THE ROLE OF HYPOTHALAMIC PITUITARY GONADAL AXIS IN AQUEOUS EXTRACT OF CANNABIS SATIVA INDUCED MALE REPRODUCTIVE DYSFUNCTION OF ALBINO WISTAR RATS

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ABSTRACT
The recent study investigates the role of hypothalamic-pituitary gonadal axis in Cannabis sativa induced male reproductive dysfunction in 21 male albino Wistar rats (body weight 150 to 200g). The rats were assigned into three groups of control, low dose and high dose. They were fed with aqueous extract of Cannabis sativa via orogastric feeding at dose of 0.4mg/100g and 0.8mg/100g body weight respectively for 28 days. The result was analysed using One way ANOVA, followed by post hoc multiple comparisons and level of significance set at p<0.05. Gonadotropin releasing hormone in low dose was significantly lower (p<0.001) than control. Follicle stimulating hormone was significantly (p<0.01) reduced and increased percentage of spermatozoa with abnormal morphology in low dose group than control group. Luteinizing hormone was significantly (p<0.001) reduced in both low dose and high dose groups compared to control, although high dose group had a significant increase (p<0.01) than low dose. Testosterone was significantly (p<0.001) elevated in low dose than control. Sperm count, motility, viability and rapid progressive forward were significantly (p<0.05) reduced and increased percentage of spermatozoa with abnormal morphology in Cannabis sativa fed groups. The histological investigation of hypothalamus and pituitary gland show no histopathological changes. However, testicular and epididymal tissues of rats fed with low dose Cannabis sativa show no visible deterioration, whereas, there were noticeable derangements on rats fed with high dose Cannabis sativa. These alterations suggest that aqueous extract of Cannabis sativa has more effect on the gonads than at higher centers and its ingestion even as tea could impair male reproductive function.

KEYWORDS: Cannabis sativa, Hypothalamic-Pituitary- Gonadal axis, Male Reproductive Dysfunction.

INTRODUCTION
Over the years infertility was assumed to be divine punishment on humans and mostly attributed to females primarily to save men from social stigma of impotency. This has raised several questions concerning its cause.[1] When a man is unable to pregnant a fertile woman after one year of unprotected intercourse is called male infertility.[2] Infertility accounts for 40-50% in humans.[3] It affects approximately about 7% of all men.[4] Different causes of male infertility have been indicated which include, low sperm count, poor sperm quality, abnormal sperm shape and function or blockage that prevent sperm delivery.[5] Erectile dysfunction and other key risk factors such as varicoceles (presence of varicose vein in scrotum), anatomical problems and obesity have also been indicated.[6] Hormonal imbalance, genetic defects, Bicycle and horseback riding, aging, sexually transmitted diseases, long-term exposure to chemicals, toxins and medications which may be dangerous to sperm production like Chemotherapy, Cimetidine, and anabolic steroids or those that may reduce sperm quality and follicle stimulating hormone (FSH) release such as phenytoin, nitrofurantoin and sulfasalazine, smoking and substance abuse which are life style activities may sperm quality negatively.[7]

The hypothalamic–pituitary–gonadal axis (HPG axis) refers to the effect of the hypothalamus, pituitary gland and gonads as a single system that regulates body’s reproductive system. Fluctuation of the hormones results in changes in the production of the hormones by each gland resulting to changes in body’s function.[8] The system controls reproduction, animal aging and growth. Hypothalamic neurons produces gonadotropin–releasing hormone (GnRH) that stimulate anterior pituitary to release luteinizing hormone (LH) and follicle–stimulating hormone (FSH) and gonads to release estrogen and testosterone.[9] Cannabis is an annual,
dioecious and flowering herb. It grows well at low temperature. The most important product of Cannabis in food and drugs are Hemp seed, Oil and Hashish. Infertility has also been attributed to the use of Cannabis via its ability to alter endocrine system. This effect was suggested to be mediated by binding to the endogenous cannabinoid receptor within the hypothalamus.

MATERIALS AND METHODS

Materials: Syringes, orogastric cannula, plain and EDTA sample bottles, distilled water, water bath, chloroform anesthesia, bucket centrifuge machine and light microscope (B-Bran Scientific and Instrument Company, England), slides and cover slips and rat specific kits for testosterone, luteinizing hormone, gonadotropin releasing hormone, and follicle stimulating hormone.

Aqueous preparation of Cannabis sativa

Permission to use Cannabis sativa was approved and provided by National Drug Law Enforcement Agency (NDLEA) Calabar, Cross River State Command Nigeria and was sent to Botany Department University of Calabar for identification and issued a Herbarium number 7. It was ground to powder and was dissolved in 500mls of distilled water for 48 hours then filtered with a filter paper and evaporated to dryness in water bath (B-Bran Scientific and Instrument Company, England) at 60°C. The brownish residue were weighed and kept in an air tight bottle in the refrigerator until use. This method was recently used by.

Laboratory animals

Twenty one (21) male albino Wistar rats aged 8 weeks and weighing 150–200g were used for this study. The animals were housed in the Department of Physiology Animal house, University of Calabar, Nigeria. Standard animal cages with wood dust as bedding were used in keeping the animals. They were allowed ad libitum access to rat chow and clean water, and exposed to 12/12-hr light/dark cycle. The animals were acclimatized for 7 days. The animals were kept in line with laid down principles for animal care as prescribed in Helsinki’s 1964 declaration. The animal ethics committee of the University of Calabar approved our study protocol graciously.

Experimental design and Cannabis sativa Extract Administration

The animals were randomly assigned into three (3) groups of seven animals each. First group serves as control; second group (low dose) and third group (high dose) were fed with aqueous extract of Cannabis Sativa at the doses of 0.4mg/100g and 0.8mg/100g body weight respectively. Administration of aqueous extract of Cannabis sativa was done via orogastric feeding once daily for 28 days while the control group received normal saline as vehicle after which the animals were sacrificed under chloroform anesthesia and the left testis and epididymis were carefully harvested for semen analysis, histological examination and blood sample was collected for hormonal assay.

Assessment of sperm motility

Sperm motility was determined by placing 10µl of sperm suspension collected from the left epididymis on a clean pre-warmed slide, covered with a cover slip and examined using a light microscope (Leica DM 750, Switzerland) equipped with a heated stage (37°C), at 100x magnification.

Determination of epididymal sperm count

Estimation of epididymal sperm count was done using the method described by Freud and Carol (1964). The left cauda epididymis from each rat was placed in 2 ml of normal saline, pre-warmed to 37°C. Small cuts were made in the cauda epididymis and spermatozoa were obtained and suspended in saline solution. Two hundred microlitres of the suspension was transferred to both chambers of a Neubauer haemocytometer using a Pasteur pipette by touching the edge of the cover slip and allowing each chamber to be filled by capillary action. The epididymal sperm count for each animal was then obtained and recorded.

Assessment of sperm viability and morphology

Twenty microlitres of 0.05% eosin Y–nigrosin was added to an equal volume of sperm suspension and incubated at room temperature for 2 min. After incubation, all slides were viewed under a light microscope (Leica DM 750) at magnifications of ×100 and ×400. Live spermatozoa were not stained, while dead spermatozoa were stained pink. For each assay, 400 spermatozoa were counted and viability percentages were calculated.

Assessment of Serum Reproductive Hormones

Blood was collected via cardiac puncture, under chloroform anesthesia using a 5 ml syringe and a 21 G needle. The blood samples were introduced into plain capped sample bottles and allowed to stand for 2 hours, after which they were centrifuged at 1,000 rpm for 5 minutes using a bucket centrifuge (B-Bran Scientific and Instrument Company, England). Serum settled on top and was then used for reproductive hormonal assay. Serum testosterone, luteinizing hormone, follicle stimulating hormone and gonadotropin-releasing hormone (GnRH) concentrations were determined using the ELISA kit for rats, method as used by.

Principle: The ELISA method is based on the principle of high specificity of antibodies to bind molecules, which in this case are the different reproductive hormones. The antibody is tagged with an enzyme, since the enzyme–labelled antibody reacts with the hormone. The concentration of hormone present in the sample is obtained by introducing a substrate for the enzyme which forms a coloured product. The intensity of the colour seen is proportional to the concentration of the bound hormone.
Histological Studies

The testes and epididymis of the control and Cannabis sativa fed rats were removed carefully, cleared of connective tissues and fixed in Bouin’s fluid [0.2% picric acid/2% (v/v) formaldehyde in PBS] while pituitary gland and hypothalamus of the control and treated rats were isolated after fixing whole skull with 10% buffered formaldehyde for 48 hours. Sections were obtained and stained with hematoxylin and eosin (H & E) stains. The microscopic slides were labeled appropriately. Photomicrographs were taken at magnifications of x100 and x400 using a light microscope (Leica DM 750, Switzerland).

Statistical Analysis

All results are presented as mean ± standard error of mean (SEM). One way analysis of variance (ANOVA) was utilized in comparing the difference within groups, followed by post hoc multiple comparisons. The level of significance was placed at p<0.05.

RESULTS

Table 1: Effect of aqueous extract of Cannabis sativa on serum reproductive hormone concentrations after 4 weeks of ingestion.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Low dose</th>
<th>High dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonadotropin releasing hormone (GnRH) (ng/l)</td>
<td>1.62±0.00</td>
<td>1.44±0.01 ***</td>
<td>1.66±0.02</td>
</tr>
<tr>
<td>FSH (ng/ml)</td>
<td>1.02±0.07</td>
<td>1.88±0.07 ***</td>
<td>0.86±0.05</td>
</tr>
<tr>
<td>LH (U/ml)</td>
<td>4.78±0.04</td>
<td>4.12±0.08 ***</td>
<td>4.42±0.07 **b</td>
</tr>
<tr>
<td>Testosterone (nmol/ml)</td>
<td>12.82±0.19</td>
<td>15.06±0.20 ***</td>
<td>12.64±0.05 *</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± SEM, n = 7.

***p<0.001 versus control; b=p<0.01 versus control; c=p<0.001 versus low dose.

Table 2: Effect of aqueous extract of Cannabis sativa on sperm characteristics after 4 weeks of ingestion.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Low dose</th>
<th>High dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm count (millions/ml)</td>
<td>10.67±0.78</td>
<td>3.48±0.79 ***</td>
<td>0.36±0.14 ***b</td>
</tr>
<tr>
<td>Sperm viability (live/dead ratio) (%)</td>
<td>74±4.30</td>
<td>31±3.32 ***</td>
<td>13±2.00 ***b</td>
</tr>
<tr>
<td>Abnormal morphology (%)</td>
<td>4.4±0.51</td>
<td>6.2±1.07</td>
<td>13.6±1.86 ***c</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>73±3.39</td>
<td>37±5.83 ***</td>
<td>4±1.00 ***c</td>
</tr>
<tr>
<td>RPFM (%)</td>
<td>46±1.87</td>
<td>6.6±1.44</td>
<td>0±0.00 ***</td>
</tr>
<tr>
<td>SPFM (%)</td>
<td>25±2.74</td>
<td>20.6±3.85</td>
<td>4±1.00 ***c</td>
</tr>
<tr>
<td>RM (%)</td>
<td>14±1.87</td>
<td>15±1.58</td>
<td>4±1.00 ***c</td>
</tr>
<tr>
<td>Motionless (%)</td>
<td>15±3.54</td>
<td>57.8±3.80 ***</td>
<td>9±1±1.22 ***c</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± SEM, n = 7.

***p<0.001 versus control; b=p<0.01 versus control; c=p<0.001 versus low dose.

Histological Examination of the testes

![Histological images](image_url)
Fig 1: Photomicrographs of H and E stained testes (Magnification: x100).

**Key**

(Fig 1A) Control group shows normal appearance of the general tissue structure with normal appearance of the seminiferous tubule and a good population of spermatocytes.

(Fig 1B) Low dose shows testicular tissue with various seminiferous tubules with adequate population of spermatozoa and spermatocytes. The basement membranes of the seminiferous tubule are lined with seminiferous epithelium which contains the sertoli cells. No visible distortion of the general tissue seen.

(Fig 1C) High dose shows numerous seminiferous tubule with most of the seminiferous tubule showing adequate population of spermatozoa while some shows noticeable reduction in the production of sperm cells than the low dose.

**Histological Examination of the epididymis**

Fig 2: Photomicrographs of H and E stained epididymis (Magnification: x100).
Key
(Fig 2A) Control group shows normal appearance of the general tissue structure with the lumen heavily populated with adequate spermatozoa.

(Fig 2B) Low dose shows epididymis tissue with tall columnar cells of the epithelium. The epididymis is covered by a two layered pseudostratified epithelium. The lumen reveals numerous spermatozoa. No visible distortion or inflammation seen.

(Fig 2C) High dose shows epididymis tissue with ducts that are highly coiled. The epididymis is covered by a two layered pseudostratified epithelium. The lumen are empty with no spermatozoa inside the lumen than low dose.

Histological Examination of the hypothalamus

Fig 3: Photomicrographs of H and E stained hypothalamus (Magnification: x400).

NCB= Neuronal cell bodies, NF= Neural filaments.
(Fig 3A) Control group shows moderately populated neuronal cell bodies (NCB) with numerous neuronal processes. The neuronal cells ranges from small, intermediate to large neuronal cells having prominent deeply stained nuclei with coarse chromatin pattern with moderate cytoplasm.

(Fig 3B) Low dose shows prominent neuronal cell bodies and processes within a dense neurophil. The neuronal cells are evenly distributed and are of various stages with deeply stained oval to round nuclei having regular outline and coarse chromatin pattern. No pathology seen.

(Fig 3C) High dose shows prominent neuronal cell bodies with well defined outline and deeply stained nuclei having regular contour and coarse chromatin pattern and prominent nucleoli. The intervening neuronal processes are abundant. No neuronal damage seen.

Key
(Fig 4A) Control group shows clusters of round to oval cells separated by thin walled capillaries. The cells have regular nuclear contour and fine chromatin patterns. Some of the cells have abundant acidophilic cells and few have basophilic cytoplasm.

(Fig 4B) Low dose shows clusters of round to oval cells and elongated spindle cells with densely stained nuclei separated by thin walled capillaries. The cells are closely packed and their nuclear contours are regular and coarse chromatin patterns. Some of the cells have abundant acidophilic cytoplasm and enlarged nuclei. No cellular damage seen. Features suggestive of increased secretion of hormones.

(Fig 4C) High dose shows clusters of round to oval cells separated by thin walled capillaries. The cells have regular nuclear contour and fine chromatin patterns. The cells are mildly swollen with abundant cytoplasm. Some of the cells have acidophilic cytoplasm and few basophilic cytoplasm. No cellular damage seen.

DISCUSSION
The most commonly abused substance in today’s world is Cannabis due to its addictive potentials when compared with heroin, nicotine or metamphetamine. The recreational abuse of Cannabis was suggested to be due to its psychoactive effect such as highness, relaxation, euphoria and increased libido which has led to its repeated use. Undesired effect such as infertility has been attributed to Cannabis. This present study was based on tracing the role hypotalamic pituitary gonadal axis in Cannabis sativa induced male reproductive dysfunction of albino Wistar rats since it is commonly abuse today in Nigeria.

The reproductive function parameters assessed in this study were serum concentration of male reproductive hormones, sperm analysis and histological examination of testes, epididymis, pituitary and hypothalamus. The aforementioned parameters were assessed in both the control and Cannabis sativa fed groups.

After four weeks of Cannabis sativa aqueous extract ingestion, there were significant alterations in male reproductive hormone concentrations in serum. Gonadotropin releasing hormone (GnRH) was significantly reduced in low dose (LD) (0.4mg/100g body weight) fed group compared with control whereas it was markedly raised in high dose (HD) (0.8mg/100g body weight) fed group compared to LD. The reduction in serum GnRH in LD may be attributed to increased serum level of testosterone in this group that may have negative feedback effect to hypothalamus to stop secretion of GnRH. Furthermore, the raised serum GnRH level in HD may be attributed to low level of testosterone in this group. The decreased GnRH in LD corresponds to the work done by Smith et al., (1979a) where, they reported decreased GnRH in THC treated monkeys. High level of serum follicle stimulating hormone (FSH) in low dose fed rats may be due to reduced GnRH in this group which may have inhibited the hypothalamus via short loop negative feedback effect, and may also be attributed to development of tolerance to Cannabis as regards long term administration. Furthermore, low level of FSH in HD fed group may be due to reduced testosterone levels in this group.

The significant decrease in luteinizing hormone (LH) recorded in both LD and HD, especially in LD may be attributed to reduced GnRH and increased testosterone recorded in this group which may have inhibited GnRH release from hypothalamus via negative feedback mechanism and further suppressed LH secretion. This corresponds with the evidence provided by Symons et al., (1976), that there was marked reduction in LH levels in Cannabis fed rats. This effect is suggested to be mediated via cannabionoid (CB1) receptors in the hypothalamus. The significant increase on mean serum testosterone recorded in LD fed group may be attributed to reduced GnRH via long loop feedback effect suppressing GnRH secretion and may also be due to marked increase in HDL-c and triglyceride in this group that are cholesterol necessary in steroid hormone synthesis. Increased FSH may likely be the cause of raised testosterone in this group.

However reduced testosterone concentration recorded in HD may be attributed to reduce FSH, LH and triglyceride in this group. This is consistent with the work done by Jakubovic, McGeer, & McGeer, (1979), who reported that high doses of Cannabinoids may directly affect the leydig cells to inhibit testosterone synthesis affecting spermatogenesis. Following four weeks of the feeding, the alteration in serum concentrations of reproductive hormones in the fed groups may likely contribute to the possibility of developing fertility problems in males who indulge in recreational use of Cannabis sativa.

The sperm analysis is the key marker in assessing male reproductive indices. Significant decrease in sperm count was recorded on both Cannabis sativa fed groups, but the probable lower level of sperm count in HD fed group may be attributed to reduced testosterone and FSH levels in this group since FSH directly induces spermatogenesis by stimulation of sertoli cells. It was documented that sertolic cells enzymes in seminiferous tubules necessary for maturation of sperm are affected by cannabionoids. However, its significant increase in LD may be due to elevated levels of testosterone and FSH in that group. This is consistent with the report that Cannabis reduces sperm count. This consequence was suggested to occur via “CB1 receptor” found in testis that may directly affect the gonads.

Sperm motility was reduced significantly on both fed groups than the control, but the reductions were majorly on HD than the LD, which may be due to decreased
sperm count on the fed groups. A proposal by world health organization (WHO) that at least about 25 percent of spermatozoa on healthy male must swim with rapid forward movement, then an extra 50 percent spermatozoa swim although may be sluggish. In spite of this recommendation, percentage of spermatozoa with rapid forward movement in Cannabis sativa fed groups was far below WHO recommendation. The percentage sperm viability reduced in both fed groups compared with control. Furthermore both fed groups show increased number of abnormal spermatozoa compared with control. Although the abnormal spermatozoa was highest in HD which was consistent with previous reports.

After four weeks ingestion of Cannabis sativa, there were no visible distortion in photomicrographs of testes and epididymis of rats fed with LD Cannabis sativa compared with control. The invisible distortion may be due to elevated testosterone and FSH levels on this group, which are necessary for numerous spermatozoa. This is contrary to the report by Thompson et al., (1973) that low dose Cannabis sativa degenerate seminiferous tubule. However, the noticeable reduction in the production of sperm cells and damage to seminiferous tubules in photomicrograph of testes and epididymis (especially epididymis) in HD fed group may be attributed to decreased sperm count in this group. Furthermore low levels of FSH and testosterone in HD may be responsible for the decreased spermatozoa density in testes. The reduced spermatozoa density in epididymis in HD further confirmed the testicular damage since matured spermatozoa are stored in the epididymis until ejaculation. The damage to testicular tissue (especially seminiferous tubule) is suggested to be direct effect of cannabinoid on the testes via CB1 receptors. There was no significant neural damage in the hypothalamus and pituitary glands of groups fed on both LD and HD of Cannabis sativa. However there seem to show a trace of mildly swollen cells with abundant cytoplasm in the pituitary gland of rats fed on HD Cannabis sativa. This may be pointing to the fact that continuous ingestion may eventually lead to damage especially in the pituitary.

CONCLUSION
Ingestion of rats with aqueous extract of Cannabis sativa, at low dose and high dose caused significant alteration of reproductive hormones levels (GnRH, FSH, LH and testosterone) in male rats. There was significant reduction of sperm count, motility, viability and increased percentage of abnormal spermatozoa in Cannabis sativa fed rats. The high dose majorly affects serum lipid profile, FSH, testosterone, sperm count, motility, viability and morphology. Therefore, if these results are applicable in humans, the excessive consumption of Cannabis in whatever form can alter the normal reproductive function and should be seriously discouraged.

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REFERENCES


