

Female Reproduction: Roles of Cannabis and Oxidative Stress

Ghosh S^{1,*} and Ahmad R²

¹Assistant Professor, Department of Zoology, Rajendra College, India

²Department of Zoology, Rajendra College, Jai Prakash University, India

*Corresponding author: Dr. Somenath Ghosh, Assistant Professor, Department of Zoology, Rajendra College, Chapra, 841301, Bihar, India

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Abstract

Cannabis sativum has long been used globally for hallucination. But, detrimental effects on female reproduction had never been studied. The aim of the present chapter to evaluate cannabinoid induced oxidative stress and reproductive containment in female mice. Fifteen adult female Parke's strain mice were randomly chosen from institutional animal house (n= 5/group) with ad libitum access to water and food. Animals were grouped into control (vehicle treated), treated with 6mg of cannabis/100g of body weight, 12mg of cannabis/100g of body weight. After 15 days all animals were sacrificed and tissues were collected for histology, immunohistochemistry and estimations of different parameters. Histology and immunohistochemistry (of Cannabinoid Receptor 1; CB1) were performed following standardized protocols. All parameters were estimated either by standard biochemical protocols or by kit following manufacturer's protocol. Stress parameters (Super Oxide Dismutase; SOD, Catalase, CAT; Malonaldehyde, MDA and Glutathione Peroxidase; GPx), apoptotic parameters of thecal cells (by Caspase-3 assay), serum level of Estrogen (E2), steroidogenic parameters (3 β Hydroxy-steroid dehydrogenase; 3 β HSD and 17 β Hydroxy-steroid dehydrogenase; 17 β HSD) and expression of CB1 were noted in ovary. Data were analyzed by One way Analysis of Variance (One way ANOVA) followed by Duncan's Multiple Range Post-Hoc Test. We found significant (P < 0.05) decrease in steroidogenic parameters and significant increase (P < 0.05) in free radical and apoptotic parameters and CB1 receptor expressions upon dose dependent cannabis treatment. We may conclude that chronic treatment of cannabis causes reproductive containment in females which has never been addressed previously.

Keywords: Cannabis; CB1; Female mice; Impairment; Reproduction; Stress

Introduction

Cannabis which is a type of marijuana has been used by the people of Indian sub-continent from time unknown [1]. They not only use this herb as a part of holy practice but also use it for recreational purposes [2]. Irrespective of sex, this hallucinogenic agent is used by most part of the world particularly by the populations of South America, India, Bangladesh and Pakistan from a long time ago [3]. Reports suggesting the roles of Cannabis causing systemic neuropathy [4], neuronal disability [5], impaired fetal development [6] and mal-functioning of male reproductive system [7-10] are documented. But no reports available are depicting the effects of marijuana in female reproductive system.

The main causative agent of marijuana/ cannabinoids is the endocannabinoid. This is a neutral lipid and highly conserved molecule throughout evolutionary history [11]. They are having different derivatives like anandamide [12], 2-arachidonoyl-glycerol [13] and Δ 9- tetrahydrocannabinol (THC) [14]. However, among all of the fatty acid derivatives of cannabinoids or endocannabinoids (eCBs) the Δ 9-tetrahydrocannabinol (THC) has now been established as the most important hallucinogenic agent of this molecule [15]. There are literatures suggesting the role of this Δ 9-tetrahydrocannabinol (THC) in regulation of functions of central nervous system and thus regulating the

reproductive functions by affecting/ modulating hypothalamo-pituitary- gonadal axis (HPG-axis) [16] via its receptor CB1 and CB2 [17]. Now it has been reported that CB1 receptors are localized mostly in whole vertebrate Central Nervous System (CNS) and some peripheral tissues, whereas CB2 receptors are mostly expressed in peripheral tissues and immune cells, however, they have recently been found also in the CNS [18]. But, with all the advancement in psycho-neuro-endocrine research, till date it is a matter of debate how THC is going to regulate reproductive system at peripheral level. Some literatures suggest that, there is a general agreement on the inhibitory effect exerted by cannabinoids and eCBs on GnRH release [19] Thus, it is affecting the subsequent FSH and LH release in females and impairing female reproduction [20].

But all the above-mentioned reported phenomenon are occurring in the central nervous system and no definitive proof has been reported till date how the endocannabinoids are affecting peripheral reproductive performances in females (in terms of gonadal activity, steroidogenesis, receptor expressions, free radical generations). Thus, aim of the present study was to note the cannabinoid (particularly endocannabinoid) induced oxidative stress and reproductive impairments in female mice specifically taking peripheral reproductive organs (ovary) in consideration.

Subjects and Methods

Animals and maintenance:

In bred adult (12–15 weeks of age) female Parkes strain mice were used for this study. Mice were maintained under hygienic conditions in a well-ventilated room with 12-hr photoperiod (8 AM to 8 PM, light) with $50 \pm 20\%$ relative humidity, $25 \pm 2^\circ\text{C}$ temperature and were fed pelleted food (Mona Laboratory Animal Feeds, Varanasi, India); drinking water was available ad libitum. Five mice in each group were housed in polypropylene cages ($430\text{mm} \times 270\text{mm} \times 300\text{mm}$), with dry rice husk as the bedding material. General health condition and body weight of the animals were monitored regularly during the entire tenure of the experiment. All experiments were conducted in accordance with principles and procedures approved by Departmental Research Committee under supervision of Committee for the Purpose of Control and Supervision of Experiments on Animals, (CPCSEA), Govt. of India (2007).

Preparations of different doses of Cannabis extracts:

Leaves and flowers of fresh Cannabis plant (100 g cannabis plant) were extensively ground in mortar and pestle with 1ml autoclaved double distilled water. From the 1 g/ml paste, 12mg was weighed and further dissolved in 1ml autoclaved double-distilled water to make a stock solution of 12 mg/ml. This solution was filtered to get a clear solution. Finally, the mice were gavaged Cannabis by means of a 100 μl micro-pipette using the 12 mg/ml stock.

Purity assessment of Cannabis preparations:

The dry-weight ratio of D9-Tetrahydrocannabinol (THC) to Cannabidiol (CBD) and the percent CBD and THC in the cannabis variant found in this region of the world has been previously reported [21]. The proportion of high THC/CBD chemotype plants in most accessions assigned to *C. sativa* was of 25% (Hillig and Mahlberg, 2004 [21]).

Experimental design:

Mice were randomly allocated into three groups (groups 1–3). Each group comprised of five female mice ($n=5/\text{group}$). Group 1 was treated with distilled water (vehicle treated; controls); Group 2 was gavaged with 6 mg/100g body weight/day aqueous Cannabis preparation; Group 3 was gavaged with 12 mg/100g bodyweight/ day aqueous Cannabis preparation. The mode of oral delivery of extracts were following the protocol published previously (21). The tips used for this purpose to deliver the dose from the micro-pipette had the pointed surface cut to avoid any injury in the mouth of the mouse. The micro-pipette was used to deliver a small volume of (~ 20 or $40\ \mu\text{l}$) dose. The study was continued for 30 days.

Collection of desired tissues:

Mice were weighed before the start of experiment as well as before killing. The animals were etherized to death and blood was collected from heart. Subsequently serum was separated and was stored at -20°C until biochemical estimations of total serum cholesterol and estradiol by ELISA. Both the ovaries and uterine horns were excised, blotted free of blood and fat tissues and were weighed. The ovary on one side of the animal was fixed in Bouin's fluid for histology and Immunohistochemical localization of CB1 receptor. The contra-lateral ovary of each mouse was stored at -20°C until used for enzyme assays (for steroidogenesis, Caspase-3 and free radical parameters) and western blot analysis of CB1 receptor.

Antibodies and reagents:

All of the chemicals used for the present study were of analytical grade and were purchased either from Sigma Aldrich (St. Louis, MO, USA) or from Merck (Germany). For western blot analysis, polyclonal primary antibody against CB1 receptor was purchased from Affinity BioReagents (Rockford, IL, USA) and horseradish peroxidase (HRP)-linked secondary antibody was purchased from Bangalore Genei Pvt. Ltd. (Bangalore, India). For immunohistochemistry (IHC), ABC Kit was purchased from ABC staining kit (Universal Elite, Vector Laboratories, Burlingame, CA). For 3β HSD and 17β HSD assays, pregnenolone was purchased from Sigma Aldrich (St. Louis, MO, USA).

Experimental Approaches

Histological preparations:

Ovaries were embedded in paraffin wax and serially sectioned of $6\ \mu\text{m}$ using a microtome (Leica, Germany). One set of slides was prepared and was further processed for haematoxylin and eosin staining following the protocol published elsewhere [22]. The permanent slides were prepared by mounting with DPX (Distyrene Plasticizer Xylene, SRL, India), after 24 hrs were observed under microscope (Leitz MPV3 with photo-automat software) and were documented for general histology.

Immunohistochemistry of CB1 receptor:

Immunohistochemistry for CB1 receptor was performed following the protocol published elsewhere [21]. Ovaries of both treated and untreated adult mice were paraffin embedded, and 6 mm sections were analyzed by immunohistochemistry, for CB1-receptor to show where, CB1, receptor is localized in mice ovaries and to have a generalized idea about the receptor expression pattern. For the secondary antibody and enzyme conjugates, ABC staining was used. Briefly after deparaffinization and hydration, and blocking of endogenous peroxidase with 3% H_2O_2 in methanol, sections were incubated with blocking serum for 1 hr, followed by incubation with primary antibody (CB1 at a dilution of 1:50) for 1 hr at room temperature. The sections were then washed and incubated with the biotinylated secondary antibody for 30 min at room temperature, followed by another 30 min with horse radish avidin-peroxidase conjugated. After washing, sections were incubated with the chromagen substrate (0.1% 3,3-diaminobenzidine tetrahydrochloride, DAB, Sigma-Aldrich, USA) in 0.05M Tris buffer, pH 7.6, and 0.01% H_2O_2 for 10 min and then counterstained with Elrich's hematoxylin. The permanent slides were prepared by mounting with DPX (Distyrene Plasticizer Xylene, SRL, India), after 24 hrs were observed under microscope (Leitz MPV3 with photo-automat software) and were documented.

Estimation of total serum cholesterol:

The total serum cholesterol was estimated by commercial cholesterol estimation kit following manufacturer's protocol (Span Diagnostics, Surat, Gujarat, India).

3β hydroxy steroid dehydrogenase enzyme activity:

3β HSD (EC 1.1.1.145) enzyme was assayed according to the protocol of Shivanandappa and Venkatesh (1997) [23] using ovarian homogenate. Ten percent tissue homogenate was prepared in 0.1M Tris-Cl buffer (pH 7.8). The homogenate was centrifuged at $12,000 \times g$ at 4°C and the supernatant was used as the source of enzyme. The enzyme was assayed in 0.1M

Tris-Cl buffer (pH 7.8) containing 500mM NAD, 100mM pregnenolone as substrate and enzyme (50 ml) in a total volume of 3.0 ml and incubated at 37°C for 1 hr. The reaction was stopped by the addition of 2.0 ml of phthalate buffer (pH 3.0) and the absorbance was noted at 490 nm. The enzyme activity was calculated from the standard curve of NADH and expressed as nmol NADH formed/h/mg protein.

17 β hydroxy steroid dehydrogenase enzyme activity:

17 β HSD (EC 1.1.1.62) activity was measured by following the protocol of Jarabek et al., (1962) [24]. In brief, ten percent homogenate of the ovarian tissues were prepared in normal phosphate Buffered Saline (PBS; pH 7.4) and 250 μ l of the supernatant was mixed with 250 μ l of 440 μ M sodium pyrophosphate buffer (pH 10.2), 10 μ l ethanol containing 0.3 μ M estradiol (Sigma, St. Louis, USA) and 240 μ l of 25 mg% BSA. Enzyme activity was measured after addition of 50 μ l of 0.5 μ M NAD to the mixture in a spectrophotometer at 340 nm against a blank (without NAD). One unit of enzyme activity was the amount causing a change in absorbance of .001/min at 340 nm.

Evaluation of SOD activity in ovary:

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed following the method of Das et al. (2000) [25]. Just after sacrifice, 10% homogenates of all ovarian tissues from group-I and set-III mice were prepared in 150 mM phosphate buffered saline (PBS, pH 7.4) and centrifuged for 30 min at 12,000 g at 4 °C. The supernatant was again centrifuged for 60 min at 12,000 g at 4 °C and then processed for enzymic activity based on a modified spectrophotometric method using nitrite formation by superoxide radicals. A 0.5 ml of homogenate was added to 1.4 ml of reaction mixture comprised of 50 mM phosphate buffer (pH 7.4), 20 mM L-methionine, 1% (v/v) Triton X- 100, 10 mM hydroxylamine hydrochloride, 50 mM ethylene diamine tetraacetic acid (EDTA) followed by a brief pre-incubation at 37 °C for 5 min. Next, 0.8 ml of riboflavin was added to all samples along with a control containing buffer instead of sample and then exposed to two 20W fluorescent lamps fitted parallel to each other in an aluminium foil coated wooden box. After 10 min of exposure, 1 ml of Greiss reagent was added and absorbance of the colour formed was measured at 543 nm. One unit of enzyme activity is defined as the amount of SOD inhibiting 50% of nitrite formation under assay conditions.

Estimation of Catalase activity in ovary:

Catalase (CAT; EC 1.11.1.6) activity was measured following the procedure of Sinha (1972) [26]. This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H₂O₂ with the formation of perchromic acid as an unstable intermediate. The chromic acetate thus produced is measured calorimetrically. The catalase preparation is allowed to split H₂O₂ for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H₂O₂ is determined by measuring chromic acetate calorimetrically after heating the reaction mixture. There is production of green color at the end of the process. Immediately after sacrifice, 20% homogenate of ovarian tissues from group-I, group-II and group-III were prepared in PBS (10 mM; pH=7.0) and then centrifuged at 12,000 g for 20 min at 4 °C. Supernatant was taken for enzyme estimation. 5 ml of PBS was added to 4 ml of H₂O₂ (200 mM) and then 1 ml of enzyme extract was added. After 1 min 1 ml of this solution was taken in a tube and 2 ml of K₂Cr₂O₇ (5%) solution was added. Then it was boiled

for 10 min and absorbance was measured at 570 nm. The activity of CAT was expressed as amount of H₂O₂ degraded per minute.

Estimation of lipid peroxidation (LPO) assay by thiobarbituric acid reactive substances (TBARS) level estimation in ovary:

After sacrifice of the mice of all the groups, the ovarian tissues were dissected out on a sterile watch glass placed in ice box, cleaned from adherent tissues and processed immediately for estimation of lipid peroxidation. Ovarian tissues of group-I, group-II and group-III experimental mice were weighed and homogenized in a tenfold excess of 20 mM Tris-HCl buffer (pH 7.4) and the 10% homogenates were centrifuged for 15 min at 3000X g at 4 °C. The supernatant was subjected to thiobarbituric acid (TBA) assay by mixing with 8.1% sodium dodecyl sulphate (SDS), 20% acetic acid, 0.8% TBA and then digested it for 1 h at 95 °C. The reaction mixture was immediately cooled in running water, vigorously shaken with 2.5 mL of n- butanol and pyridine reagent (15:1) and centrifuged for 10 min at 1500X g (Ohkawa et al., (1978) [27]. The absorbance of the upper phase was measured at 534 nm. Total thiobarbituric acid reactive substances (TBARS) were expressed as malondialdehyde (MDA; nmol/g tissue weight) taking 1,1,1,1-tetraethoxy propane (TEP) as standard. The standard curve was calibrated using 10 nM TEP.

Glutathione Peroxidase (GPx) estimation in ovary:

Glutathione peroxidase (GPx; EC 1.11.1.9) activity was assayed as described by Mantha et al. (1993) [28]. The reaction mixture (1 ml) contained 50 μ l sample, 398 μ l of 50 mM phosphate buffer (pH 7.0), 2 μ l of 1 mM EDTA, 10 μ l of 1 mM sodium azide, 500 μ l of 0.5 mM NADPH, 40 μ l of 0.2 mM GSH and 1 U glutathione reductase. The reaction mixture was allowed to equilibrate for 1 min at room temperature. After this, the reaction was initiated by addition of 100 mM H₂O₂. The absorbance measured kinetically at 340 nm for 3 min. The GPx activity was expressed as nmol of oxidized NADPH oxidized to NADP⁺ per min per mg of protein using an extinction coefficient (6.22 mM⁻¹ cm⁻¹) for NADPH.

Caspase 3 activity assay:

Thecal cell suspension was prepared following the protocol of Sharma et al., 2008 [29]. In brief, thecal cell suspensions from all the groups were prepared by mincing the entire ovary in ice-cold 1 \times PBS, at 4°C. After washing, cell pellets were collected by centrifugation at 500 g for 10 min at 4°C and the supernatant was gently removed. Cell pellets were lysed by the addition of 50 ml of cold lysis buffer (5 mM Tris, 20 mM EDTA, 0.5% Triton-X 100, pH 6.0) per 2 \times 6 10⁶ cells and incubated on ice for 10 min. Lysates were centrifuged at 10,000 g for 1 min at 4°C, and the supernatant was transferred to a fresh tube and processed for caspase-3 (EC 3.4.22.xx) activity using a caspase-3 colorimetric assay kit, according to manufacturer's instructions (R&D Systems, Inc. MN). Each enzymatic reaction, carried out in a 96-well flat bottom microplate, required 50 ml cell lysate, 50 ml reaction buffer and 5 ml caspase-3 colorimetric substrate (DEVD-pNA). The plate was incubated at 37°C for 2 h with a substrate blank and sample blank. At the end of the incubation period, the absorbance of enzymatically released chromophore p- nitroanilide (pNA) was read at 405 nm in a microplate reader (Tecan, Spectra II-micro-ELISA plate reader, Austria). Caspase-3 activity was determined by comparing the absorbance or optical density (OD) of pNA from

apoptotic samples with the untreated control and expressed as fold increase in OD405/106 cells per ml [29].

Serum level of estradiol:

Estradiol was assayed using ELISA kit (Biotron Diagnostics Inc., USA) according to manufacturer's protocol. The coefficient of intra- and inter-assay variation was less than 4.1% and 6.4%, respectively. The analytical sensitivity was 10 pg/ml.

Western blot analysis of Cannabinoid receptor 1 (CB1) analysis:

The ovarian tissue protein pooled from six mice was extracted as described earlier [30]. For western blot analysis, 10% ovarian homogenate was prepared. Equal amounts of proteins (50 mg) determined by Bradford's method were loaded on SDS PAGE (10%) for electrophoresis. Thereafter, proteins were transferred electrophoretically to nitrocellulose membrane (NC; Sigma- Aldrich, USA) overnight at 4°C. NC was then blocked for 60 min with Tris buffered saline (TBS; Tris 50mM, pH 7.6) and then incubated with primary antiserum (CB1 at a dilution of 1:250) for 1 hr. Then, membranes were washed for 10 min each (three washes) in TBS-Tween 20. Then, NC membrane was incubated with secondary conjugated with serum immunoglobulin (1:500) for 30 min and then washed in TBS for 10 min (three times). Signals were detected using an ECL kit (Bio-Rad, Hercules, CA). Blot for each protein was repeated for three times. The densitometry analysis of blots was performed by scanning and quantifying the bands for density value by using computer-assisted image analysis (Image J 1.38X, NIH). The densitometry data were presented as the mean of the integrated density value \pm SEM. A pre-stained multicolour broad range marker (Spectra TM multicolour broad range marker; 10 to 260 kDa x SM-1841; Fermentas, MD, USA) was also run along with sample proteins to clarify the position of band obtained as published elsewhere previously to detect the specificity of the bands [30].

Statistical analyses:

The data were analyzed on Microsoft Office Excel worksheet followed by one way ANOVA. All data are expressed as mean \pm SEM. The data were considered significant if $P < 0.05$. Further, to note the level of significance between the experimental groups Duncan's multiple rangs Post-Hoc test was applied. All of the estimations were done in single lot using replicates and were repeated thrice. Analyses were done using Statistical Package for Social Sciences software version 16 for windows (SPSS, 16.0, IBM, Chicago, IL, USA) and in accordance to Brunning and Knitz (1977) [31].

Results

Histomorphology of ovary:

The ovarian sections of both 6mg/100g of body weight and 12mg/100g of body weight showed degeneration of ovarian micro-architecture in comparison to control. There was absence of corpora-lutea in the ovaries of Cannabis treated mice. The ovaries of 12mg/100g of body weight showed highest number of degenerating follicles.

Immunohistochemistry of CB1 receptor in ovary:

CB1 receptors protein was demonstrated immunohistochemically in the ovaries of the control and Cannabis treated groups of mice. The immunoreactivity of CB1 receptors was mainly observed in the granulosa cells of secondary follicles in the control group. There was a dose dependent increase in the ex-

pression of the CB1 receptor in the ovarian sections. Intense staining was also observed in the degenerating follicles and oocyte (group-3). However, negative control did not show any immunostaining.

Body weight:

We noted a significant ($P < 0.05$) decrease in body weight in a dose dependent manner following Cannabis treatment in comparison to control. However, the difference of body weight between two experimental groups were not significant ($P > 0.05$).

Ovarian weight:

We recorded the ovarian weight upon Cannabis treatment. It was observed that upon Cannabis treatment the ovarian weight was significantly low ($P < 0.01$) in dose dependent manner as compared to control. Among two experimental groups, the difference in weight was also statistically significant ($P < 0.05$).

Uterine weight:

We recorded the same result in uterine weight also where Cannabis treatment profoundly ($P < 0.01$) decreased uterine weight as compared to control. However, the difference in uterine weight between two experimental groups was not significant ($P > 0.05$).

Total serum cholesterol:

Serum cholesterol also showed significant dose dependent decrease ($P < 0.01$) in serum cholesterol level upon Cannabis treatment being lowest in 12mg/100g of body weight group as compared to control. However, the difference between two experimental groups was statistically non-significant ($P > 0.05$).

3 β HSD enzyme activity in ovary:

Significant decrease in 3 β HSD enzyme activity ($P < 0.01$) was noted in a dose dependent manner in Cannabis treated ovaries as compared to control. However, the difference between two experimental groups was statistically non-significant ($P > 0.05$).

17 β HSD enzyme activity in ovary:

Significant decrease ($P < 0.01$) in 17 β HSD enzyme activity was noted in Cannabis treated ovaries in comparison to control. The difference in decreased activity between two experimental groups was also statistically significant ($P < 0.05$).

SOD activity in ovary:

Significant increase in SOD activity was noted in Cannabis treated groups in dose dependent manner being significantly high ($P < 0.01$) in both the groups of 6mg/100g of body weight and 12mg/100g of body weight as compared to control. The level was highest in the latter group in comparison to 6mg/100g of body weight ($P < 0.05$).

Catalase activity in ovary:

Significant increase in catalase activity was noted in Cannabis treated groups in dose dependent manner ($P < 0.01$) as compared to control. But, among the treated groups the level was not significant ($P > 0.05$).

Malondialdehyde level in ovary:

Significant decrease in ovarian malondialdehyde levels were noted in a dose dependent manner following Cannabis treatment being lowest in 12mg/100g of body weight dose ($P < 0.01$). The level in the 6mg/100g of body weight dose was interme-

diate with significantly lower level ($P < 0.05$) than control. Among the treated groups, group-3 showed least level of MDA activity ($P < 0.05$).

GPx level in ovary:

Glutathione peroxide (GPx) level was found to be significantly high ($P < 0.01$) in both the treatment groups when compared to control. Among 6mg/100g body weight and 12mg/100g body weight groups, the latter showed significantly high level ($P < 0.01$).

Caspase 3 activities in ovarian thecal cells:

Caspase 3 activity was assayed in the ovarian thecal cells upon cannabis treatment. We noted a significant increase of caspase 3 in the thecal cells in dose dependent manner being highest in 12mg/100g of body weight dose ($P < 0.01$) in comparison to control. Further, among the treated groups, group 3 presented the highest level of caspase 3 activity ($P < 0.01$).

Serum level of estradiol:

Serum level of estradiol was found to be significantly low ($P < 0.05$) in 6mg/100g of body weight dose, however, the level was further significantly low ($P < 0.01$) in 12mg/100g of body weight dose as compared to control which was recorded to be significantly low among the treated groups ($P < 0.05$).

Western Blot analysis of CB1 receptor in ovaries of mice:

We noted a significant increase ($P < 0.05$) in Cannabinoid receptor type 1 (CB1) in 6mg/100g of body weight treatment group. The level was further significantly high ($P < 0.01$) in 12mg/100g of body weight group as compared to control group. Further, the level of expression was highest in group 3 ($P < 0.05$) as compared among the treated groups.

Discussions

The present study was confined on the role of chronic Cannabis induced oxidative stress and reproductive impairment in female mice. In the recent years, there are several literatures available depicting the role of Cannabis in neuro-degeneration [32], neuro-myopathy [33] and different other neurological disorders [34]. But, till date there are no data or reports are available depicting the role of Cannabis treatment in regulating/modulating the female reproduction, however, it had been predicted from prolonged time that Cannabis is potent enough to interfere in reproduction in males [35] and females.

Our study, in relation to the dose dependent effect of Cannabis treatment in the female reproduction is the preliminary and elaborated study depicting the deleterious and detrimental effects of Cannabis in female reproduction. Our study is divided into two different parts addressing the role of Cannabis in reproductive impairments in female mice due to oxidative stress and loss in the functions of steroidogenesis.

We noted a significant decrease in body weight, ovarian and uterine weight upon Cannabis treatment suggesting the first clue in reproductive impairment upon Cannabis treatment. The results were further supported by degeneration in ovarian histomorphology and increase in expressions of CB1 receptors in ovaries of different treatment groups. Cumulatively, the histological and immunohistochemical data suggest a dose dependent impairment in ovarian as well as reproductive functions which are in agreement with previous reports in where Cannabis causes reproductive impairment in males [36]. We

have also studied the different aspects of free radical as well as reproductive enzyme activities (3β HSD and 17β HSD). The SOD, catalase and GPx levels were significantly high in ovary tissues whereas MDA level was significantly low. The increased results of free radical scavenging enzyme activities suggest the reproductive impairment in mice is may be due to high generation of free radicals and also due to different physiological malfunctions which are yet to be traced out [37-40].

Further, significant decrease in total serum cholesterol levels, estradiol levels in circulation, 3β HSD and 17β HSD enzyme activities in ovarian tissues upon Cannabis treatment were noted. Thus, we may suggest that upon Cannabis treatment reproduction in females was impaired by Cannabis treatment by generation of free radicals in female reproductive tissues. The results were also discussed in light of apoptosis in thecal cells by Caspase 3 activity assays and it was found to be significantly high in different doses of Cannabis treatments. To delineate the possible molecular mechanism of Cannabis function in ovary, we checked the CB1 receptor expression in ovarian tissues and we also found that the CB1 receptor expressions were significantly high in both the 6mg/ 100g of body weight and 12 mg/100g of body weight groups which are in agreement with the reports published earlier [41-43].

Thus, we may suggest that Cannabis treatments were not only impairing the reproduction in females but also chronic duration of doses is responsible for high fecundity in terms of reproductive malfunctions.

Conclusion

This study, for the first time showed the effect of administration of cannabis, in controlling the reproductive process in female mice. It also showed the interrelation between the exogenous administrations of cannabis, the possible mechanism that was not dealt by earlier workers showing anti-fertility effect of cannabis for females in particular.

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