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RESEARCH

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Cannabidiol impairs sperm quality and function in adult mice

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Abstract

Background Considering the growing therapeutic use of cannabidiol as well as the presence of cannabinoid receptors in sperm and its possible genotoxic activity, the effect of cannabidiol on sperm quality and function was examined.

Methods Thirty male NMRI mice were randomly divided into three groups: control (no injection), sham (intraperitoneal (IP) injection of DMSO daily for 34 days), and cannabidiol (IP injection of cannabidiol 30 mg/ml daily for 34 days). Following 35 days after the last injection, sperm parameters, chromatin integrity (CMA3 staining), acrosome reaction (FITC-PNA method), fertility-related genes (*IZUMO1*, *PLCζ*), and blastulation rate of the embryos obtained from the oocytes fertilized with the mentioned sperms was investigated.

Results Count, motility, and morphology of sperm were not significantly affected by cannabidiol. $CMA3^{+}$ sperms (protamine deficiency) were significantly higher in the cannabidiol group compared to the control group ($P = 0.03$). The acrosomal reaction and fertility-related genes (*IZUMO1*, *PLCζ*) in the cannabidiol group did not differ significantly compared to the control group. Also, there was no significant difference between the cannabidiol group and the control group in the two-cell and the eight-cell stages but the rate of blastocyst formation was significantly lower in the cannabidiol group compared to other groups ($P < 0.0001$).

Conclusions Our results showed that cannabidiol leads to negative effects on the male reproductive system through an effect on sperm chromatin and the rate of reaching the blastocyst stage of the embryo.

Keywords Cannabidiol, Sperm, Acrosomal reaction, Chromatin integrity

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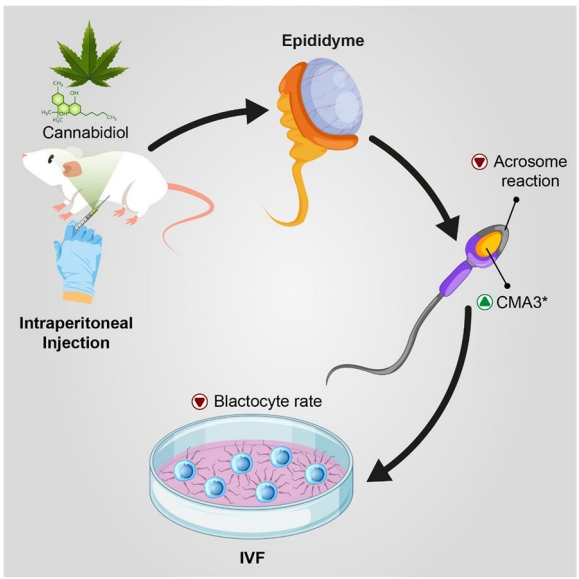
Graphical Abstract



Background

The endocannabinoid system (ECS) is a biological system that mediates the cannabinoid effects, and it consists of two receptors, *CB1R* and *CB2R*, endogenous ligands, and enzymes that synthesize and degrade these ligands. Endocannabinoids are lipophilic chemical messengers of this system that are like exogenous cannabinoids. ECS is widely distributed throughout the human body and affects many biological processes. This system has a key role in mammals' reproductive system [1]. The ECS is intricately involved in the female reproductive system and a precise balance between endocannabinoid production and degradation and CBR activity is essential for the proper functioning of the reproductive system and the hypothalamic-pituitary axis [2]. Recent studies have also indicated that ECS is important in multiple processes of the male reproductive system, since CB1R is expressed in different parts such as vas deferens, prostate, testis, and Leydig cells, and CB2R is expressed in prostate epithelium, testis, and Sertoli cells. The presence of these receptors has been reported in the sperm of a human, mouse, rat, frog, and boar [3]. In addition, ECS is a key inducer of mitosis to meiosis transition in male germ cells [1].

Cannabidiol (CBD) is obtained from the *Cannabis sativa* plant, known as marijuana, and is one of the main exogenous cannabinoids with medicinal properties, the therapeutic potential of which has been evaluated in cardiovascular diseases, diabetes, cancer, etc. This phytocannabinoid is a CB2 receptor agonist and by binding to it, it induces vasodilation in type 2 diabetes. Also due to its antioxidant and anti-inflammatory activity, this substance has been tested in the prevention and treatment of diseases whose development is associated with redox imbalance and inflammation [4]. CBD is FDA approved for the treatment of epilepsy and has had anti-anxiety, and anti-psychotics in animal studies and clinical trials, although its mechanism of action has not yet been determined [5].

Considering the various uses of this substance, it seems necessary to investigate the effect of these substances and similar compounds on the reproductive system. In sea urchin sperm treated with CBD, the sperm's ability to fertilize the egg was reduced through a negative effect on the acrosome reaction [6, 7]. The researchers' study showed that consuming cannabis by one cigarette per week, with a history of 1 year, has detrimental effects on sperm DNA integrity [8]. In addition, researchers in in vitro studies showed that CBD can lead to DNA damage, nuclear anomalies, and induce necrosis and apoptosis [9]. Since the researchers have shown a significant negative relation between sperm DNA abnormality and embryo development [10], Investigating the effect of cannabidiol on sperm chromatin and embryo health seems necessary. But information about CBD genetic damage in vivo is limited.

Cannabidiol also acts through TRPV1 receptors. This receptor is present in the tail [11, 12], head and apical region, and postacrosome region of sperm [13]. On the

other hand, anandamide, which is an endocannabinoid, can pair with TRPV1 and play a role in sperm capacity by activating this receptor [14]. Studies have shown that cannabidiol may work by inhibiting the breakdown of anandamide [15]. Further investigation in this area suggests a biphasic nature of CBD action, so that, lower doses of CBD may increase endocannabinoid concentrations indirectly, mainly by inhibiting the breakdown of anandamide and increasing its supply to cannabinoid receptors, while higher doses of CBD can block these receptors [9].

Therefore, considering the growing therapeutic use of CBD as well as the presence of cannabinoid receptors in sperm and its possible genotoxic activity and its interactions with the endocannabinoid system, the effect of cannabidiol on sperm parameters, chromatin status, acrosomal reaction, expression of fertilization-related genes and blastocyst embryo formation was examined.

Methods

Animals and study groups

This study was approved by the ethics committee of Iran University of Medical Science (IR.IUMS.REC.1399.905). Experiments performed on animals were also in accordance with national guidelines and protocols of the Animal Ethics Committees (AECs). In this study, 30 adult male (6–8 weeks) NMRI mice were kept in environmentally controlled conditions (23–25 °C, 50% humidity, 12/12-h light/dark cycle, and free access to drinking water). All animals were 13 to 15 gr of weight. Animals were divided into three groups (each containing 10 mice): control (no injection), sham (intraperitoneal (IP) injection of DMSO daily for 34 days), and cannabidiol (IP injection of cannabidiol 30 mg/ml daily for 34 days). Following 35 days after receiving the last injection, animals were sacrificed by cervical dislocation [16]. Since there is a wide range of CBD doses for the treatment of children and adolescents with comorbidities [17, 18], and because of the complex relationship between human doses and pharmacokinetic differences in mice, the doses used in this study were based on CBD concentrations, which were previously used in studies with this animal model [16, 19].

Semen analysis

To collect sperm, cauda epididymis was dissected and cut to release dense sperm. Packaged sperm were placed in a preheated petri dish with 0.5 ml of HTF medium containing 4 mg/ml BSA. The sperm were incubated for 10 min at 37 °C to disperse the sperm into the environment. The count, motility, and morphology were analyzed for at least 200 sperm of each animal. Makler Chamber and hemocytometer were used for sperm motility and count analysis respectively. For motility analysis, progressive sperms were counted. Progressively motile sperm were identified by moving forward in a straight line or large circles. The Diff-Quik kit (BRED Life Science Technology Inc., China) and the Kruger classification were used for sperm morphology analysis. Analysis was done under a light microscope (Motic BA410, Kowloon, Hong Kong) at 400× magnification. One experienced technician performed all analyses, while blinded to the study [20].

Sperm chromatin evaluation

During spermatogenesis, the degree of sperm chromatin condensation is profoundly altered when histones are replaced by protamines. This type of nuclear condensation protects the sperm genome against external damage such as oxidative stress, high temperature, and DNA denaturation caused by acid, and disruption of this process leads to male infertility. The CMA3 method is used to determine protamine deficiency in the nucleus. CMA3 is a fluorochrome that competes with protamine binding sites in the minor groove of the DNA strand. Therefore, the binding of this substance to the DNA strand and creating a light yellow color indicates the deficiency of protamine in the DNA strand [21]. In this study, method CMA3 was used to investigate the damage to DNA.

Twenty microliters of the semen sample and 20 µl of Carnoy's solution were kept in a vial for 5 min at 4 °C. Then, the smear was prepared and dried. The slide was treated with 150 µl of CMA3 (0.25 mg/ml) in the McIlvain buffer and was kept at room temperature for 20 min. Then, the samples were washed with PBS and mounted with buffered glycerol. For chromatin evaluation, 200 sperms were analyzed under a fluorescent microscope. Light yellow sperms were considered as CMA3 positive (protamine deficiency) and pale yellow sperms were considered as CMA3 negative (normal protamine) [22, 23].

Acrosomal reaction

Acrosome status was evaluated by FITC-PNA. Briefly, after smear preparation and drying, slides were fixed via methanol at RT for 20 min and washed with PBS. In the following, 100 µg/ml lectin from Arachis hypogaea (peanut) (L7381, Sigma-Aldrich) was used for 30 min at RT. After washing with PBS, DAPI staining was used (Sigma-Aldrich). at least 200 sperm were evaluated by a fluorescence microscope (Olympus AX70, Tokyo, Japan). Sperms were divided into two groups based on their fluorescence pattern. The green fluorescent acrosome area was classified as intact sperm and the low fluorescence or non-fluorescent acrosome area was classified as reacted sperm [24].

QRT-PCR analysis

The RNeasy Mini Kit (Qiagen) was utilized to extract RNA from sperm cells. Technical replicates were carried out for all samples in triplicates. All samples were incubated with DNase I to eliminate the contamination of genomic DNA (IPTG, MBI Fermentas GmbH, St. LeonRot, Germany). The samples were checked spectrophotometrically by the A260/A280 ratio technique for determination of the RNA concentration (purity and yield). cDNA was synthesized using the QuantiNova Reverse Transcriptase Kit (Qiagen). In each PCR cycle, using the conditions mentioned before, a reverse transcription control was performed without the superscript II enzyme [25]. For quantification of *IZUMO1* (izumo sperm-oocyte fusion 1), *PLCζ* (phospholipase C zeta) gene expression levels, *GAPDH* was used as an internal control for normalization. Negative controls were used for all tests. Primer sequences were PLCζ (phospholipase C zeta) (forward primer (5′-3′): CCCAAAGCAACA AGAGAC, reverse primer (5′-3′): GCAGATCCATGG GCAGACC); IZUMO1 (izumo sperm-oocyte fusion 1) (forward primer (5′-3′): ACAGTGATGTAAAAGGCG ATC, reverse primer (5′-3′): GTAGGACTTTCGACA AGCGTG); and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (forward primer (5′-3′): GCAGGG ATGATGTTCTGG, reverse primer (5′-3′): CTTTGG TATCGTGGAAGGAC). The method for analyzing relative changes in mRNA levels was $2^{-\triangle\triangle Ct}$ method. The qPCR reactions were performed based on the mentioned details previously.

In vitro fertilization

Superovulation of adult female mice was performed by intraperitoneally injecting 7.5 IU of Folligan pregnant mare serum gonadotropin (PMSG, MSD, Intervet, Australia). After 48–50 h, 10 IU of human chorionic gonadotropin (hCG, Daro Farseh, Tehran, Iran) was used. Cumulus-oocyte complexes (COCs) were collected from oviducts after 14–16 h following human chorionic gonadotropin (hCG) injection. COCs were placed in an FHM medium and then in drops (50 µl) of human tubal fluid (HTF) containing 4 mg/ml BSA. Five mice sperm samples from each group were used for gene expression. To collect sperm, cauda epididymis was dissected and cut to release dense sperm. Packaged sperm were placed in a preheated petri dish with 0.5 ml of HTF + 4% BSA. The sperm were incubated for 10 min at 37 °C to disperse the sperm into the environment. Then the suspension of sperms was centrifuged for 3 min at 3000 rpm. The pellet of sperms was placed in 1 ml of HTF medium and incubated for 60 min in 5% CO2 at 37 °C for capacitation and swim-up. Finally, 5×10^6 capacitated sperms were added to droplets of HTF media with COCs for 6 h. Then, the zygotes were cultured in KSOM (potassium simplex optimized medium) (Sigma Chemical Company) + 4% BSA medium to blastocyst stage [26]. To evaluate the rate of fertilization and development, 100 oocytes were assayed in each group.

Statistical analysis

Following the normality test of Shapiro-Wilk, data were analyzed through analysis of variance (ANOVA) by post hoc Tukey test in SPSS software (version 16). If the data were not normal, the Kruskal-Wallis test was used. Developmental competence rate analysis was performed using the chi-square test. Data were reported as mean ± standard deviation (SD), and $P \leq 0.05$ was considered significant.

Result

Sperm parameters

Sperm parameters including sperm count, motility, and morphology were analyzed. There was no significant difference in sperm count. motility and morphology between the cannabidiol group compared to other groups. Therefore, no adverse effect of cannabidiol treatment was seen on sperm parameters ($P > 0.05$) (Table 1).

Table 1 Sperm count, motility and morphology analysis

Parameter	Groups		
	Control	DMSO	Cannabidiol
Count ($\times 10^4$)	34.10 ± 2.38	41.66 ± 4.61	36.20 ± 4.24
Prog motility (%)	41.29 ± 4.45	48.05 ± 3.99	44.13 ± 3.94
Ab morphology (%)	16.7100 ± 0.18248	16.4400 ± 0.51069	17.2733 ± 0.25325

No statistically significant differences were observed between the control group and other groups

Data was presented as mean ± SD. $P < 0.05$ was considered as significant

Prog progressive, *Ab* abnormal

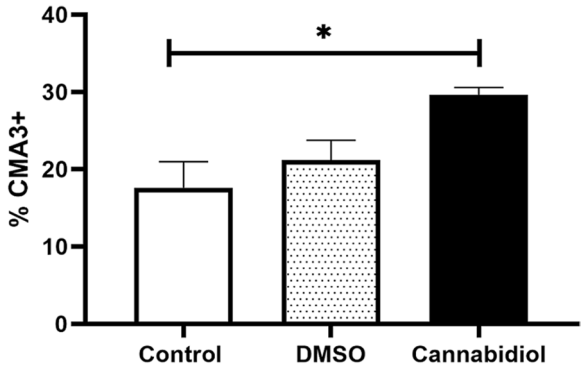


Fig. 1 CMA3 staining for sperm chromatin protamination. There was a statistically significant difference between the cannabidiol group and the control group ($P = 0.03$). Data was reported as mean ± SD. * $P < 0.05$ was considered as significant

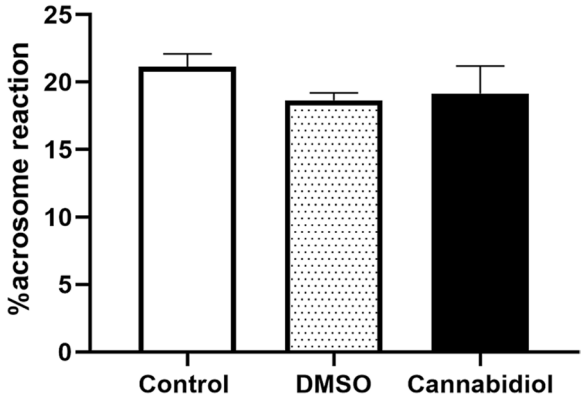


Fig. 2 Evaluation of acrosomal reaction by FITC-PNA. There was no significant difference between the cannabidiol group and the control group. Data was reported as mean ± SD

Chromomycin A3 staining

In the CMA3 test, light and pale yellow sperms were considered $CMA3^+$ (protamine deficiency) and $CMA3^-$ (normal protamine), respectively. Results showed that the number of $CMA3^+$ cells and protamine deficiency of sperm were significantly higher in the cannabidiol group compared to the other groups (Fig. 1) ($P = 0.03$).

Acrosomal reaction

In this study, the acrosomal reaction was evaluated by FITC-PNA. The results showed that there was no significant difference between the cannabidiol group and the control group. Therefore, no adverse effect of cannabidiol treatment was seen on sperms' acrosomal reaction (Fig. 2) ($P > 0.05$).

Gene expression by qRT-PCR

According to Fig. 3, *IZUMO1* and *PLCζ* gene expressions were not significantly different in the cannabidiol group compared to other groups (Fig. 3) ($p = 0.061$ for *IZUMO1* gene and $p = 0.055$ for *PLCζ* gene) ($P > 0.05$).

Effect of cannabidiol on developmental competence

According to Table 1, 220, 200, and 212 oocytes were used for the control group, DMSO, and cannabidiol groups, respectively. The two-cell stage rate for the control group was 87.01%, whereas it was 79.26% for the DMSO group and 75.65% for the cannabidiol group. The eight-cell stage rate for the control group was 87.40%, whereas it was 67.61% for the DMSO group and 65.24% for the cannabidiol group. No significant difference was evident in the two-cell stage and eight-cell stage among study groups. But in the blastocyst stage, cannabidiol treatment adversely affected the number of blastocysts and we found a statistically significant decrease in blastulation rate in the cannabidiol group compared to other groups (Fig. 4) ($P \leq 0.0001$).

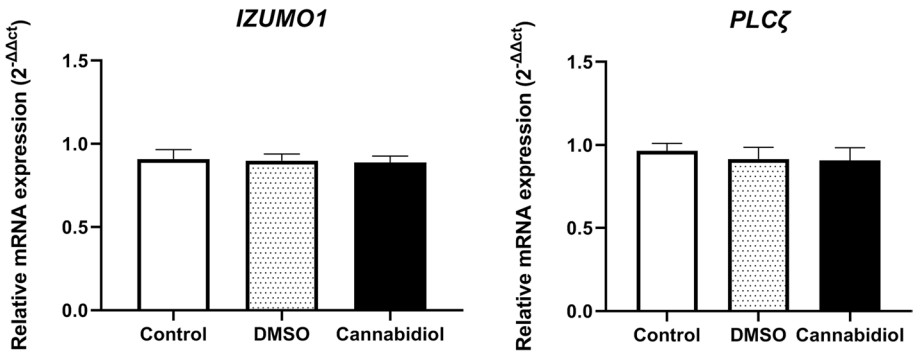


Fig. 3 Relative expression of IZUMO1 and PLCζ gene. Semen samples related to 30 mice from three groups were used for qRT-PCR. No significant difference was found in the cannabidiol group compared to other groups. Data was reported as mean ± SD. * $P < 0.05$ was considered as significant

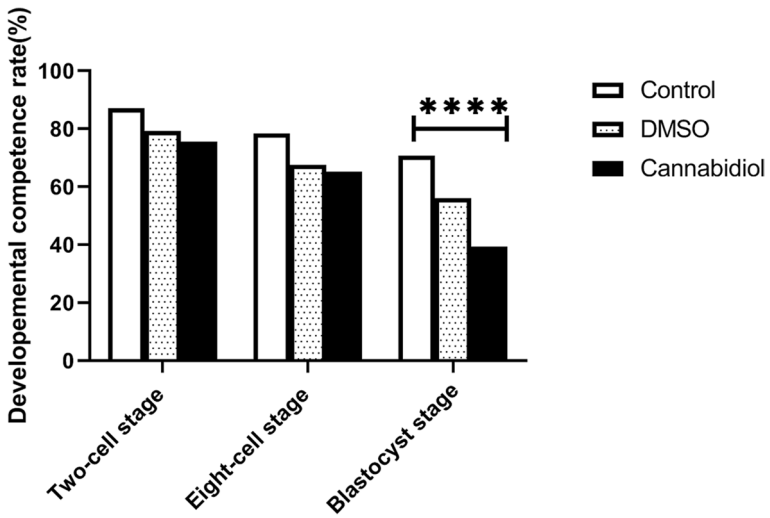


Fig. 4 The effect of cannabidiol on the developemental competence rate. No statistically significant difference was evident in the two-cell stage and eight-cell stages among study groups, but the results showed a significant decrease in blastulation rate in the cannabidiol group compared to other groups. **** $P < 0.0001$ was considered as significant

Discussion

Considering the growing therapeutic use of CBD, we investigated the effect of CBD on sperm parameters, chromatin integrity, acrosome reaction, fertility-related gene expression, and in vitro fertilization of oocytes with CBD-treated sperms of male mice. The results of our study showed that sperm parameters — count, motility, and morphology — were not affected by cannabidiol. Acrosome reaction and fertility-related genes were also not significantly affected in the cannabidiol group compared to the control group but $CMA3^{+}$ sperms were significantly higher in the cannabidiol group compared to the control group and the rate of blastocyst formation was also significantly lower in the cannabidiol group compared to other groups.

It has been identified that major functions of cannabimimetic agents in reproductive physiology may be the result of interactions with cannabinoid receptors of CB1 or suppression of enzymatic metabolism of endogenous ligands such as anandamide [27]. In the reproductive system, these receptors exist in the testis [28]. It has been shown that cannabis use is closely and causally associated with testicular cancer rates and cannabinoid genotoxicity replicates all major steps to testicular carcinogenesis and accelerates this pathway by several decades [29]. Our study indicated that sperm parameters (count, motility, and morphology) were not adversely affected by cannabidiol. Also, in Zimmerman et al. [30] study, no adverse effect of CBD on sperm parameters was reported. However in another study, CBD led to defects in sperm morphology and reduced sperm count [31].

In our study, acrosomal reaction was assessed by FITC-PNA — an efficient method reported previously [32] — and was not negatively affected by CBD treatment. First studies regarding acrosomal reaction showed that in sea urchins following CBD treatment, reduced sperm capacity and suppressed acrosomal reaction in a time- and dose-dependent manner led to fertility suppression [33]. Franco et al. [34] reported a spontaneous acrosomal reaction caused by an endocannabinoid — 2-arachidonoylglycerol — which leads to an early acrosomal reaction before contacting the oocyte causing the sperm to lose its ability of fertilization. CBD can affect early acrosomal reaction in mice by enzymatic suppression and anandamide aggregation on CB1 receptors or through reaction with TRPV1 receptors [27]. These studies show the adverse effects of cannabinoids, although we did not see negative effects on acrosomal reactions.

Fusion of the two gametes is achieved through a two-step mechanism in which the protein IZUMO1 on the sperm recognizes its receptor, JUNO, on the surface of the oocyte. This connection leads to the fusion of the two plasma membranes [35]. Defect in *IZUMO1* protein leads to male infertility [36], and IZUMO1 −/− mice are infertile [37]. PLCζ plays a critical role in mammalian fertilization. *PLCζ* is a cytosolic protein that is transferred from the fertile sperm to the cytoplasm of the oocyte and releases intracellular calcium ions following entering, leading to oocyte activation which involves meiotic arrest regulation, exocytosis of cortical granules of the oocyte, utilizing maternal mRNA, formation of pronucleus, polyspermia prevention and beginning of the embryo development. Many recent genetic and clinical reports have

associated male infertility cases with reduced expression and mutated forms of this specific sperm protein [38, 39]. Primary immunocytochemistry localization studies indicate *PLCζ* protein in acrosomal and postacrosomal areas in human sperm [38]. In this study, these two important genes in the fertilization process were investigated. However, gene expression investigation did not show any significant changes indicating a probable different pathway for CBD to influence the sperm through other genes. In this study, due to limitations, only two genes were investigated. Since hundreds of genes play a role in male fertility, other key genes may have been affected, and in this sense, further investigations are necessary. It is suggested to use the microarray technique to investigate sperm transcriptome and epigenome in finding the most effective gene in the field of cannabidiol toxicity on male infertility.

Despite sperm with normal parameters, damage in its DNA can lead to defects in fertility. Replacement of histone with protamine during spermatogenesis in addition to protecting chromatin, may also be needed to turn off the paternal genome and plan to imprint the pattern of the paternal genome. This process is a sperm-specific epigenetic mechanism [40]. In the present study we used the CMA3 method for the detection of sperm protamine deficiency and following treatment with CBD, the number of $CMA3^{+}$ sperms increased significantly compared to the control group which shows the adverse effect of CBD on DNA protamination. Any factor that affects this process leads to changes in the epigenetic signature of sperm. Although sperm protamines are replaced by oocyte histones during the fertilization process, damage to the protamination process leads to sperm epigenetic abnormalities that are associated with poor embryogenesis [40]. Studies have reported that abnormal protamination leads to abnormal chromatin density and increases the sensitivity of sperm DNA to external stress that triggers an oxidative attack [41]. Also, deficiencies in protamine levels are often associated with severe spermatogenesis defects, increased sperm chromatin defects, and decreased fertilization ability [42]. DNA integrity is of importance since its defects will be transferred to the zygote and lead to embryos of reduced quality that are not qualified to develop. Other studies show that a damaged sperm's DNA leads to apoptosis [43]. A few data is available in regard to the cannabidiol effect on sperm's DNA, although the *Cannabis sativa* plant can lead to changes in sperm DNA methylation which is caused by Tetrahydrocannabinol [44]. Chioccarelli et al. (2010) showed that cannabinoid receptor 1 activation regulates the chromatin remodeling of spermatids by increasing the level of transfer proteins or increasing histone displacement [45]. Carvalho's study also found that the chronic effect of 30 mg/kg cannabidiol causes a defect in spermatogenesis and DNA damage [46]. But Paria et al. [47] sought the effect of various doses of 6.4, 32, and 160 nanomolar of CBD on mouse embryo development and reported no adverse effect. However, because there is a significant relationship between sperm chromatin health and male fertility potential, more studies are needed in this area. In our study, no significant difference was evident in the two-cell stage and eight-cell stage among study groups. But in the blastocyst stage, cannabidiol treatment adversely affected the number of blastocysts, and a significant decrease was found in blastulation rate in the cannabidiol group compared to other groups. The reduced number of blastocysts following CBD treatment in this study was probable because there is a direct correlation between sperm chromatin defect and blastocyst formation [48]. Chromatin integrity defects, which were seen in the treated group of our study, are the most studied parameter of sperm quality which is associated with pregnancy failure [49], implantation failure [50], and reduced embryo quality [51]. The lack of significant difference between CBD group and other groups in the two-cell and eight-cell stages is that the effect of sperm DNA damage manifests just before or during blastocyst stage [49]; therefore, in this study, the rate of blastocyst formation is significantly decreased in CBD group. In one study, circular RNA biogenesis in sperm was investigated. circCNOT6L was selected. This RNA is transferred from the sperm to the oocyte during fertilization and has been proposed as an active regulator of the zygote transition to the 2-cell stage. Cannabinoid receptor type-1 knock-out (Cb1−/−) male mice were used for this study. The results showed that the Cb1 receptor has a role for the biogenesis of this RNA during sperm maturation [52]. Therefore, through these receptors, cannabidiol may indirectly interfere with the processes that affect the results of fertilization or embryo formation at different stages. It is suggested that researchers investigate the effect of CBD on the epididymosome function and incorporation in the caudal epididymis and its effect on blastocyst development.

Conclusion

Regarding the adverse effects of CBD on sperm chromatin integrity and the resulting low rate of blastocyst formation, sufficient care and further studies should be taken into account when using CBD.

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Authors' contributions

MM and FMK: conceptualization, funding acquisition, supervision, writing — original draft, writing — review and editing; AG and SE: conceptualization,

data curation, formal analysis, methodology, writing — original draft, writing — review and editing; MA, FSA, and MN: data curation, formal analysis.

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Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon reasonable request (mehdizadeh.m@iums.ac.ir).

Declarations

Ethics approval and consent to participate
This study was approved by the ethics committee of Iran University of Medical Science (IR.IUMS.REC.1399.905).

Consent for publication
All authors consent to the publication of this study.

Competing interests
The authors declare that they have no competing interests.

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