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Review Article

Cannabinoids, Oxidative Stress and Female Reproduction: Where do we stand?

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Abstract

Cannabis sativum has long been used globally for hallucination. However, detrimental effects on female reproduction have never been studied. The aim of the present paper is to evaluate cannabinoid-induced oxidative stress and reproductive containment in female mice. Fifteen adult female Parkes strain mice were randomly chosen from institutional animal houses (n = 5/group) with *ad libitum* access to water and food. Animals were grouped into control (vehicle-treated), treated with 6mg of cannabis/100 g of body weight, and 12 mg of cannabis/100 g 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; CB_1) were performed following standardized protocols. All parameters were estimated either by standard biochemical protocols or by kit following the 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 (3β Hydroxy-steroid dehydrogenase; 3β HSD and 17β Hydroxy-steroid dehydrogenase; 17β 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 a significant ($p < 0.05$) decrease in steroidogenic parameters and a significant increase ($p < 0.05$) in free radical and apoptotic parameters and CB_1 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.

More Information

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Introduction

Cannabis which is a type of marijuana has been used by the people of the Indian subcontinent for 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 has been used by most parts of the world particularly by the populations of South America, India, Bangladesh, and Pakistan for a long time ago [3]. Reports suggesting the roles of *Cannabis* causing systemic neuropathy [4], neuronal disability [5], impaired fetal development [6], and malfunctioning of the male reproductive system [7-10] are documented. However, no reports available depict the effects of marijuana on the 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 have different derivatives like anandamide [12], 2-arachidonoylglycerol [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 is literature suggesting the role of this Δ^9-tetrahydrocannabinol (THC) in the regulation of functions of the central nervous system and thus regulating the reproductive functions by affecting/ modulating hypothalamo-pituitary- gonadal axis (HPG-axis) [16] *via* its receptor CB_1 and CB_2 [17]. Now it has been reported that CB_1 receptors are localized mostly in the whole vertebrate Central Nervous System (CNS) and some peripheral tissues, whereas CB_2 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 advancements in

psycho-neuro-endocrine research, to date, it is a matter of debate about how THC is going to regulate the reproductive system at the 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 phenomena are occurring in the central nervous system and no definitive proof has been reported to date on how the endocannabinoids are affecting peripheral reproductive performances in females (in terms of gonadal activity, steroidogenesis, receptor expressions, free radical generations). Thus, the 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) into consideration.

Subjects and methods

Animals and maintenance

Inbred 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 a 12-hr photoperiod (8 AM to 8 PM, light) with 50 ± 20% relative humidity, 25 ± 2 °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 mm × 270 mm × 300 mm), with dry rice husk as the bedding material. The 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 the Departmental Research Committee under the supervision of the 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, 12 mg was weighed and further dissolved in 1 ml 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 [20]. The proportion of high THC/CBD chemotype plants in most accessions assigned to *C. sativa* was 25% [21].

Experimental design

Mice were randomly allocated into three groups (groups 1 – 3). Each group comprised of five female mice (n = 5/group). Group I was treated with distilled water (vehicle-treated; controls); Group 2 was gavaged with 6 mg/100 g body weight/day aqueous *Cannabis* preparation; Group 3 was gavaged with 12 mg/100 g body weight/ day aqueous *Cannabis* preparation. The mode of oral delivery of extracts was 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 µl) dose. The study was continued for 30 days.

Collection of desired tissues

Mice were weighed before the start of the experiment as well as before killing. The animals were etherized to death and blood was collected from the heart. Subsequently, the 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 weighed. The ovary on one side of the animal was fixed in Bouin's fluid for histology and Immunohistochemical localization of the CB_1 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 the CB_1 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, a polyclonal primary antibody against the CB_1 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), the ABC Kit was purchased from the 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 6 µm using a microtome (Leica, Germany). One set of the slides was prepared and was further processed for hematoxylin 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 a microscope (Leitz MPV3 with photo-automat software) and were documented for general histology.

Immunohistochemistry of CB_1 receptor

Immunohistochemistry for the CB_1 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 the CB_1 receptor to show where, the CB_1, 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 (CB_1 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 0.05 M 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 a microscope (Leitz MPV3 with photo-automat software) and were documented.

Estimation of total serum cholesterol

The total serum cholesterol was estimated by a commercial cholesterol estimation kit following the 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 [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 X g at 4 °C and the supernatant was used as the source of the enzyme. The enzyme was assayed in 0.1 M Tris-Cl buffer (pH 7.8) containing 500 mM 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.

17β hydroxy steroid dehydrogenase enzyme activity

17β HSD (EC 1.1.1.62) activity was measured by following the protocol of Jarabek, et al. [24]. In brief, ten percent homogenate of the ovarian tissues was prepared in normal phosphate Buffered Saline (PBS; pH 7.4), and 250 µl of the supernatant was mixed with 250 µl of 440 µM sodium pyrophosphate buffer (pH 10.2), 10 µl ethanol containing 0.3 µM estradiol (Sigma, St. Louis, USA) and 240 µl of 25 mg% BSA. Enzyme activity was measured after the addition of 50 µl of 0.5 µM 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 the ovary

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed following the method of Das, et al. [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 aluminum 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 the ovary

Catalase (CAT; EC 1.11.1.6) activity was measured following the procedure of Sinha [26]. This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2 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 H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of a dichromate/acetic acid mixture and the remaining H_2O_2 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 H_2O_2 (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 $K_2Cr_2O_7$ (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 the amount of H_2O_2 degraded per minute.

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

After the sacrifice of the mice of all the groups, the ovarian tissues were dissected out on a sterile watch glass placed in the 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 sulfate (SDS), 20% acetic acid, and 0.8% TBA and then digested for 1 h at 95 °C. The reaction mixture was immediately cooled in running water, vigorously shaken with 2.5 mL of n- n-butanol and pyridine reagent (15:1), and centrifuged for 10 min at 1500X g [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 the ovary

Glutathione peroxidase (GPx; EC 1.11.1.9) activity was assayed as described by Mantha, et al. [28]. The reaction mixture (1 ml) contained 50 µl sample, 398 µl of 50 mM phosphate buffer (pH 7.0), 2 µl of 1 mM EDTA, 10 µl of 1 mM sodium azide, 500 µl of 0.5 mM NADPH, 40 µl of 0.2 mM GSH and 1 U glutathione reductase. The reaction mixture was allowed to equilibrate for 1 minute at room temperature. After this, the reaction was initiated by the addition of 100 mMH_2O_2. The absorbance was 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×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 × 6 10^6 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 a fold increase in $OD405/10^6$ cells per ml [29].

Serum level of estradiol

Estradiol was assayed using an ELISA kit (Biotron Diagnostics Inc., USA) according to the 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 (CB_1) 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 50 mM, pH 7.6) and then incubated with primary antiserum (CB_1 at a dilution of 1:250) for 1 hr. Then, membranes were washed for 10 min each (three washes) in TBS-Tween.

20. Then, the NC membrane was incubated with secondary conjugated serum immunoglobin (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). The blot for each protein was repeated 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 ± SEM. A pre-stained multicolor broad range marker (Spectra TM multicolor 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 the band obtained as published elsewhere previously to detect the specificity of the bands [30].

Statistical analyses

The data were analyzed on a Microsoft Office Excel worksheet followed by one-way ANOVA. All data are expressed as mean ± SEM. The data were considered significant if $p < 0.05$. Further, to note the level of significance between the experimental groups Duncan's multiple range post-hoc test was applied. All of the estimations were done in single lots 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 by Brunning and Knitz [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 an absence of corpora-lutea in the ovaries of *Cannabis* treated mice. The ovaries of 12 mg/100 g of body weight showed the highest number of degenerating follicles.

Immunohistochemistry of CB_1 receptor in the ovary

CB_1 receptor protein was demonstrated immunohistochemically in the ovaries of the control and *Cannabis* groups of mice. The immunoreactivity of CB_1 receptors was mainly observed in the granulose cells of secondary follicles in the control group. There was a dose-dependent increase in the expression of the CB_1 receptor in the ovarian sections. Intense staining was also observed in the degenerating follicles and oocytes (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 differences in body weight between the 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 the 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 the two experimental groups was not significant ($p > 0.05$).

Total serum cholesterol

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

3β HSD enzyme activity in the ovary

A significant decrease in 3β 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 the two experimental groups was statistically non-significant ($p > 0.05$).

17β HSD enzyme activity in the ovary

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

SOD activity in the ovary

A significant increase in SOD activity was noted in *Cannabis* treated groups in a dose-dependent manner being significantly high ($p < 0.01$) in both the groups of 6 mg/100 g of body.

Weight and 12 mg/100 g 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 the ovary

A significant increase in catalase activity was noted in *Cannabis* groups in a dose 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 the ovary

Significant decreases in ovarian malonaldihyde levels were noted in a dose-dependent manner following *Cannabis* treatment being lowest in 12 mg/100 g of body weight dose ($p < 0.01$). The level in the 6 mg/100 g of body weight dose was intermediate with a significantly lower level ($p < 0.05$) than control. Among the treated groups, group 3 showed the lowest level of MDA activity ($p < 0.05$)

GPx level in the ovary

Glutathione peroxide (GPx) level was found to be significantly high ($p < 0.01$) in both the treatment groups when compared to the control. Among the 6 mg/100 g body weight and 12 mg/100 g body weight groups, the latter showed a 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 dose-dependent manner being highest in 12 mg/100 g of body weight dose ($p < 0.01$) in comparison to the 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 6 mg/100 g of body weight dose, however, the level was further significantly low ($p < 0.01$) in 12 mg/100 g 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 CB_1 receptor in ovaries of mice

We noted a significant increase ($p < 0.05$) in Cannabinoid receptor type 1(CB_1) in 6 mg/100 g of body weight treatment group. The level was further significantly higher ($p < 0.01$) in the 12 mg/100 g body weight group as compared to the control group. Further, the level of expression was highest in group 3 ($p < 0.05$) as compared to the treated groups.

Discussion

The present study was confined to the role of chronic *Cannabis* induced oxidative stress and reproductive impairment in female mice. In recent years, there have been several kinds of literature available depicting the role of *Cannabis* in neuro-degeneration [32], neuro-myopathy [33], and different other neurological disorders [34]. But, to date there are no data or reports are available depicting the role of *Cannabis* treatment in regulating/modulating female reproduction, however, it had been predicted for a 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 female reproduction, is a 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 an increase in expressions of CB_1 receptors in the 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 which *Cannabis* causes reproductive impairment in males [36]. We have also studied the different aspects of free radicals as well as reproductive enzyme activities (3β HSD and 17βHSD). The SOD, catalase, and GPx levels were significantly high in ovary tissues whereas the MDA level was significantly low. The increased results of free radical scavenging enzyme activities suggest the reproductive impairment in mice may be due to the high generation of free radicals and also due to different physiological malfunctions that are yet to be traced [37-40].

Further, significant decreases 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 the ovary, we checked the CB_1 receptor expression in ovarian tissues and we also found that the CB_1 receptor expressions were significantly high in both the 6 mg/ 100 g of body weight and 12 mg/100 g 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 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 the 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 with by earlier workers showing the anti-fertility effect of cannabis for females in particular.

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