



# EFFECTS OF CANNABIS SATIVA EXTRACT AND ITS COMBINATION WITH VITAMIN-C ADMINISTRATION ON HISTOMORPHOLOGICAL CHANGES IN THE TESTICULAR TISSUE OF MALE MICE

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## ABSTRACT

**OBJECTIVE:** To observe morphological changes in testicular tissue of adult male BALB/c mice after administration of Cannabis sativa extract and to investigate the protective role of vitamin-C against cannabis-induced histopathological changes.

**METHODS:** Eighteen BALB/c mice (31-40 g) were divided into 3 groups. Group-I was control group, Group-II received oral cannabis extract (14 mg/kg) and Group-III received cannabis extract (14 mg/kg) orally along with vitamin-C (100 mg/kg) for 45 days. At the end of experiment, variations in body weight, testicular weight and histological changes in testicular tissues of mice were observed.

**RESULTS:** Average increase in weight was  $7.000 \pm 0.77$  gm,  $4.833 \pm 0.87$  gm &  $5.833 \pm 0.79$  gm in group-I, group-II and group-III respectively. Weight reduction in right and left testis was  $42.30 \pm 0.465$  mg and  $42.45 \pm 0.424$  mg in group-I,  $38.85 \pm 0.523$  mg and  $39.35 \pm 0.520$  mg in group-II, and  $42.38 \pm 0.465$  mg and  $42.23 \pm 0.424$  mg in group-III animals respectively ( $p < 0.01$ ). Mean seminiferous epithelium thickness was  $4.367 \pm 0.302$   $\mu$ m in group-II,  $5.683 \pm 0.104$   $\mu$ m and  $6.417 \pm 0.116$   $\mu$ m in Group-I and group-III respectively ( $p < 0.001$ ). Average germ cells count (cells/tubule) in group-II was reduced ( $256.25 \pm 6.58$ ) as compared to group-I ( $373.50 \pm 4.063$ ) and group-III ( $366.25 \pm 3.442$ ). Histological changes observed after the treatment were decreased height of germinal epithelium, basal lamina disruption, seminiferous tubules shrinkage, scanty cytoplasm with shrunken nuclei and germinal epithelium detachment from basal lamina in group-II animals as compared to group-I and group-III.

**CONCLUSION:** The cannabis administration significantly declines the weight, size of testes, germ cells count and the height of seminiferous epithelium in animals. Vitamin-C had a protective role on cannabis induced toxic changes in the testes of the mice.

**KEY WORDS:** Cannabis (MeSH); Cannabis Sativa (MeSH); Vitamin C (MeSH); Ascorbic Acid (MeSH); Fertility (MeSH); Testes (MeSH); Histology (MeSH); Seminiferous Tubules (MeSH); Spermatogenesis (MeSH).

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testosterone and gonadotropins with administration of THC, which is due to inhibition of the gonadotropin releasing hormone (GnRH) in the hypothalamus. Marijuana has also been associated with the development of gynecomastia in both men and rodents.<sup>5</sup> Researches also indicate that with cannabis extract administration, there is a decrease in the size of testes, shrinkage of the seminiferous tubules, epididymis, vas-deference and it affects the process of spermatogenesis in experimental animals.<sup>6</sup>

Vitamin-C (ascorbate) is a strong water-soluble antioxidant in biological fluids, physiologically relevant reactive oxygen (RO) and nitrogen species.<sup>1</sup> Vitamin-C is nutritive anti-oxidants, which can neutralize the free radicals and react directly with peroxides and execute an important antioxidant function in humans and animals.<sup>7</sup> Additionally, it is involved in the regeneration of other small antioxidants molecule ( $\alpha$ -tocopherol, glutathione, urate, and  $\beta$ -carotene) and its potential pro-oxidant activity also been well documented.<sup>1,8</sup>

This study was designed to compare the morphological changes caused by Cannabis sativa extract with the protective role of vitamin-C against cannabis induced histopathological changes in the testes of adult male BALB/c mice strain.

## INTRODUCTION

Cannabis sativa is the most commonly used illegal drug throughout the globe.<sup>1</sup> It is a complex plant containing more than 400 chemical entities and more than 60 belong to cannabinoid.<sup>1,2</sup> The main ingredient having psychoactive activity is marijuana THC (delta-9-tetrahydrocannabinol) and its amount determines its strong effects.<sup>3</sup> Marijuana

smoking disrupts the hormonal balance of both male and female. Higher usage of cannabis responsible for various endocrine changes such as, reduced testosterone levels, decreased sperm counts in males, declined luteinizing hormone and prolactin levels during luteal phase in females which ultimately leads to shorter periods and more anovulatory cycles.<sup>4</sup> Studies in male rodents exposed to marijuana show significant decrease in both

**TABLE I: BODY WEIGHT VARIATION AND BODY WEIGHT GAIN IN DIFFERENT EXPERIMENTAL GROUPS**

Groups	Experimental Days				Body Weight Gain	
	I	15	30	45	gm	%
Group I (Control)	37.33±1.11	42.50±0.71	43.00±0.93	44.33±0.88	7.000±0.77	19.02±2.40
Group II (Cannabis)	35.50±1.76	38.67±1.74	39.17±1.62	40.33±1.62	4.833±0.87	14.01±2.87
Group III (Vit-C+Cannabis)	34.17±0.70	38.50±1.45	39.17±1.57	40.00±1.36	5.833±0.79	16.95±2.10

**TABLE II: TESTICULAR WEIGHT (RIGHT & LEFT) OF DIFFERENT EXPERIMENTAL GROUPS AT 45 DAYS**

Treatment Groups	Weight of right testis (mg)	Weight of left testis (mg)
Group I (Control)	42.30±0.465mg	42.45±0.424mg
Group II (Cannabis)	38.85±0.523mg	39.35±0.520mg
Group III (Vit-C+Cannabis)	42.38±0.465 mg	42.23±0.424 mg

## METHODS

Eighteen BALB/c mice (31–40 g) were obtained from the National Institute of Health, Islamabad, Pakistan. They were housed at 18–26°C (68–75.2°F) temperature with relative humidity of 40–70%. Study animals were provided with food and water ad-libitum and maintained at a 12/12 hours light/dark cycle. Light within the cages was diffuse and uniform to avoid glare, heat clusters and fluctuating lighting conditions. All experiments were examined approved by Ethical Committee of the Department of Pharmacy, University of Peshawar, Pakistan and conducted according to the principles of "United Kingdom Animal Scientific Procedure Act 1986".

For the collection and extraction of Cannabis sativa leaves, the procedure of Ullah et al.<sup>9</sup> were followed. Female plants of Cannabis sativa were collected at a farm with permission from Pladura area of Charsadda at its bloom season. The plant was authenticated by personnel of the Department of Botany, University of Peshawar, Peshawar. Leaves and flowering tops were separated. The plant material was shade dried and coarsely grinded. The grinded plant material was then extracted with n-hexane to obtain a yield of 500gm. The extract was then filtered and concentrated under reduced pressure at 45°C in a rotary evaporator. A yield of 50 ml was obtained. For oral administration, the extract was dissolved in absolute ethanol, mixed with emulsifier and made the volume

with distilled water in such a way that the final mixture consists of ethanol: emulsifier: distilled water in a ratio of 5:5:90.

After one-week acclimatization to their new environment with standard diet and water given ad-libitum, the 18 male BALB/c mice were randomly divided in a blinded fashion into 3 oral treatment groups. All animals were kept in the animal house under standard conditions at a room temperature (18°C to 26°C) for 45 days duration. The details of the group as follows:

Group I (control): Consisted of 6 mice that received solvent system, plain water and commercial standard mash feed orally for 45 days.

Group II: Consisted of 6 mice and received cannabis extract (14 mg/kg) orally once daily for a period of 45 days.

Group III: Consisted of 6 mice that were given cannabis extract at a dose of 14mg/kg orally once daily for period of 45 days along with vitamin-C 100mg/kg of body weight.

The concentration of 1 mg/ml of Cannabis sativa solution was administered at a dose of 14 mg/kg according to Dixit<sup>10</sup> and 4 mg/ml of vitamin-C was dissolved in normal saline and, was administered at a dose of 100 mg/kg according to Hsu and Guo.<sup>11</sup> Cannabis sativa was administered orally through an oral gavage tube. At the end of the experiment, after 45 days, the control and experimental animals were sacrificed by cervical dislocation. After cervical dislocation, testes from each

animal were removed and the weight of animal body and their testes were recorded as quickly as possible to avoid any pathological changes due to necropsy and were transferred to labelled jars containing 10% neutral buffered formalin for preservation. After fixation the testicular tissue samples were dehydrated using increasing grades of alcohol, cleared in xylene and embedded in paraffin. Tissue sections (5µm) were cut using a microtome and stained with Harris Hematoxylin and eosin (H & E) and mason trichrome for histological analysis. Photomicrographs of tissues sections were taken on a digital camera attached with the microscope. Quantitative methods were used for the evaluation of all slides. Parameters including height of the germinal epithelium and germ cell count were noted. For the calculation of height of germinal epithelium, ocular micrometer was used, which was calibrated with stage micrometer.<sup>13</sup>

Paired testicular/body weight ratio (PTWR) was determined by dividing the total weight of the two testes by the final body weight of the animal.

Data was analyzed with different tests using Statistical Package for Social Science (SPSS) for window version 13. Data were presented as the mean±SEM. P-Value ≤0.05 were measured statistically significant.

## RESULTS

The average weight of animal at the beginning of the study and at the end of the study is listed in Table I. However, there was an increase in relative body weights of all the groups. Average increase in weight of control group I was 7.000±0.77 gm, in group II was 4.833±0.87 gm and in group III was 5.833±0.79 gm respectively (Table I).

Significant reduction was observed in the weight of right ( $38.85 \pm 0.523\text{mg}$ ) and left ( $39.35 \pm 0.520\text{mg}$ ) testis of group-II animals as compared to right ( $42.30 \pm 0.465\text{mg}$ ) and left ( $42.45 \pm 0.424\text{mg}$ ) testis of animals in group-I (Table II).

Histological evaluation of testis in solvents systems, cannabis alone and cannabis plus vitamin-C groups after 45 days by H & E method, for routine histological studies and Masson Trichrome for observing fibrosis are presented in Figure 3. Histological changes observed after the treatment were decreased height of germinal epithelium, basal lamina disruption, seminiferous tubules shrinkage, scanty cytoplasm with shrunken nuclei and germinal epithelium detachment from

basal lamina in group-II animals as compared to group-I and group-III.

A significant decrease ( $p < 0.001$ ) was observed in the thickness of germinal epithelium of cannabis alone ( $4.367 \pm 0.302$ ) animals as compared to group I ( $5.683 \pm 0.104$ ) animals (Table III).

Mean germ cells count (cells/tubule) in group-II was  $256.25 \pm 6.58$  as compared to  $373.50 \pm 4.063$  and  $366.25 \pm 3.442$  in Group-I and group-III respectively ( $p < 0.001$ ).

## DISCUSSION

The present study using male BALB/C mice yielded information on the toxic effects of Cannabis sativa on the seminiferous tubules of the testes and

the protective role of the vitamin-C against cannabis induced testicular toxicity. The findings revealed that, after 45 days of the experiment our study did not reveal any significant decrease in the mean body weight of group II (cannabis alone) animals. These results were supported by the previously published study in which cannabis was administered to rats in the dose of 20 (mg/kg/body weight) for 21 consecutive days.<sup>14</sup> However, findings of this study were deviated from the studies conducted by Lotfi et al.<sup>15</sup>, and Banerjee et al.<sup>16</sup> Author reported the significantly decreased in body weight of cannabis group as compared to the control group animals. The variation in the result of this study from previously conducted studies may be due to the use of lower cannabis dose (14 mg/kg/body weight).

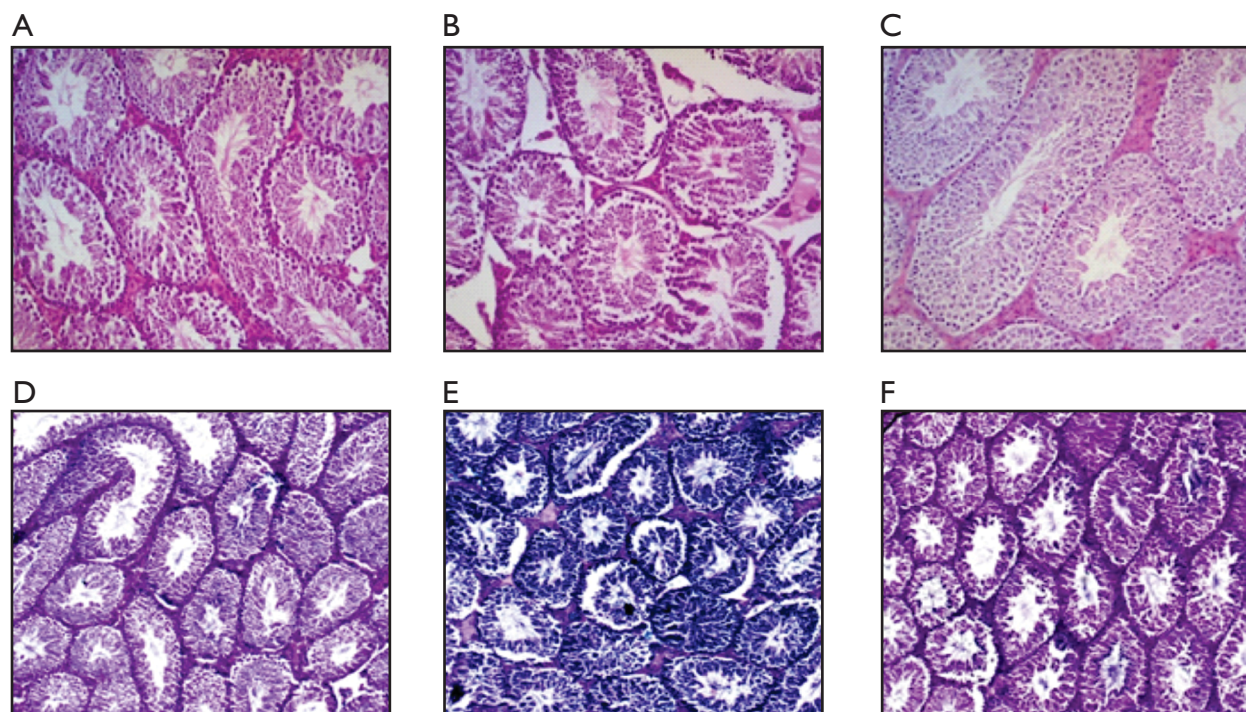


Figure 3: Histological evaluation of testis in solvents systems, cannabis and cannabis plus Vit-C after 45 days. A. Photomicrograph of  $5\mu\text{m}$  thick H and E stained section of testis of a control group showing normal histological appearance of seminiferous tubules. B: H and E stained section of testis of a group II (cannabis only) showing histopathological changes (changes in Morphology, thickness, number of spermatogenic cells and damage in basement membrane or basal lamina of seminiferous tubules) in the germinal epithelium of seminiferous tubules. C: H and E stained section of testis of a group III (cannabis plus Vitamin-C) showing normal histological appearance of seminiferous tubules. D. Photomicrograph of  $5\mu\text{m}$  thick Masson trichrome stained section of testis of a control group showing normal histological appearance of seminiferous tubules. E. Masson trichrome stained section of testis of a group II (cannabis only) showing histopathological changes (changes in Morphology, thickness, number of spermatogenic cells and damage in basement membrane or basal lamina of seminiferous tubules) in the germinal epithelium of seminiferous tubules. F. Masson trichrome stained section of testis of a group III (cannabis plus Vitamin-C) showing normal histological appearance of seminiferous tubules.

This study regarding the protective role of vitamin-C suggested that animals in group III were treated with vitamin-C retained the body weight loss, which was induced by chronic administration of cannabis. However, this protection in body weight was not statistically significant. Our study have analogous results with the studies conducted by Mandal and Das<sup>17</sup> and Nwangwa et al.<sup>14</sup> who showed that treatment with vitamin-C at a dose of (100mg/kg/body weight) for 21 consecutive days restored the cannabis induced decrease in body weight.

The finding revealed a reduction in testes size and weight of group II (cannabis alone) animals and thus the mean weight of paired testes of these animals were significantly less than those of control group. The reason of reduction of testes weight of group II animals could have been due to degeneration and shrinkage of germinal epithelium of seminiferous tubules of the testes. Therefore, finding of this study was in accord with the result of a study conducted by Bergamaschi MM et al.<sup>18</sup> in which the effects of cannabis oral treatment at doses of 30, 100, or 300 (mg/kg/body weight) daily for 90 days, observed reduction in testicular weight. Furthermore, the present study also supports the study conducted by Banerjee et al.<sup>16</sup> who administered cannabis to adult male mice at a dose 3 or 6 (mg/kg/body weight) daily orally for 36 days and revealed that mice exposed to cannabis showed a significant decline in the weight of testes with increasing dose of cannabis. The reduction in the testicular weight with the higher dose was greater than with the lower dose of cannabis. They correlated the reduction of testes weight with the decrease in number of germ cells.

This study with regard to the protective role of vitamin-C revealed that the testicular weight was reduced in treated group II (cannabis only) and was significantly restored in group III (cannabis along with vitamin-C). Comparable results with this study also reported by Mandal and Das<sup>17</sup> and Nwangwa et al.<sup>14</sup> whose observed that treatment with vitamin-C in the dose of (100mg/kg/body weight) for 21 consecutive days protected the

cannabis induced decrease in testicular weight.

The recent study reported that testes of the cannabis treated animals were associated with degeneration and separation of germinal epithelium from the basal lamina and sloughing of degenerated germ cells into the lumen of seminiferous tubules. Similar finding was also reported by Tijani AA et al.<sup>19</sup>, Yassa HA et al.<sup>20</sup> and Banerjee et al.<sup>16</sup>. They found that cannabis exposure was associated with tubular degeneration, characterized by germinal epithelium degeneration. The present study revealed that testes of the cannabis treated animals were associated with the loss and degeneration of Leydig cells in the interstitial connective tissue and widening of the intertubular space. These findings also supported by the study of Banerjee et al.<sup>16</sup> and Khan and Hassan.<sup>21</sup> A decrease in height of the seminiferous epithelium of group II (cannabis alone) as compared to control group and this change was not observed in group III. Our finding is in accord with the result of a studies conducted by Reuhl J et al.<sup>22</sup> Nwangwa et al.<sup>14</sup> and Lotfi et al.<sup>15</sup> who showed decrease in height of seminiferous epithelium.

The germ cells count in the cannabis alone (group II) was markedly decreased as compared to control (group I). A study conducted by Khan and Hassan<sup>21</sup> and Lotfi et al.<sup>15</sup> also reported a decreased in germ cell count. Whereas, in group III there was recovery of germ cells, after being treated with antioxidants (Vitamin-C) along with cannabis during experiment. These findings were supported by Yasmina et al.<sup>7</sup> and Nwangwa et al.<sup>14</sup> whose showed that treatment with vitamin-C had restored the cannabis induced decrease in germ cells in terms of count. Therefore, our current study has similar results which showed that treated animals with Vitamin-C significantly protected cannabis induced decrement germ cells count.

## CONCLUSION

Administration of cannabis had toxic effect on mouse testes which were significantly prevented by the concurrent administration of Vitamin-

C. The cannabis administration significantly declines the weight, size of testes, germ cells count and the height of seminiferous epithelium in animals. However, cannabis along with vitamin-C administration significantly restored the weight, size of testes, germ cells count and the height of seminiferous epithelium in animals.

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### AUTHORS' CONTRIBUTIONS

Following authors have made substantial contributions to the manuscript as under:

**ZSK:** Acquisition, analysis and interpretation of data, drafting the manuscript, final approval of the version to be published

**MS:** Conception & study design, critical review, final approval of the version to be published

**SMH:** Analysis and interpretation of data, drafting the manuscript, final approval of the version to be published

*Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.*

### CONFLICT OF INTEREST

Authors declared no conflict of interest

### GRANT SUPPORT AND FINANCIAL DISCLOSURE

NIL

### DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request



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