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Melatonin and vitamin C exacerbate Cannabis sativa-induced testicular damage when administered separately but ameliorate it when combined in rats

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Abstract

Background: The mechanisms involved in the spermatotoxic effect of Cannabis sativa are inconclusive. The involvement of oxidative stress in male factor infertility has been well documented, and the antioxidative potential of melatonin and vitamin C in many oxidative stress conditions has been well reported. This study sought to investigate whether melatonin and vitamin C will ameliorate C. sativa-induced spermatotoxicity or not.

Methods: Fifty-five (55) male albino rats (250–300 g) were randomly divided in a blinded fashion into five oral treatment groups as follows: group I (control, n=5) received 1 mL/kg of 10% ethanol for 30 days; groups IIa, IIb, and IIc (n=5 each) received 2 mg/kg C. sativa for 20, 30, and 40 days, respectively; groups IIIa, IIIb, and IIic (n=5 each) received a combination of 2 mg/kg C. sativa and 4 mg/kg melatonin for 20, 30, and 40 days, respectively; groups IVa, IVb, and IVc (n=5 each) received a combination of 2 mg/kg C. sativa and 1.25 g/kg vitamin C for 20, 30, and 40 days, respectively; group V (n=5) received a combination of 2 mg/kg C. sativa, 4 mg/kg melatonin, and 1.25 g/kg vitamin C for 30 days.

Results: Cannabis treatments reduced the Johnsen score, sperm count, motility, morphology, paired testicular/body weight ratio, and total antioxidant capacity, but increased lactate dehydrogenase activity. In addition, supplementation of cannabis-treated rats with either melatonin or vitamin C exacerbates the effect of cannabis on those parameters, whereas combination of melatonin and vitamin C reversed the trend to the level comparable to control.

Conclusions: This study further showed the gonadotoxic effect of C. sativa, which could be mediated by oxidative stress. It also showed that melatonin and vitamin C exacerbate C. sativa-induced testicular damage when administered separately but ameliorate it when combined in rats.

Keywords: Cannabis sativa; melatonin; oxidative stress; sperm parameters; vitamin C.

Introduction

Infertility, the inability to conceive (by women) or impregnate (by men) after 12 months of regular unprotected sexual intercourse, is found in about 15% of couples [1]. Male factor infertility, a consequence of male reproductive disorders, has been reported to be the sole cause of infertility in approximately 20% of infertile couples, with an additional 30%–40% secondary to both male and female factors, thereby constituting an approximately half of all infertility cases [2]. An increase in the incidence of male reproductive disorders has been reported in the past decade [3]. This has been attributed to a combination of lifestyle factors, prenatal exposure to environmental chemicals with endocrine-disrupting properties, and genetic susceptibility [4].

Cannabis sativa (marijuana), the most widely used illicit drug in the world, is a complex plant with over 400 chemical entities, of which more than 60 are cannabinoid compounds. After a period of decline in the last decade, its use has been increasing among young people since 2007, corresponding to a diminishing perception of the drug’s risks that may be associated with increased public debate over the drug’s legal status. Its use has been associated with various mental health problems, particularly in the young [5], although not everyone who uses it is affected in the same way. It has been shown that Δ⁹-tetrahydrocannabinol, the main active compound in cannabis, is a partial agonist and resembles anandamide in
its cannabinoid 1 receptor (CB1R) affinity, albeit with less efficacy than anandamide (an endocannabinoid agonist), while displaying even lower efficacy in the cannabinoid 2 receptor (CB2R) than in CB1R in vitro [6].

Previous work has found a complete endocannabinoid signaling system in sperm. For instance, sperm from mice, humans, pigs, and frog express CB1R, whereas CB2R has been detected in boar and human sperm [7]. Although it is well known that chronic marijuana use transiently decreases male fertility in animal models and humans [8], the mechanism involved remain unclear. Some studies have implicated reduction in some male reproductive parameters such as testosterone secretion, sperm production, sperm motility, sperm viability, luteinizing hormone (LH), and follicle-stimulating hormone as possible culprits [9, 10].

Melatonin (N-acetyl-5-methoxy-tryptamine) is the main pineal hormone synthesized from tryptophan, predominantly at night [11]. Melatonin is critical for the regulation of circadian and seasonal changes in various aspects of physiology and neuroendocrine function [12]. Being the most potent naturally occurring antioxidant, its beneficial effect on male fertility would be expected, as there are numerous links between oxidative stress and male factor infertility. However, decline in progression of spermatogenesis, testosterone, human chorionic gonadotropin-binding sites, and LH at the end of juvenile period has been reported in immature male rats treated with melatonin [13]. Even in female mammals, it has been evaluated as an oral contraceptive due to its ability to inhibit pre-ovulatory LH surge [14].

Vitamin C (ascorbate) acts as a potent water-soluble antioxidant in biological fluids by scavenging physiologically relevant reactive oxygen species (ROS) and reactive nitrogen species [15]. In addition, it can regenerate other small molecule antioxidants, such as α-tocopherol, glutathione, urate, and β-carotene, from their respective radical species [15]. However, its pro-oxidant potentials have also been well documented [16].

Because melatonin is produced endogenously and occurs naturally in some food, it can be sold as a dietary supplement in the USA under the Dietary Supplement Health and Education Act of 1994 without pre-market approval from the Food and Drug Administration. Vitamin C has also been grossly abused by users because of its wide perception as an antioxidant. Moreover, legislation and decriminalization of cannabis possession are increasing worldwide. This will lead to an increasing use of cannabis and consequently an increase in infertility. There is therefore the need to be proactive in solving the reproductive health problems that may arise from increasing use of cannabis due to its decriminalization. This study was designed to investigate whether or not melatonin and vitamin C will ameliorate the adverse effects of cannabis on sperm parameters. This information, which this study sought to provide, is important especially because male factor infertility accounts for half of infertility cases globally.

Materials and methods

Fifty-five male albino rats (250–300 g) were obtained from the animal house of the Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, Kwara State, Nigeria. They were housed at room temperature with free access to food and water and libitum and were maintained on a 12-h light/dark cycle, with the lights on from 7:00 a.m. The “Principles of Laboratory Animal Care” (NIH Publication No. 85-23, revised 1985) was followed. All experiments have been examined and approved by our Institutional Ethics Committee.

Extraction of *C. sativa* leaves

Extraction of *C. sativa* leaves, which was kindly donated by the National Drug Law Enforcement Agency (NDLEA), Nigeria, for research purpose only, was done with 98% ethanol in Soxhlet apparatus for 4–8 h as described earlier [17, 18]. The extract was evaporated to dryness in a rotary evaporator under vacuum, and the percentage yield was 21.2%.

Experimental protocol

After 2 weeks acclimatization to their new environment with standard laboratory diet and water given ad libitum, the 55 animals were randomly divided into five oral treatment groups as follows:

- **Group I** was the control and consisted of five rats that received 1 mL/kg of 10% ethanol for 30 days.
- **Group II** consisted of 15 animals that were subdivided into three subgroups (n=5 each) and received 2 mg/kg cannabis extract each for 20, 30, or 40 days.
- **Group III** also consisted of 15 animals that were subdivided into three subgroups (n=5 each) and received combination of 2 mg/kg cannabis extract and 4 mg/kg melatonin for 20, 30, or 40 days.
- **Group IV** also consisted of 15 animals that were subdivided into three subgroups (n=5 each) and received combination of 2 mg/kg cannabis extract and 1.25 g/kg vitamin C for 20, 30, or 40 days.
- **Group V** consisted of 5 animals that received combination of 2 mg/kg cannabis extract, 4 mg/kg melatonin, and 1.25 g/kg vitamin C for 30 days.

The 2-mg/kg dose of *C. sativa* was arrived at as the tenth of its LD$_{50}$, the lethal dose that killed 50% of the treated animals [19]. The cannabis extract was given to animals in a dissolved form in ethanol, whereas melatonin and vitamin C were dissolved in normal saline. Animals were sacrificed a day after the last treatment under ketamine anesthesia. The testes of each rat were harvested, weighed, and preserved in separate formalin bottles for histological examination; the epididymis was harvested for estimation of semen parameters, whereas plasma was collected for the estimation of lactate dehydrogenase (LDH) and total antioxidant capacity (TAC).
Johnsen score for testicular histology: All slides were evaluated using the quantitative methods described in [20].

Estimation of epididymal sperm parameters: Sperm count, motility, and morphology were estimated as previously described [21].

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

Estimation of plasma LDH and TAC: LDH activity was assayed spectrophotometrically (Spectramax Plus; Molecular Devices, Sunnyvale, CA, USA) following the kit manufacturer’s procedures (product code BXC0243; Fortress Diagnostics, UK).

TAC was assayed with OxiSelect TAC assay kit (catalog no. STA360; Cell Biolabs, San Diego, CA, USA) using a standard 96-well spectrophotometric microplate reader (Spectramax Plus; Molecular Devices) following the kit manufacturer’s procedures.

Data processing: Data were analysed with one-way ANOVA using GraphPad prism Version 5.0.3.0 (San Diego, CA, USA), followed by a post-hoc least significance difference (LSD) test for multiple comparisons. Data were presented as the mean±SEM. p-Values ≤0.05 were considered statistically significant.

Results

Effects of cannabis with and without melatonin and/or vitamin C on the Johnsen score in rats

Twenty-day administration of cannabis with and without melatonin or vitamin C caused nonsignificant reduction in the Johnsen score of rats when compared with control. At 30 days of treatment, cannabis significantly (p<0.05) reduced the Johnsen score when compared with control, whereas supplementation of cannabis-treated rats with melatonin or vitamin C caused further reductions in the Johnsen score when compared with control (p<0.001), cannabis 20 days (p<0.05 and p<0.001), cannabis+vitamin C 20 days (p>0.05 and p<0.01), and cannabis+melatonin+vitamin C 30 days group (p<0.001). At 40 days, cannabis treatment reduced the Johnsen score significantly when compared with control (p<0.01), cannabis 20 days (p<0.05), and cannabis+melatonin+vitamin C 30 days (p<0.01). Supplementation of cannabis-treated rats with melatonin for 40 days caused further significant reductions in the Johnsen score when compared with all the treatment groups (p<0.001). However, supplementation of cannabis-treated rats with vitamin C for 40 days caused significant increase in the Johnsen score when compared with cannabis+melatonin 30 days (p<0.01), cannabis+vitamin C 30 days (p<0.001), cannabis 40 days (p<0.01), and cannabis+melatonin 40 days (p<0.001), whereas supplementation of cannabis-treated rats with both melatonin and vitamin C abolished the cannabis-induced reduction in the Johnsen score that was worsened by either melatonin or vitamin C only (Figures 1 and 2).

Effects of cannabis with and without melatonin and/or vitamin C on sperm count in rats

Twenty-day administration of cannabis with and without melatonin or vitamin C caused reductions in the sperm...
count of rats, which was only significant with vitamin C (p<0.01) when compared with control. At 30 days of treatment, cannabis significantly (p<0.01) reduced the sperm count when compared with control, whereas supplementation of cannabis-treated rats with melatonin or vitamin C caused further reductions in sperm count when compared with control (p<0.001), cannabis 20 days (p<0.001 and p<0.05), cannabis+melatonin 20 days (p<0.01 and p>0.05), and cannabis+melatonin+vitamin C 30 days group (p<0.001 and p<0.01). At 40 days, cannabis treatment caused insignificant change in sperm count that was only significantly higher than cannabis+melatonin 30 days (p<0.05) but not other groups. Supplementation of cannabis-treated rats with melatonin for 40 days caused further significant reductions in sperm count when compared with all the treatment groups (p<0.001). However, supplementation of cannabis-treated rats with vitamin C for 40 days caused significant reduction in sperm count when compared with control (p<0.001), cannabis 20 days (p<0.01), cannabis+melatonin 20 days (p<0.05), cannabis+melatonin+vitamin C 30 days (p<0.001), cannabis 40 days (p<0.01), but significantly higher than cannabis+melatonin 40 days (p<0.001). Lastly, supplementation of cannabis-treated rats with both melatonin and vitamin C abolished the cannabis-induced reduction in sperm count that was worsened by either melatonin or vitamin C only (Figure 3).

**Effects of cannabis with and without melatonin and/or vitamin C on sperm motility in rats**

Twenty-day administration of cannabis with and without melatonin or vitamin C caused significant reductions in
the sperm motility of rats \((p<0.01)\) when compared with control. At 30 days of treatment, cannabis with and without melatonin or vitamin C caused significant reductions in the sperm motility of rats when compared with control \((p<0.05\) and \(p<0.001\)) and cannabis + melatonin + vitamin C 30 days \((p<0.05\) and \(p<0.001\)). At 40 days, cannabis treatment caused significant reduction in the sperm motility of rats when compared with control \((p<0.01)\) and cannabis + melatonin + vitamin C 30 days \((p<0.001)\). Supplementation of cannabis-treated rats with melatonin for 40 days caused further significant reductions in sperm motility when compared with all the treatment groups \((p<0.001)\), except cannabis 40 days \((p>0.05)\). However, supplementation of cannabis-treated rats with vitamin C for 40 days caused significant reduction in sperm motility when compared with control \((p<0.001)\), cannabis + melatonin + vitamin C 30 days \((p<0.001)\), but significantly higher than cannabis + melatonin 40 days \((p<0.001)\). Lastly, supplementation of cannabis-treated rats with both melatonin and vitamin C abolished the cannabis-induced reduction in sperm motility that was worsened by either melatonin or vitamin C only (Figure 4).
Effects of cannabis with and without melatonin and/or vitamin C on sperm morphology in rats

Twenty-day administration of cannabis with and without melatonin or vitamin C caused significant reductions in the sperm morphology of rats \( p < 0.01 \) when compared with control. At 30 days of treatment, cannabis with and without melatonin or vitamin C caused significant reductions in the sperm morphology of rats when compared with control \( p < 0.001 \) and cannabis + melatonin + vitamin C 30 days \( p < 0.001 \). Vitamin C supplement in cannabis-treated rats tends to improve the sperm morphology above that of cannabis 30 days group \( p < 0.05 \). At 40 days, cannabis with and without melatonin treatment reduced sperm morphology when compared with control \( p < 0.01 \) and cannabis + melatonin + vitamin C \( p < 0.001 \). Moreover, rats that received cannabis for 40 days had significantly higher sperm morphology than those that received it for 30 days \( p < 0.05 \). However, rats that received cannabis and vitamin C had significantly higher sperm viability than cannabis 20 days \( p < 0.05 \), cannabis + melatonin 20 days \( p < 0.01 \), cannabis + vitamin C 20 days \( p < 0.05 \), cannabis 30 days \( p < 0.001 \), cannabis + melatonin 30 days \( p < 0.01 \), and cannabis + melatonin 40 days \( p < 0.001 \). Lastly, supplementation of cannabis-treated rats with both melatonin and vitamin C significantly increased the sperm morphology above cannabis 20 days \( p < 0.01 \), cannabis + melatonin 20 days \( p < 0.001 \), and cannabis + vitamin C \( p < 0.01 \), but no significant difference from the control. This showed that supplementation of cannabis-treated rats with both melatonin and vitamin C abolished the reduction in sperm morphology caused by treatment with cannabis alone and cannabis with either melatonin or vitamin C only (Figure 5).

Effects of cannabis with and without melatonin and/or vitamin C on PTWR in rats

There were significant reductions in the PTWR of cannabis-treated rats that received melatonin \( p < 0.01 \) or vitamin C \( p < 0.05 \) for 20 days but not in cannabis only \( p > 0.05 \). Rats that received cannabis with and without melatonin or vitamin C had significantly reduced PTWR when compared with control \( p < 0.001 \), cannabis 20 days \( p < 0.01 \), cannabis + vitamin C 20 days \( p < 0.05 \) and \( p < 0.01 \), and cannabis + melatonin + vitamin C 30 days \( p < 0.001 \). Forty-day treatment with cannabis also reduced the PTWR when compared with control \( p < 0.01 \). Moreover, supplementation of cannabis-treated rats with melatonin for 40 days further caused significant reduction in PTWR when compared with control \( p < 0.001 \), cannabis 20 days \( p < 0.001 \), cannabis + melatonin 20 days \( p < 0.001 \), cannabis + vitamin C 20 days \( p < 0.001 \), cannabis + melatonin + vitamin C 30 days \( p < 0.001 \), cannabis 30 days \( p < 0.001 \),

![Figure 5: Sperm morphology in rats given cannabis with and without melatonin and/or vitamin C. Values are expressed as mean ± SEM (n=5). *p < 0.05, †p < 0.01, and ‡p < 0.001 vs. control; *p < 0.05 and †p < 0.01 vs. cannabis 20 days; †p < 0.01 and ‡p < 0.001 vs. cannabis + melatonin 20 days; *p < 0.05 and †p < 0.001 vs. cannabis + vitamin C 20 days; †p < 0.05 and ‡p < 0.001 vs. cannabis + melatonin + vitamin C 30 days; *p < 0.05 and †p < 0.001 vs. cannabis 30 days; †p < 0.001 vs. cannabis + melatonin 30 days and ‡p < 0.001 vs. cannabis + vitamin C 30 days; *p < 0.001 vs. cannabis + melatonin 40 days.](image-url)
cannabis+melatonin 30 days (p<0.05), cannabis+vitamin C 30 days (p<0.05), cannabis 40 days (p<0.001). However, supplementation of cannabis-treated rats with vitamin C for 40 days significantly decreased PTWR when compared with control (p<0.05), but increased it when compared with cannabis 30 days (p<0.001), cannabis+melatonin 30 days (p<0.001), cannabis+vitamin C 30 days (p<0.001), and cannabis+melatonin 40 days (p<0.001). Lastly, supplementation of cannabis-treated rats with both melatonin and vitamin C significantly increased the PTWR above those that were supplemented with either melatonin or vitamin C alone but not significantly different from the control. This showed that supplementation of cannabis-treated rats with both melatonin and vitamin C abolished the reduction in PTWR caused by treatment with cannabis alone and cannabis with either melatonin or vitamin C only (Figure 6).

**Effects of cannabis with and without melatonin and/or vitamin C on LDH activity in rats**

There were significant increases in LDH activity after 20 (p<0.05), 30 (p<0.001), and 40 (p<0.01) days of cannabis treatment when compared with control. Supplementation of cannabis-treated rats with melatonin and/or vitamin C caused insignificant increase in LDH activity when compared to control, but lower than the increase caused by cannabis alone. In addition, the LDH activity in rats that received cannabis for 30 days was significantly higher than its activity in cannabis+melatonin 20 days (p<0.05), cannabis+vitamin C 20 days (p<0.01), cannabis+melatonin+vitamin C 30 days (p<0.01), cannabis+vitamin C 30 days (p<0.01), cannabis+melatonin 40 days (p<0.05), and cannabis+vitamin C 40 days (p<0.001) (Figure 7).

**Effects of cannabis with and without melatonin and/or vitamin C on TAC in rats**

Cannabis treatment caused insignificant reductions in the TAC of rats at 20 and 30 days but not at 40 days (p>0.05), whereas melatonin or vitamin C supplement caused further reductions that were significant at 30 (p<0.05 and p<0.01, respectively) and 40 days (p<0.01 and p<0.05, respectively) when compared with control. Supplementation of cannabis-treated rats with both melatonin and vitamin C caused increased TAC that was significant when compared with cannabis+vitamin C 30 days (p<0.01) and cannabis+melatonin 40 days (p<0.01) but not when compared with others. Cannabis treatment for 40 days caused significantly higher TAC than cannabis+vitamin C 20 days (p<0.05), cannabis+melatonin 30 days (p<0.05), cannabis+vitamin C 30 days (p<0.01), cannabis+melatonin 40 days (p<0.01), and cannabis+vitamin C 40 days (p<0.05). Combination of melatonin and vitamin C in cannabis-treated rats for 30 days reversed the TAC to the level that is not different from control (p>0.05) (Figure 8).

**Discussion**

Male germ cell development or spermatogenesis is a complex process that involves the mitotic proliferation of...
spermatogonial stem cells, meiotic division of spermatoocytes, and dramatic morphological changes from haploid spermatids to highly specialized sperm through spermiogenesis [22]. The Johnsen score offers a convenient and rapid method for quantitative analysis of spermatogenesis [20]. Cannabis treatment for 20 days caused disorganization of the germinal epithelium, with marked sloughing or obliteration of the lumen, whereas all the cells of the spermatogenic lineage including spermatozoa were present in large number. However, 30- or 40-day treatment with cannabis caused a significant reduction in the Johnsen score, with evidence of large number of other spermatogenic cells, but few (<5) or no spermatozoa, respectively. Similar pattern was observed for sperm count, motility, morphology, and PTWR, but not sperm viability following cannabis administration for those treatment days.

The data from cannabis treatment in this study not only agree with previous spermatotoxic reports by others [10], but further supports the contention that cannabis also hinders spermatogenesis and reduces PTWR [18], another index of reproductive toxicity. This is consistent with previous observation that disruption of spermatogenesis may result in changes in the proportion of sex cell types in the testis, cause decline in the number and quality of sperm cells, and decrease in the number of germ cells [23]. In addition, the close association between PTWR and sperm parameters agrees with previous report that observed abnormal spermatogenesis and reduced sperm production and quality in mice with reduced PTWR [24].

A balance called oxidative stress status normally exists between ROS production and antioxidant scavenging system in the male reproductive tract [25]. Small
physiological levels of ROS are essential for the regulation of normal sperm functions such as sperm capacitation, the acrosome reaction, and sperm-oocyte fusion [26]. However, production of excessive amounts of ROS in semen, for instance, in smokers, and during exposure to environmental pollutants, genitourinary tract infection, chronic diseases, leukocytospermia, and inflammation can overwhelm the antioxidant defense mechanisms of spermatozoa and seminal plasma, resulting in oxidative stress. The oxidative stress caused by an excessive generation of ROS is one of the main factors involved in the pathogenesis of male infertility [27]. In high quantities, the superoxide, peroxynitrite, hydroxyl radicals, and the hydrogen peroxide, through their interaction with membrane lipids, proteins, and nuclear, and mitochondrial DNA, affect motility, viability, and the functions of the sperm cell [28]. Free radicals play an important role in the apoptosis process as well through the activation of the caspases [29]. In normal conditions, abnormal sperm cells are eliminated from the semen by apoptosis, but in the case of excessive ROS production, more sperm cells are affected, leading to the decrease in their number and implicitly to the lowering of fertility [30].

Now, is oxidative stress a mechanism for cannabis-induced gonadotoxicity? Although previous studies have attributed the spermatotoxic effect of cannabis to reduction in LH and testosterone, Mandal and Das [18] were the first to associate it with oxidative stress by reporting increased lipid peroxidation and reduced antioxidant enzymes. The main places of ROS generation in the sperm cell are mitochondria and the plasmatic membrane. Quantitatively, the mitochondrion (the NADH regions) is the major production site for the superoxide radical \( \left( \text{O}_2^\cdot \right) \) and hydrogen peroxide \( (\text{H}_2\text{O}_2) \) [31]. The sperm cells are very rich in mitochondria because they constantly need an energy supply to maintain their motility [30]. At the level of the plasmatic membrane, ROS generation occurs in the route of NADPH oxidase [32]. In general, sperm cells produce ROS when spermiogenesis (cytoplasmic drops) defects occur, with a positive correlation between immature sperm cells and ROS production [33]. The immature sperm cells seem to be more susceptible to oxidative deteriorations induced by NADPH and materialized as lower number of sperm cells and with affected motility and morphological anomalies [34]. During spermatogenesis, a reduction in the cytoplasm content occurs to allow the sperm cell to regain its elongated shape. The teratozoospermic immature sperm cells are often characterized by the presence of cytoplasmic residues at the middle piece. Moreover, the immature sperm cells with cytoplasmic drops have high levels of cytosolic enzymes (LDH, creatine phosphokinase, glucose-6-phosphate dehydrogenase), the activity of these enzymes being correlated to sperm dysfunctions [35]. The enzyme glucose-6-phosphate dehydrogenase controls the rate of glucose flow and the intracellular production of NADPH through the hexose-monophosphates shunt. NADPH will fuel the enzymatic route of NADPH oxidase (located at the level of the spermatic membrane) for the generation of ROS [34]. As a result, these immature sperm cells will produce high amounts of ROS, compared with normal sperm cells [36]. Thus, the positive relationship between sperm abnormality and LDH in this study suggests that cannabis-induced sperm abnormality led to an increase in the activity of LDH, which could have played a significant role in the generation of NADPH, a fuel for ROS generation.

Furthermore, it is necessary to know if antioxidant supplements will reverse cannabis-induced sperm toxicity and oxidative stress or not. Infertile men exhibit higher ROS and/or lower antioxidant capacity within their seminal plasma than fertile men [37]. As sperm oxidative stress and DNA damage are recognized as significant factors in male infertility, there is a clear rationale behind antioxidant treatment for infertile men. For instance, vitamin C has been shown to have an antioxidant efficacy on sperm oxidative stress and thus beneficial effect on various semen parameters in infertile group [38]. The present study further investigated the involvement of oxidative stress in cannabis-induced gonadotoxicity by supplementing the cannabis-treated rats with melatonin and/or vitamin C (both with widely reported antioxidant properties). Surprisingly, supplementation of cannabis-treated rats with melatonin or vitamin C for 20 or 30 days further caused damage to the spermatogenic cells and consequent incomplete spermatogenesis, as there is no evidence of spermatozoa in the histology. The deleterious effect of melatonin in cannabis-treated rats was more pronounced in 40 days, as there was evidence of only spermatogonia in the section, whereas other cells including spermatocytes, spermatids, and spermatozoa were absent. However, supplementation of cannabis-treated rats with vitamin C only for 40 days or with both melatonin and vitamin C abolished the cannabis-induced deleterious effects on spermatogenesis. This is evident from the presence of complete spermatogenesis with numerous numbers of spermatozoa comparable to what was observed in the control group.

It has been reported that seminal plasma from a fertile male has a higher TAC than seminal plasma from infertile male [39]. In most cases, reduction in TAC is associated with increase in oxidative stress. The reduction in TAC in cannabis-treated rats that received melatonin or vitamin C...
alone further showed that oxidative stress is involved in their gonadotoxic effect. This is consistent with the increase in LDH activity in these animals, which could have led to accumulation of ROS.

The deleterious effect of melatonin or vitamin C when administered separately to cannabis-treated rats was surprising. The pro-oxidant potential of vitamin C has been well reported. Its large dose has been shown to increase ROS production in the presence of certain cations such as iron or copper [40]. Moreover, previous studies have established the pro-oxidant role of melatonin. Its pro-oxidant potential has been related to its known pro-apoptotic ability in tumor cells [41]. For example, melatonin reportedly promotes apoptotic cell death in several cancer cells including human myeloid HL-60 cells, B-lymphoma cells, HT-29 human colorectal cancer cells, and rat pituitary pro-lactin-secreting tumor cells [42]. The increased oxidative stress and reduced TAC in rats that received melatonin or vitamin C in this study could be a reason for their gonadotoxic effects in cannabis-treated rats. This negative finding with vitamin C and melatonin in cannabis-treated rats in this study is comparable to the harmful effect of vitamins C and E on male reproductive organs of rats chronically exposed to sodium nitrate [43].

However, the ameliorative effects of combined vitamin C and melatonin in cannabis-treated rats in this study showed the synergistic effect of these two known antioxidants. This is evident from the improvement of semen parameters, the Johnsen score, PTWR, TAC, and reduction in LDH activity previously. It has been shown that vitamins C and E alleviate germ cell loss and oxidative stress in cryptorchidism when administered separately but not when combined in rats [44]. It is thus of interest to note that a known antioxidant may not display its potential until combined with another one. The mechanism for the pro-oxidant property of melatonin and vitamin C when administered separately but antioxidant potential when combined is not known and could be investigated in future study.

In conclusion, this study showed that cannabis has gonadotoxic effect that was partly mediated by oxidative stress. Moreover, melatonin and vitamin C deteriorated cannabis-induced gonadotoxicity when administered separately but ameliorated it when combined in rats.

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