis-induced reduction in LH. This resistance or lack of responsiveness of the pituitary gland to the increased GnRH elicited by co-administration of cannabis with either melatonin or vitamin C shows that the source of cannabis-induced hypogonadotropic hypogonadism is from the pituitary gland. It also provides additional information that this condition can neither be ameliorated by melatonin nor vitamin C when administered separately.

The next thing is to justify what could have accounted for the selective effect of cannabis on LH but not FSH. The hypothalamic-pituitary-gonadal (HPG) axis consists of three parts: gonadotropin-releasing hormone (GnRH) neurons projecting from the hypothalamus of the brain, gonadotropes in the anterior pituitary gland (adenohypophysis) which secrete the gonadotropins (LH and FSH), and the Leydig and Sertoli cells in the testis of males (Gingrich, 2010). GnRH is secreted in pulses from the terminals of GnRH neurons and acts on the gonadotropes to induce secretion of both LH and FSH, which then act on their respective target cells in the gonads (LH on Leydig cells; FSH on Sertoli cells) (Kimura and Funabashi, 1998; Terasawa, 1998). Consequently, gonadal sex steroids (testosterone and estradiol) stimulated by LH and the protein hormone inhibin B stimulated by FSH are released into the bloodstream and provide feedback to the hypothalamus and pituitary gonadotropes to reduce the secretion of GnRH, LH, and FSH, with inhibin selectively inhibiting FSH and the sex steroids inhibiting LH secretion (Crowley *et al.*, 1991). The responses of pituitary LH and FSH secretion to pulsatile hypothalamic GnRH release are radically different, with LH release being stimulated very acutely (in pulses) by the GnRH pulses, whereas the response of FSH is extremely sluggish and takes many hours (Crowley *et al.*, 1991; Bousfield *et al*., 1994). This discrepancy has been shown to stem from fundamental differences in GnRH-induced synthesis, packaging, and release of LH and FSH. Similarly, although the sex steroids (testosterone and estradiol) negatively regulate LH secretion via effects on both GnRH secretion and gonadotrope function, they also exert some little negative feedback on FSH secretion; while inhibin selectively inhibits FSH secretion. This physiological description of the loop provides an explanation to the lack of significant change in FSH concentration despite reduction in GnRH and testosterone level following cannabis treatment. However, measurement of inhibin B, which is a limitation of this study, will provide further explanation to the unchanged FSH observed with cannabis in this study.

Endocrine disruptors are estrogen-like and/or anti-androgenic chemicals in the environment that have potentially hazardous effects on male reproductive axis resulting in infertility and on other hormonal dependent reproductive functions causing erectile dysfunction (ED) (Sikka and Wang, 2008). Endocrine disruptors are characterized by causing increased estrogen or reduced androgen or combination of both effects. Treatment with various kinds of endocrine disruptors have been reported to cause germ cell and Leydig cell damage, down-regulation of HPG axis, and reduction of fertility, libido, spermatogenesis, and accessory sex glands weight (Sikka and Wang, 2008). The present study further demonstrates that the cannabis-induced infertility reported by us, the mechanism of which was partly explained by us through oxidative stress (Alagbonsi *et al.*, 2016), could additionally be as a result of its endocrine disrupting ability due to the observed estrogenic and anti-androgenic effects. This study further adds cannabis to the comprehensive lists of endocrine disruptors. It is also noteworthy that the cannabis-induced endocrine disruption could only be reversed by melatonin and vitamin C when combined but not when administered separately in rats.

The observation of increased GnRH and reduced LH elicited by melatonin or vitamin C in cannabis-treated rats was surprising to us. Although it is generally held that testosterone, the major secretory product of the testis, is the primary inhibitor of LH secretion in men, a number of testicular secretory products, including estrogens and other androgens, have the ability to inhibit LH secretion. Estradiol, a potent estrogen, is produced both from the testis and from peripheral conversion of androgens and androgen precursors and is a much more potent inhibitor of LH secretion (approximately 1000-fold) in males. Also, testosterone acts primarily to feedback at the level of the hypothalamus whereas estrogens provide feedback to the pituitary to modulate the gonadotropin secretion response to each GnRH surge (Gingrich, 2010). In this study, co-administration of cannabis with either melatonin or vitamin C maintained the testosterone to the level lower than the control but comparable to the

rats that received cannabis only, whereas reversed the trend for GnRH. In addition, we observed reductions in LH (except in rats that received cannabis and melatonin for 40 days) simultaneously with increased estradiol. This showed that the increase in GnRH by melatonin and vitamin C in cannabis-treated rats in this study is a consequence of reduced testosterone caused by these two agents, probably because of the absence of feedback inhibition of GnRH by testosterone, and strongly support the claim that testosterone is a potent feedback regulator of GnRH secretion. Similarly, the reductions in LH which could be accounted for by increased estradiol concentration closely agree with the contention that estradiol negative feedback on the pituitary gonadotropin secretion is stronger than testosterone feedback (Gingrich, 2010).

Hyperprolactinemia has been extensively shown to be a common cause of infertility in males and females (Buvat, 2003; Ciccarelli *et al*., 2005). Previous study had reported indices of sexual dysfunctions in 88% of hyperprolactinemic men**,** including erectile dysfunction, reduced sexual desire, delayed or absent orgasm, and retrograde ejaculation (Buvat *et al*., 1985). Hyperprolactinemia was also reported to account for infertility in around 11% of oligospermic males. These have been associated with the inhibition of pulsatile secretion of gonadotropin releasing hormone, which causes decreased release of follicle stimulating hormone, luteinizing hormone, and testosterone, which in turn causes spermatogenic arrest, impaired sperm motility, and altered sperm quality (Masud *et al.*, 2007). The present study provides additional information that the mechanism of gonadotoxic effect of CS reported by us, which we partly explained by oxidative stress (Alagbonsi *et al.*, 2016), could further be associated with cannabis-induced hyperprolactinemia.

Since HPG axis and prolactin have been established to play role in male fertility, we were curious to know if the infertility that might result from cannabis-induced hyperprolactinemia be associated with the HPG axis. The down-regulation of the hormones of the hypothalamic-pituitary-gonadal axis simultaneously with hyperprolactinemia following cannabis treatment in this study is in agreement with previous study of Masud *et al.* (2007) that associated hyperprolactinemia with reduced pulsatile secretion of GnRH, gonadotropins (FSH and LH), and testosterone. This tends to support the contention that the axis is involved in the hyperprolactinemia-induced infertility seen in cannabis treatment.

There are many studies suggesting that hyperprolactinemia is one of the reversible causes of infertility (Soler *et al.*, 1990; Buvat, 2003; Masud *et al*., 2007). Medications such as bromocriptine and cabergoline, which normalizes serum prolactin levels, have been reported to restore gonadal function, reverse infertility caused by hyperprolactinemia, and induce reduction in prolactinoma size in the majority of patients (Buvat, 2003). On the contrary, co-administration of melatonin or vitamin C with cannabis in the present study reversed cannabis-induced hyperprolactinemia without concomitantly reversing cannabis-induced gonadotoxicity in our previous study (Alagbonsi *et al*., 2016).

Since the cannabis-induced hyperprolactinemia is related to HPG axis, we were amazed to observe that the reduction in prolactin was not followed by increased LH and FSH after melatonin or vitamin C was separately administered with cannabis. Hyperprolactinemia has been reported to directly influence spermatogenesis and steroidogenesis by acting on prolactin receptors present in Sertoli cells and Leydig cells in testis, and produces primary hypogonadism and infertility (Masud *et al.*, 2007). In addition, it has been shown that oligospermic or azoospermic patients with normal serum levels of gonadotropins show relatively higher serum levels of prolactin, providing a role of prolactin in gametogenesis, which is independent of gonadotropins (Soler *et al.*, 1990; Masud *et al*., 2007). Similarly, a direct, testosterone-independent effect of hyperprolactinemia on men's sexual behavior has been documented (Bancroft *et al.*, 1984). For instance, serum testosterone was reported to be in the normal range in nearly half of the erectile dysfunction patients with marked hyperprolactinemia. During treatment of hyperprolactinemic men with the prolactin-lowering agent bromocriptine, sexual improvement correlates better with serum prolactin decrease than with testosterone increase (Buvat *et al*., 1985). Though melatonin and vitamin C reversed the cannabis-induced hyperprolactinemia and reduction of GnRH when administered separately and when combined, the cannabis-induced reductions in LH and testosterone were reversed by melatonin and vitamin C only when combined but not when administered separately. Those previous reports and our present data do not rule out the fact that the infertility associated with cannabis-induced hyperprolactinemia is associated with HPG axis, but rather shows that combination of melatonin and vitamin C is required for the reversal. This is similar to our recent findings that show that melatonin and vitamin C exacerbate *Cannabis sativa*-induced testicular damage when administered separately but ameliorate it when combined in rats (Alagbonsi *et al.*, 2016).

In conclusion, this study showed that cannabis causes down-regulation of hypothalamic-pituitary-gonadal axis, endocrine disruption, and hyperprolactinemia. In addition, these effects (except hyperprolactinemia) can be reversed by melatonin and vitamin C only when combined but not when administered separately.

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