

Possible mechanisms for the toxic effects of marijuana smoke on the reproductive axis of male albino rats

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ABSTRACT

The aim of this study was to assess the possible mechanisms for the toxic impact of marijuana smoke on the level of male reproductive hormones and their related genes expression. Male albino rats were enrolled in this study and divided into: (I) control group; (II) and (III) Marijuana smoke exposed groups in which the animals were exposed to marijuana smoke daily for two and four weeks respectively. Serum free testosterone, FSH and LH were assayed. Brain follicle stimulating hormone gene (FSH-B) and luteinizing hormone gene (LH-B) as well as testicular cytochrome P19 (CYP19) genes expression were evaluated. Histological examination of brain and testis tissues was done. Marijuana smoke produced significant reduction in serum reproductive hormones levels. Similarly, it caused significant down-regulation in the expression of reproductive hormones related genes. Histological investigation of brain tissue of marijuana smoke exposed rats revealed marked deterioration in medulla oblongata and cerebellum. While, histological examination of their testis tissues showed no histopathological changes. The present findings suggest that marijuana smoke adversely affects male reproductive function due to its ability to suppress gonadotropin output from pituitary in addition to its impact on the structure and/or function of DNA nucleotide sequences of reproductive hormones related genes. Marijuana smoke could impair fertility in male rats through alteration in circulating levels of reproductive hormones and down-regulation of the expression level of reproductive hormones related genes.

INTRODUCTION

Marijuana, derived from the ubiquitous plant, *Cannabis sativa*, is the most widely used illicit drug worldwide and became the second most commonly smoked substance after tobacco, with an estimated 160 million users (3.8% of 15–64 yrs. olds) (UNODC, 2008).

People who are dependent on marijuana frequently have other comorbid mental disorders. Population studies reveal an association between marijuana use and increased risk of depression, and anxiety. There are now sufficient data indicating that marijuana may trigger the onset or relapse of schizophrenia in people predisposed to it, perhaps also intensifying their symptoms (NIDA, 2010). A growing body of evidence suggests that marijuana use during adolescence increases the risk of developing

psychosis (Moore *et al.*, 2007).

D-9-tetrahydrocannabinol (D-9-THC) and cannabidiol (CBD), the two main ingredients of the *Cannabis sativa* plant, have distinct symptomatic and behavioral effects (Bhattacharyya *et al.*, 2010). Exposure to marijuana, both acutely and chronically, is associated with changes in brain activity, many paralleling those found with other substance abuse disorders. These findings predominantly involve frontal, limbic and also cerebellar regions, with generally increased activity during exposure and generally decreased activity during abstinence (Quickfall and Crockford, 2006). In healthy individuals, D-9-THC can induce psychotic symptoms, anxiety and impair memory (D'Souza *et al.*, 2004). In patients with schizophrenia, D-9-THC may exacerbate existing psychotic symptoms, anxiety and memory impairments (D'Souza *et al.*, 2005). In contrast, CBD has anxiolytic (Crippa *et al.*, 2004) and possibly antipsychotic properties (Zuardi, 2008). It does not impair memory or other cognitive functions (Fadda *et al.*, 2004).

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Functional imaging studies, including positron emission tomography (PET) and magnetic resonance (MR) perfusion, in the acute stage of marijuana abuse demonstrate an overall decrease in regional cerebral blood flow in the frontal, parietal, temporal, and occipital lobes (O'Leary *et al.*, 2002). Smoking marijuana leads to changes in the brain similar to those caused by the use of cocaine and heroin (Lundqvist, 2005).

In Egypt, drug addiction is considered one of the most serious problems that worry both people and government. It affects young people within their productive years and it may lead to many problems such as social maladaptation, decreased works productivity and job loss (El-Akabawi, 2004).

Drug addiction is a chronic, relapsing brain disease characterized by compulsive drug seeking and use despite often devastating consequences. It results from a complex interplay of biological vulnerability, environmental exposure, and developmental factors as with many other diseases, vulnerability to addiction stems partly from a person's genetic makeup. Scientists estimate that genetic factors account for 40–60% of an individual's vulnerability to addiction, with environmental and developmental variables influencing whether and how particular genes are expressed. Additional factors, such as conditions at home, at school, or in the neighborhood, can heighten addiction vulnerability. Several studies demonstrated that early drug use increases the likelihood of addiction and that people with psychiatric disorders have a higher risk of drug abuse and addiction than others (NIDA, 2010).

Although addiction affects both men and women of all ages, some findings reported that men are two to three times higher than women with highest rates among people aged 15 to 24 (Statistics Canada, 2003).

There is a problem central to understand *Cannabis sativa*-induced effects on a broad spectrum of biological processes since numerous modifications in cell structure and function, which have been reported to be associated with abused substances. Within this context, the main objective of the present study was to assess the possible mechanisms for the toxic effects of marijuana smoke on male reproductive hormones and to evaluate the impact of marijuana smoke on the expression level of reproductive hormones related genes in adult male albino rats. Also, this study was extended to examine the pathological effects of this smoke on brain and testis using histopathological technique.

MATERIALS AND METHODS

Materials

Extraction of Cannabis sativa

Ten gram of dried *Cannabis sativa* was ground with mortar and pestle, and then 10 ml of analytical grade chloroform was added and allowed to react for 1 h. The dried cannabis was extracted three times and the fractions were combined and filtered using Millex Millipore filters (0.45mm). The filtrate was evaporated under a gentle stream of nitrogen (on ice and protected from light). The remaining resin was resuspended in 2 ml of 100%

methanol, flushed with nitrogen and stored under vacuum at 41 °C and protected from light (in an aluminum –covered container) (Turner and Mahlberg, 1984).

Exposure protocol

Rats were transferred to the inhalation chamber and marijuana cigarettes were individually lit using negative pressure bulb and fixed to smoking machine, within one minute (Nelson *et al.*, 1999). The machine was immediately switched on and kept working for 15 minutes (Ortega *et al.*, 1994), this time was approximate time for one cigarette to completely burn down to the filter in the smoking machine ensuring total delivery of smoke to the inhalation chamber. Cigarette ashes and animal waste was removed after each exposure. The apparatus was ventilated for about 5 minutes after each exposure period to minimize any interference between doses. The dose was equivalent to 3 cigarettes each weighed about 990-1000 mg for 10 rats daily (Rosenkrantz and Monique, 1973).

Animals

This study was conducted on forty adult male albino rats weighting about 100-120g were obtained from the Animal Breeding House of the National Research Center, Dokki, Giza. Animals were provided standard pellets (Al Nasr Co., 20% proteins, 5% fats and 1% multivitamins) and water *ad-libitum*, housed under normal environmental conditions of average room temperature (25-30°C) and humidity (28-30%) and were used after being housed for one week to adapt to laboratory environment. This study was carried out in compliance with the guidelines for care and use of laboratory animals of the Ethical Committee for Medical Research of the National Research Centre, Cairo, Egypt.

Experimental design

Forty rats were divided into three main groups. Negative control group (Group I) consisted of twenty rats which were further divided into two equal subgroups (Group Ia) and (Group Ib). While, the other two groups (Group II) and (Group III) each consisted of ten adult male albino rats. Group (Ia) represents healthy animals kept in the same environment of other groups without exposure to marijuana and sacrificed after two weeks. Group (Ib) represents healthy animals similar to those in group (Ia) but sacrificed after four weeks. Group (II) represents animals exposed daily to marijuana cigarettes in a dose of 3 cigarettes/day for two weeks. Group (III) represents animals that were sacrificed after four weeks from daily exposure to marijuana cigarettes in a dose of 3 cigarettes/day. At the end of each experimental period, the animals were anaesthetized using diethylether and blood was withdrawn using heparinized capillary tube from retrobulbar plexus of veins, then immediately sacrificed by cervical dislocation. Brain and testis was dissected and weighed, then one portion was preserved in formalin buffer for histopathological examination and the other portion was stored at -80°C for molecular genetic study.

Methods

Hormones analyses

Quantitative estimation of free testosterone was carried out in serum using enzyme linked immune sorbent assay (ELISA) according to McCann and Kirkish (1985). Quantitative estimation of serum follicle stimulating hormone (FSH) was carried out using enzyme linked immune sorbent assay (ELISA) according to Knobil (1980). Quantitative estimation of luteinizing hormone (LH) in serum was carried out using enzyme linked immune sorbent assay (ELISA) according to Wakabayashi (1977). The kits used for determination of free testosterone, follicle stimulating hormone and luteinizing hormone in rat serum were obtained from Gloryscience. Co., Ltd, USA.

Gene expression analysis

Expression of FSH-B, LH-B and CYP19 genes.

I- Isolation of total RNA

According to Elmegeed *et al.* (2011), total RNA was isolated from brain and testis tissues of male rats by the standard TRIzol® reagent extraction method (Cat#15596-026, Invitrogen, Germany). Briefly, tissue samples were homogenized in 1 ml of TRIzol® reagent per 50 mg of the tissue. Afterwards, the homogenized sample was incubated for 15 minutes at room temperature. A volume of 0.2 ml of chloroform per 1 ml of TRIzol® reagent was added. Then the samples were vortexed vigorously for 15 seconds and incubated at room temperature for 3 minutes. The samples were centrifuged for no more than 12,000 x g for 15 minutes at 4 °C. Following centrifugation, the mixture was separated into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA was remained exclusively in the aqueous phase. Therefore, the upper aqueous phase was carefully transferred without disturbing the interphase into a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. A volume of 0.5 ml of isopropyl alcohol was added per 1 ml of TRIzol® reagent used for the initial homogenization. Afterwards, the samples were incubated at 15 to 30 °C for 10 minutes and centrifuged at not more than 12,000 x g for 10 minutes at 4 °C. The RNA was precipitated which was often invisible before centrifugation, formed a gel-like pellet on the side and bottom of the tube. The supernatant was removed completely. The RNA pellet was washed once with 1 ml of 75% ethanol. The samples were mixed by vortexing and centrifuged at no more than 7,500 x g for 5 minutes at 4 °C. The supernatant was removed and RNA pellet was air-dried for 10 minutes. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by passing solution a few times through a pipette tip. Total RNA was treated with 1 U of RQ1 RNase-free DNase (Invitrogen, Germany) to digest DNA residues, resuspended in DEPC-treated water.

Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT).

II. Reverse transcription (RT) reaction

The complete Poly(A)⁺ RNA isolated from male rats tissues was reverse transcribed into cDNA in a total volume of 20 µl using RevertAid™ First Strand cDNA Synthesis kit (MBI Fermentas, Germany). An amount of total RNA (5µg) was used with a reaction mixture, termed as master mix (MM). The MM was consisted of 50 mM MgCl₂, 5x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3; 10 mM of each dNTP, 50 µM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M-MuLV reverse transcriptase. The RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and the reaction was stopped by heating for 5 min at 99 °C. Afterwards, the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through semi-quantitative real time-polymerase chain reaction (sqRT-PCR).

III. Semi-quantitative real time-polymerase chain reaction (sqRT-PCR)

PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix Ex Taq™ (TaKaRa, Biotech. Co. Ltd, Germany), 0.5 µL 0.2 µM sense primers, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water, and 5 µL of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec; and (c) at 72.0 °C for 30 sec. The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C. At the end of each sqRT-PCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers. Each experiment included a distilled water control. The semi quantitative values of RT-PCR (sqRT-PCR) of FSH (FSH-F: 5'-GGG CCA GGA ACT GTG AAA TA-3', FSH-R: 5'-TCT CAG AAC TGC CGA GGT TT-3'), LH (LH -F: 5'-TCT CAC CACCAC CGT CTG TA-3', LH-R: 5'-TGC AGT CGC TGT AGT CCA TC-3') and Cyp19 (Cyp19-F: 5'-ATA CCA GGT CCT GGC TAC TG-3', Cyp19-R: 5'-TTG TTG TTA AAT ATG ATG CC-3') genes were normalized on the bases of β-actin (β-actin-F: 5'-TTG CCG ACA GGA TGC AGA A-3', β-actin-R: 5'-GCC GAT CCA CAC GGA GTA CT-3') expression. At the end of each sqRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers.

Calculation of gene expression

First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formulae (Bio-Rad 2006):

$$Ef = 10^{-1/\text{slope}}$$

$$\text{Efficiency (\%)} = (Ef - 1) \times 100$$

The relative quantification of the target to the reference was determined by using the ΔCT method if E for the target (FSH, LH and Cyp19) and the reference primers (β-actin) are the same (Bio-Rad 2006):

$$\text{Ratio (reference/ target gene)} = Ef_{CT(\text{reference})} - CT(\text{target})$$

The amplification efficiency (E) for FSH, LH and Cyp19 were 1.991 (%E=99.09), 1.993 (%E=99.25) and 1.997 (%E=99.66) respectively. Whereas, the PCR conditions indicated that the slopes of FSH, LH and Cyp19 were -3.344, -3.34 and -3.33 respectively.

Further, to ensure that the PCR efficiency ($E = 10^{-1/s} - 1$) was similar between the sample and the standard which was close to 2, we analyzed whether the addition of RT products to the reaction mixture for the standard curve which was prepared for purified RNA affected the PCR efficiency.

Histopathological examination

Autopsy samples were taken from brain and testis of rats in the different studied groups and fixed in 10% formalin buffer for 24 h. washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 °C in hot air oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4 µm by slide microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained by hematoxylin and eosin stains (Banchroft *et al.*, 1996) for histopathological examination through the electric light microscope.

Statistical analysis

In the present study, all results were expressed as Mean ±S.D of the mean. Data were analyzed by one way analysis variance (ANOVA) which performed using the Statistical Package for the Social Science (SPSS) program, version 9 (SPSS FOR Windows, 2001). Difference was considered significant when *P* value was < 0.05. Percent of changes representing the percent of variation with respect to control group was also calculated as follows: % Change = [(Marijuana exposed group-control group)/ control group] × 100.

RESULTS

Hormones Analyses

Effect of marijuana smoke inhalation on reproductive hormones of male rats

The results depicted in **Table (1)** showed the effect of marijuana smoke exposure for two and four weeks on serum levels of free testosterone, LH, and FSH of adult male rats. The results indicate that there was significant decrease in serum free testosterone level in marijuana exposed groups as compared with the corresponding control groups. The decrease in serum level of free testosterone was -69.4% at 2 weeks and -77.2% at 4 weeks from the control groups. The results of serum luteinizing hormone (LH) showed significant decrease in marijuana exposed groups as compared with the corresponding control groups. The decrease in serum level of LH was -34.8% at 2 weeks and -46.26% at 4 weeks from the control groups. In similar pattern serum follicle stimulating hormone (FSH) level showed significant decrease in rats exposed to marijuana smoke as compared with the

corresponding control groups. The decrease in serum level of follicle stimulating hormone was -72.4% at 2 weeks and -83.2% at 4 weeks from the control groups.

Table. 1: Serum level of free testosterone, LH, and FSH in control and marijuana smoke exposed rats at different time intervals.

Hormones Groups	Free testosterone (ng/L)	LH (mIU/mL)	FSH (mIU/mL)
Group Ia	5.65±1.57	0.977±0.034	0.762±0.091
Group (II)	1.71±0.69	0.637±0.091	0.21±0.1
P value	P<0.001	P<0.001	P<0.001
%	-69.4%	-34.80%	-72.4%
Group Ib	5.65±1.57	0.977±0.034	0.762±0.091
Group (III)	1.285±0.36	0.525±0.070	0.128±0.045
P value	P<0.001	P<0.001	P<0.001
%	-77.2%	-46.26%	-83.2%

The data are expressed as mean ±SD for 10 rats/group
%: percent of change

Gene Expression Analysis

Effect of marijuana smoke inhalation on reproductive related genes (FSH-B, LH-B and CYP19) of male rats

The effect of marijuana smoke exposure for different time intervals; two weeks (group II) and four weeks (group III) on the expression level of follicle-stimulating hormone (FSH-B) and luteinizing hormone (LH-B) genes in the brain of adult male rats is summarized in **Table 2**.

The expression level of FSH-B gene in brain tissue of rats in group (II), and group (III) was significantly decreased (*P*< 0.05) in comparison with the control group. The same trend was shown regarding LH-B gene, where group (II), and group (III) showed significant decrease (*P*< 0.05, *P*< 0.001, respectively) in the gene expression level of LH-B as compared with the control group.

Data in **Table 2** also represent expression level of Cyp19 gene in testis tissue of rats exposed to marijuana smoke for two weeks (group II) and four weeks (group III). The expression level of Cyp19 gene in testis tissue of group II and group III showed significant decrease (*P*< 0.05, *P*< 0.001, respectively) in comparison with the control group.

Table. 2: Effect of marijuana smoke inhalation for different time intervals on male reproductive related genes (FSH-B, LH-B and CYP19) of male rats.

Groups	Genes Relative gene expression values		
	FSH-B gene	LH-B gene	Cyp19 gene
Group (I)	1.200±0.03	1.30±0.04	1.250±0.02
Group (II)	0.600±0.02	0.85±0.02	0.800±0.01
P value	P<0.05	P<0.05	P<0.05
Group (III)	0.580±0.01	0.69±0.02	0.670±0.001
P value	P<0.05	P<0.001	P<0.001

Gene expression values are expressed as Mean ±SD for 10 rats / group.

Histopathological Examination

Histopathological examination of the brain

Histological investigation of brain tissue section of control rat showed no histopathological alteration and the normal histological structure of the covering meninges, cerebral cortex, hippocampus, cerebellum, and medulla oblongata was observed (**Fig. 1a-d**). Histopathological examination of brain tissue section

of rat after two weeks exposure to marijuana smoke showed sever congestion in the blood capillaries of the cerebrum and striatum with focal hemorrhage in the medulla oblongata. Hemorrhage was observed also in the brain fissures with degeneration in the purkenji cells of the cerebellum (**Fig. 2a-e**).

Histopathological examination of brain tissue section of rat after four weeks exposure to marijuana smoke showed congestion with oedema in the meninges, associated with vacuolization in the cerebral matrix and degeneration of the neuronal cells. Hemorrhage was observed also in the meninges covering the cerebellum, with degeneration in the purkenji cells of the cerebellum (**Fig. 3a-d**).

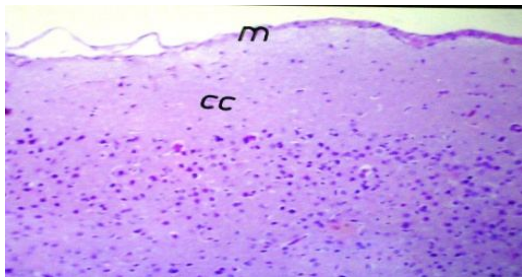


Fig. 1a: A photomicrograph of brain section of control rat showing normal histological structure of meninges and cerebral cortex

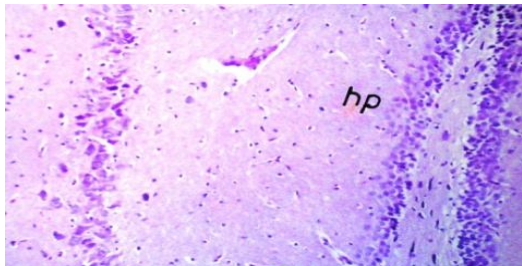


Fig. 1b: A photomicrograph of brain section of control rat showing normal histological structure of hippocampus and cerebral cortex

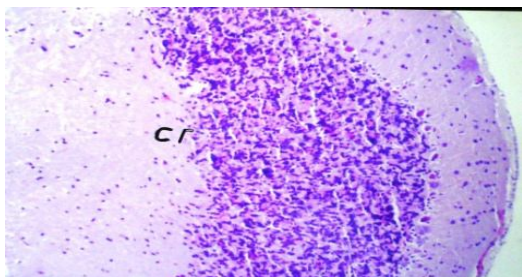


Fig. 1c: A photomicrograph of brain section of control rat showing normal histological structure of cerebellum and covering meninges

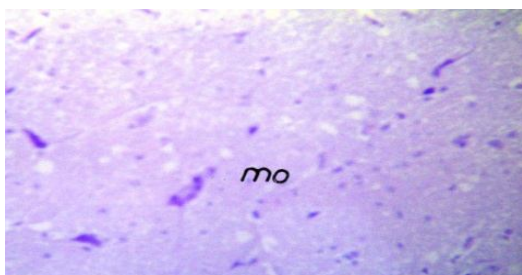


Fig. 1d: A photomicrograph of brain section of control rat showing normal histological structure of medulla oblongata

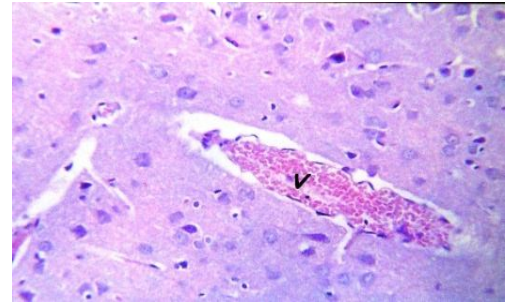


Fig. 2a: A photomicrograph of brain section of rat after two weeks exposure to marijuana smoke showing congestion in cerebral blood capillaries

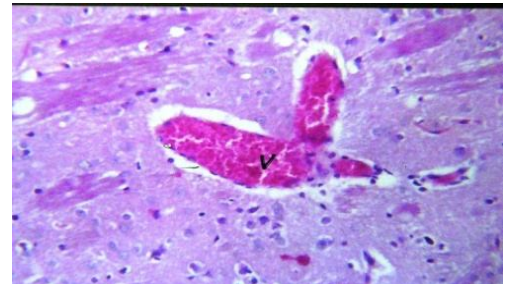


Fig. 2b: A photomicrograph of brain section of rat after two weeks exposure to marijuana smoke showing congestion in blood vessels of striatum

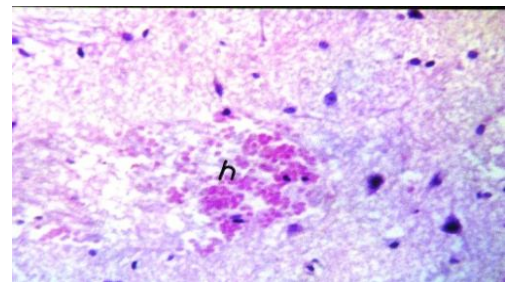


Fig. 2c: A photomicrograph of brain section of rat after two weeks exposure to marijuana smoke showing focal hemorrhage in medulla oblongata

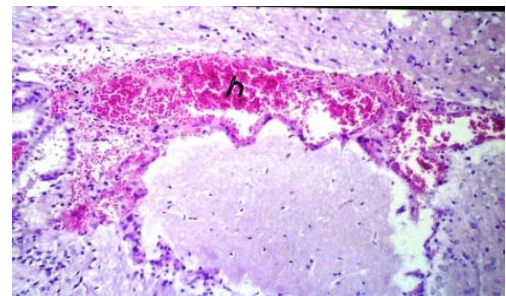


Fig. 2d: A photomicrograph of brain section of rat after two weeks exposure to marijuana smoke showing hemorrhage in the fissures

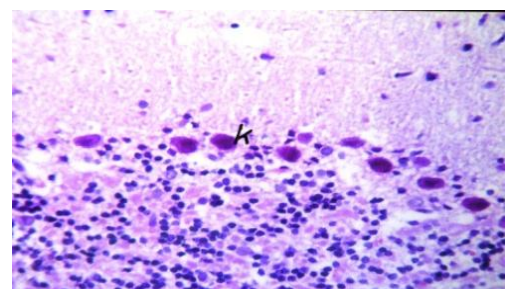


Fig. 2e: A photomicrograph of brain section of rat after two weeks exposure to marijuana smoke showing degeneration in purkenji cells of the cerebellum.

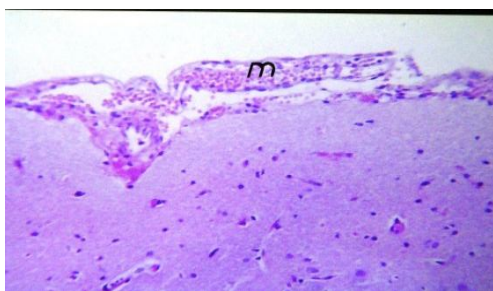


Fig. 3a: A photomicrograph of brain section of rat after four weeks exposure to marijuana smoke showing congestion and oedema in meninges covering the cerebellum .

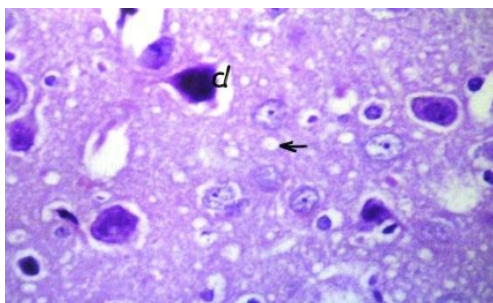


Fig. 3b: A photomicrograph of brain section of rat after four weeks exposure to marijuana smoke showing vacuolization in cerebral matrix with neural degeneration in cerebrum

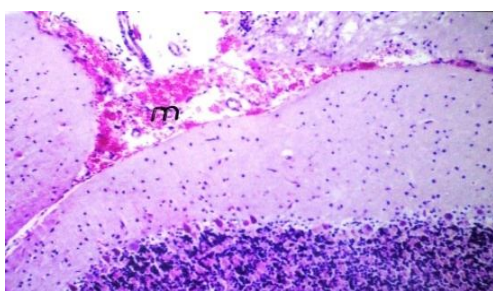


Fig. 3c: A photomicrograph of brain section of rat after four weeks exposure to marijuana smoke showing haemorrhage in meninges covering cerebellum

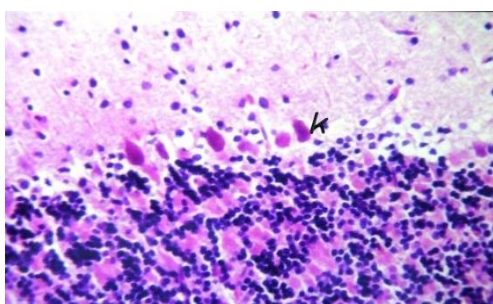


Fig . 3d: A photomicrograph of brain section of rat after four weeks exposure to marijuana smoke showing degeneration in purkenji cells of the cerebellum.

Histopathological examination of the testicle gland

Histological examination of the testicle gland tissue section of control rat showed no histopathological alteration and the normal histological structure of mature active seminiferous tubules with complete spermatogenic series was noticed (**Fig. 4**).

Histopathological examination of the testicle gland tissue section of rat after two weeks exposure to marijuana smoke showed no histopathological alteration and normal histological

structure of mature active seminiferous tubules with complete spermatogenic series was observed (**Fig. 5**).

Histopathological examination of the testicle gland tissue section of rat after four weeks exposure to marijuana smoke showed no histopathological alteration and normal histological structure of mature active seminiferous tubules with complete spermatogenic series was noticed (**Fig 6**).

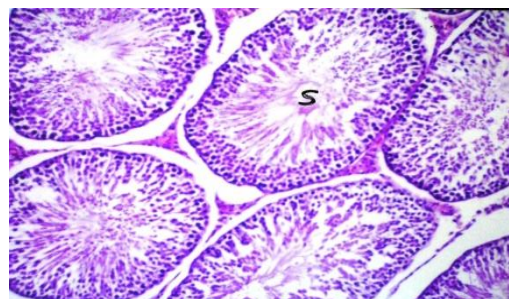


Fig. 4: A photomicrograph of testicle gland tissue section of control rat showing normal histological structure of mature active seminiferous tubules with complete spermatogenic series

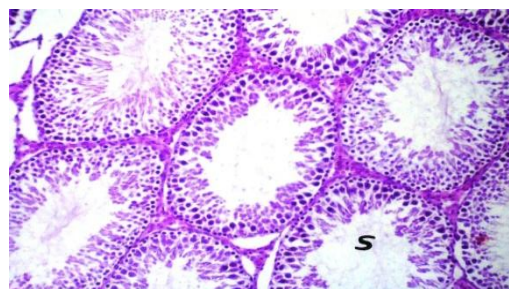


Fig. 5: A photomicrograph of the testicle gland tissue section of rat after two weeks exposure to marijuana smoke showing normal histological structure of mature active seminiferous tubules with complete spermatogenic series.

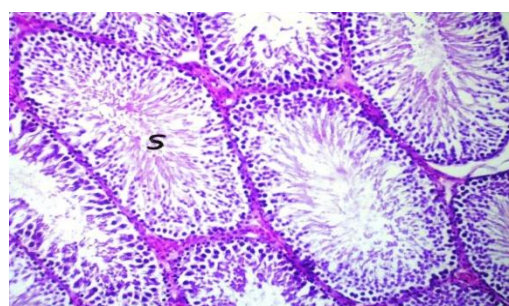


Fig. 6: A photomicrograph of the testicle gland tissue section of rat after four weeks exposure to marijuana smoke showing normal histological structure of mature active seminiferous tubules with complete spermatogenic series.

DISCUSSION

Addiction is defined as a chronic relapsing brain disease that is characterized by compulsive drug seeking and use, despite harmful consequences including change in brain structure and how brain works. These brain changes are long lasting and lead to the harmful behaviors seen in people who abused drugs (NIDA, 2011).

According to WDR (1997) marijuana is the most widely abused drug in all parts of the world, with an estimated 141

million people (2.4 percent of the world's population) consuming marijuana. This means that there is a sharp rise in marijuana smoking, particularly in young men and women (Johnston *et al.*, 1995).

The results of the current study indicate that serum levels of free testosterone, LH and FSH showed significant decrease in adult male rats exposed to marijuana smoke for either two weeks or four weeks. In agreement with our findings, Gupta and Elbracht (1983) found that chronic administration of delta 8- and delta 9-tetrahydrocannabinols to sexually developing male rats resulted in significant decrease in pubertal growth spurts, especially the second peak, testosterone, LH and FSH concentrations in the blood compared with control animals. In addition, marijuana smoking has been shown to decrease serum LH, FSH, and testosterone levels when compared to pre-smoking baseline hormone levels (Cone *et al.*, 1986) or when compared to nonsmoking individuals (Kolodny *et al.*, 1976). Dalterio *et al.* (1978) showed that oral administration of tetrahydrocannabinol (THC) to male mice resulted in a reduction of plasma testosterone, LH, and FSH levels, but that single dose of cannabinol (CBN) had no effect on any of these three hormones. They concluded that the reduction in plasma hormone levels was due to inhibition of pituitary function in producing these gonadotropic hormones as well as to a direct effect of THC on the testicular function. Moreover, Rubino *et al.* (2008) found that cannabis affects both neuroendocrine function and germ cells. THC can cause decline in pituitary hormones such as follicle stimulating hormone (FSH), luteinizing hormone (LH) and prolactin as well as it can deplete steroid hormones such as progesterone, estrogen and androgen. Furthermore, Sigmon and Howards (1998) and Gye *et al.* (2005) demonstrated that marijuana smoke and cannabinoids adversely affect male reproductive function in human and laboratory animals. They found significant decrease in both testosterone and gonadotropins with acute administration of THC because inhibition of gonadotropin release hormone pulse generator in the hypothalamus.

In general, it appears that the possible cause of significant decrease in free testosterone, LH and FSH serum level in rats exposed to marijuana smoke is that THC blocks gonadotropin releasing hormone (GnRH) release by the hypothalamus (Matsuda *et al.*, 1993). Since the pituitary remains capable of responding to exogenous GnRH in the presence of THC, it seems likely that the observed THC induced suppression of gonadotropin output arises indirectly from an action of THC upon the hypothalamus and not through a direct effect upon the pituitary (Nir *et al.*, 1973).

Another explanation of significant decrease in free testosterone, LH and FSH serum level in the present study may be due to the alteration of neural transmitter substances throughout the brain due to THC inhalation, but especially in that region of the central nervous system that is important in regulating GnRH release from the hypothalamus (Rosenkranz and Esber, 1980).

One of the main objectives of the current study was to evaluate the effect of marijuana smoke on the expression level of

male reproductive related genes; FSH-B, LH-B and CYP19 of adult male rats. The current findings revealed that marijuana smoke down-regulated the expression of FSH-B, LH-B and CYP19 genes. In agreement with our findings Harclerode (1984) reported that exposure to marijuana has significant effects upon the reproductive system where the marijuana component (cannabinoid) has equally significant effects on both male and female reproductive systems. Among the effects of cannabinoid treatment on male reproductive system are the alteration of testicular function, in the form of depressed male hormone secretion, and changes in both the quantity and quality of the sperm produced by the seminiferous tubules. Also, there are changes in the weight and in certain enzymes associated with the reproductive organs. Much research effort focused on the ability of THC to depress the secretion of the gonadotropins from the pituitary that are responsible for stimulating testosterone production by the Leydig cells of the testis and the action on the hypothalamus to depress gonadotropic releasing hormone (GnRH) (Harclerode, 1984).

The mechanism of action on marijuana on gene expression is not fully understood. Marijuana smoking is associated with inflammation, cellular atypia, and molecular dysregulation of the tracheobronchial epithelium (Sarafian *et al.*, 2005). While marijuana smoke shares many components in common with tobacco and it also contains a high concentration of THC and cannabinoid.

The study on the effect of THC on the gene expression level showed that THC could induce changes in the expression of mRNA for inflammatory mediators and antioxidant enzymes. These findings suggest a contribution of THC to DNA damage which in turn contributes to the pathological alterations associated with marijuana use (Sarafian *et al.*, 2005).

Cannabinoids may influence the structure and/or function of DNA nucleotide sequences which constitute structural genes or their components, in which case regions of the genome coding for defined proteins would not be transcribed or the transcription would not be appropriately processed and translated into functional proteins. In addition, cannabinoid induces alterations in the genetic sequences coding for the synthesis of ribosomal RNAs, transfer RNAs, or regulatory RNAs (Carchman *et al.*, 1976).

Cannabinoid may also induce alterations in the nucleotides contained within regulatory sequences or within those sequences involved in punctuating the genetic code. In an overall evaluation of the mechanisms by which cannabinoids may modify genes, one must bear in mind that there are four general categories of changes in the nucleotide bases which are prevalent--base substitutions, modifications of pre-existing bases, base additions, and base deletions. Recent evidence for additions, deletions, and amplification of nucleotide sequences, as well as rearrangements of genetic sequences in conjunction with expression, necessitates serious consideration of quantitative and qualitative modifications in DNA as potential regulatory events, and hence targets for drug-induced perturbations in gene expression. Within this context drug-mediated effects on DNA methylation, which has been

implicated in structural/transcriptional properties of genetic sequences, should not be overlooked (Stein and Stein, 1984; Sarafian *et al.*, 2005).

In present study the histopathology of the brain revealed that there was observed congestion in the blood capillaries of the cerebrum, and striatum with focal hemorrhage in the medulla oblongata. Hemorrhage was observed also in the brain fissures, with degeneration in the purkenji cells of the cerebellum. Congestion with edema was detected in the meninges, associated with vacuolization in the cerebral matrix and degeneration of the neuronal cells associated with neuronal degeneration and gliosis. These results go in line with the study of Yassa *et al.* (2009) which showed that brain histological effects of subchronic bango administration were so sever in the form of irregular shrunken cells with dense nuclei and vacuolated cytoplasm in all layers. The neuronal cells were appeared to be surrounded by irregular wide spaces. Brain is the most organ mostly affected due to exposure to marijuana because the main effects of THC are mediated through the cannabinoid receptors CB1 that dense decorate brain regions.

Regarding the histological investigation of testicle gland tissue of rats exposed to marijuana smoke at different time intervals our results showed no histopathological changes. Our results are in agreement with those of Rosenkrantz *et al.* (1975) who demonstrated that rats treated with THC orally at doses of 2, 10, or 50 mg/kg for 6 months showed no histopathological changes in the testes despite reduced growth rate and increased organ/body weight ratio.

CONCLUSION

In conclusion, marijuana smoke exposure leads to suppression of reproductive axis in males from biochemical, molecular genetic and histological points of view. The findings of this study suggest that marijuana could impair fertility in male albino rats through alteration in the circulating levels of reproductive hormones and down-regulation of the expression level of reproductive hormone related genes.

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