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Full length article

Cannabis consumption might exert deleterious effects on sperm nuclear quality in infertile males

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Abstract

Research question: Can cannabis consumption alter sperm nuclear integrity in infertile males?

Design: A retrospective cross-sectional study was conducted between July 2003 and December 2013 and included 54 males who consulted for male factor infertility. Twenty-seven infertile males who were regular cannabis users were matched to 27 infertile males who were cannabis non-users. To complement the conventional semen parameter and plasma hormone plasmatic level assessments, sperm nuclear alterations were explored using fluorescence in situ hybridization to assess numerical chromosomal abnormalities, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling to investigate DNA fragmentation, aniline blue staining to examine chromatin condensation and a Motile Sperm Organelle Morphology Examination (MSOME) to detect vacuoles in sperm heads.

Results: The rates of sperm aneuploidy ($P = 0.0044$), diploidy ($P = 0.037$), total chromosome abnormalities ($P = 0.0027$) and DNA fragmentation ($P = 0.027$) were significantly higher in cannabis users than in cannabis non-users. Furthermore, a positive correlation was found between the rate of sperm DNA fragmentation and the rates of aneuploidy ($P = 0.0144$) and total chromosome abnormalities ($P = 0.0162$) in cannabis users.

Conclusions: Cannabis consumption might have deleterious effects on sperm nuclear quality in infertile males by increasing numerical chromosome abnormalities and DNA fragmentation. Cannabis consumption induces additional detrimental effects on the progression of spermatogenesis from meiotic stages to spermiogenesis and potentially on post-testicular sperm maturation in infertile males. However, any potential findings need to be validated with larger sample size and our data are only exploratory findings.
**Key message**

Cannabis consumption might exert deleterious effects on sperm nuclear quality in infertile males by increasing numerical chromosome abnormalities and DNA fragmentation. Cannabis consumption might enhance the impairment of both spermatogenesis progression from meiotic stages to spermiogenesis and post-testicular sperm maturation.

**Key words**

Aneuploidy, cannabis, DNA fragmentation, male infertility, sperm nuclear damage

**Clinical trials registry**

None: the current study is a retrospective study.
Introduction

Recent studies investigating the evolution of sperm quality highlight a degradation of semen parameters, which has led to subsequent studies exploring the underlying aetiological factors (Swan et al., 2000). Genetic susceptibility, nutrition, weight, physical exercise, psychological stress, prenatal and postnatal exposure to environmental chemical endocrine disruptors and occupational factors are likely to have deleterious effects on male reproductive function. Similarly, addictions such as smoking, illicit drug use, as well as alcohol and caffeine consumption, can negatively affect fertility (Sharma et al., 2013).

Experimentation with Cannabis sativa (marijuana), which is the most widely consumed illicit substance, in the population aged 12 years and older has continually increased since 2002, leading to concerns regarding the potential adverse effects of this substance on health. Approximately 2.5% of the world’s population consumes cannabis (Alagbonsi et al., 2016; Azofeifa et al., 2016). Furthermore, in a sample of 1215 young adult males from the general population, 45.4% of the participants reported smoking cannabis during the previous 3 months (Gundersen et al., 2015). Up to 22% of men who intended to have children reported using cannabis within the previous 12 months (van Gelder et al., 2011).

Cannabis contains more than 460 known products, including more than 60 cannabinoids. Among these cannabinoids, the most abundant and well-studied active compound is Δ⁹-tetrahydrocannabinol (Δ⁹-THC), which is the main psychoactive ingredient in cannabis. The cloning of two specific cannabinoid receptors, type 1 (CB1) (Matsuda et al., 1990) and type 2 (CB2) (Munro et al., 1993), rendered possible not only the identification of their endogenous ligands [i.e., endocannabinoids (eCBs)], with N-arachidonoyl ethanol amide (anandamide, AEA) being the most studied due to its wide distribution (Devane et al., 1992), but also the
understanding of the functions of the eCB system (ECS). CB1 and CB2 are major actors in the eCB signalling system and can also interact with exocannabinoids such as $\Delta^9$-THC. The ECS has been identified at different levels of the male reproductive tract, and this system likely plays a physiological role in endocrine and exocrine testicular function and, consequently, in male fertility (Bari et al., 2011; Pierantoni et al., 2009; Wenger et al., 2001). In mammalian and non-mammalian vertebrates, the ECS plays a critical role in the progression of spermatogenesis from the mitotic and meiotic stages to spermiogenesis (Bovolin et al., 2014). Indeed, recent studies have reported a deregulation of this system in infertile men (Rapino et al., 2014). Therefore, exocannabinoids such $\Delta^9$-THC, the main psychoactive ingredient in cannabis, might disturb the normal signalling of the ECS and could have additional detrimental effects on spermatogenesis in infertile males (Mouslech and Valla, 2009).

$\Delta^9$-THC affects follicle stimulating hormone (FSH) and luteinizing hormone (LH) secretion in animals. Acute or chronic doses of $\Delta^9$-THC induce a decrease in testosterone levels in rats and monkeys (Brown and Dobs, 2002). Thus, cannabinoids or THC can negatively modulate the activation of the hypothalamic-pituitary-gonadal axis by acting either directly through gonadotrophin-releasing hormone (GnRH) or indirectly through other modulators (Banerjee et al., 2011; Harclerode, 1984). In addition, eCBS are potential modulators of Leydig cell proliferation and testosterone production (Cacciola et al., 2008). In men, cannabis, particularly when delivered acutely (Cohen, 1976), has been shown to reduce plasma LH and testosterone levels (Kolodny et al., 1974); however, these levels can be normalized following chronic consumption, likely via a tolerance mechanism (Block et al., 1991; Mendelson et al., 1974; Thistle et al., 2017). More recently, cannabis smokers have been shown to have higher
levels of testosterone than cannabis non-smokers. However, the plasma testosterone levels were within the same range as those observed in cigarette smokers (Gundersen et al., 2015).

In the sea urchin, high doses of Δ⁹-THC alter the fertilization ability of spermatozoa by decreasing sperm count, motility and vitality (Schuel et al., 2002), and in mice, Δ⁹-THC induces sperm morphological and chromosomal abnormalities (Banerjee et al., 2011; Dalterio et al., 1987b; Patra and Wadsworth, 1991; Zimmerman and Zimmerman, 1990). *In vitro*, high concentrations of Δ⁹-THC alter mouse sperm motility (Morgan et al., 2012) and the spontaneous acrosome reaction (Whan et al., 2006). Furthermore, in mice, an intraperitoneal injection of cannabis induces testicular oxidative stress, leading to a decrease in the seminiferous tubule diameter and impairment of the seminiferous epithelium (Mandal and Das, 2010). In addition, Δ⁹-THC inhibits copulatory behaviour in rats (Murphy et al., 1994).

In human males, daily and intensive use of cannabis leads to a decrease in sperm count, which is preceded by a decrease in the percentage of motile and morphologically normal spermatozoa in the ejaculate (Hembree et al., 1978; Pacey et al., 2014). Furthermore, men using cannabis more than once per week have significantly lower sperm concentrations and total sperm counts than non-users, and these impairments are worsened by the use of additional recreational drugs (Gundersen et al., 2015). Δ⁹-THC induces a concentration-dependent decrease in sperm motility and a marked decrease in the percentage of spermatozoa undergoing the spontaneous acrosome reaction (Whan et al., 2006). Additionally, the *in vitro* administration of supra-physiological doses of AEA decreases human sperm motility, vitality and the acrosomal reaction likely by inhibiting CB1-mediated mitochondrial activity (Aquila et al., 2010; Rossato, 2008a). In contrast, the concentration of AEA in seminal plasma is significantly lower in men with asthenozoospermia or oligoasthenoteratozoospermia than in normozoospermic men (Amoako et
al., 2013). In chronic cannabis users, the volume of seminal vesicles and ejaculate is decreased (Lotti et al., 2015). Similar to its action in animals, Δ⁹-THC alters sexual motivation and erectile function in human males (Gorzalka et al., 2010).

Recently, eCBs have been considered new actors in chromatin remodelling in mouse spermatids (Chioccarelli et al., 2010). Both heterozygous (CB1+/−) and homozygous (CB1−/−) deletions of the CB1 gene in mice result in decreased mRNA levels of transition protein 2 (TNP2) and histone displacement. Male CB1−/− mice exhibit inefficient histone displacement and produce spermatozoa with poorly condensed chromatin and damaged DNA. According to complementary tests performed in vivo in wild-type and CB1+/− mice or in vitro with wild-type mice testis, AEA could be a paracrine factor that acts locally and modulates TNP2 expression by increasing mRNA levels via the activation of CB1 (Chioccarelli et al., 2010).

These observations emphasize that the ECS plays a major role in male reproduction (Rossato et al., 2008b) and that aberrant signalling in this system could lead to spermatogenesis impairment and sperm nuclear alterations. To our knowledge, only a few studies have investigated the potential effects of illicit drugs on human fertility, because of ethical considerations and bias resulting from the frequent under-reporting of consumption. In addition, in infertile males, the impact of exocannabinoids found in cannabis on spermatogenesis and, more precisely, on sperm nuclear quality, remains unclear.

The main objective of our study was to explore the impact of cannabis consumption on sperm nuclear quality in infertile males. We performed a retrospective cross-sectional study comparing sperm nuclear integrity between two groups of infertile males, namely, cannabis users and cannabis non-users. Numerical chromosomal abnormalities [assessed by fluorescence in situ hybridization (FISH)], DNA fragmentation [assessed by a terminal deoxynucleotyl transferase-
mediated dUTP nick-end labelling (TUNEL) assay], chromatin condensation (assessed by aniline blue staining) and sperm head vacuoles [assessed by Motile Sperm Organelle Morphology Examination (MSOME)], in the spermatozoa of infertile males, grouped as cannabis users and cannabis non-users, were compared.
Materials and methods

Patients and semen samples

This retrospective cross-sectional study was conducted at the Reproductive Biology Laboratory-CECOS of Normandy Rouen University Hospital between July 2003 and December 2013. This study was approved by the ethical committee for retrospective studies of Normandy Rouen University Hospital.

The males included in the current study consulted for male factor infertility after a 12-month trial period to achieve spontaneous pregnancy and presented an alteration in at least one conventional semen parameter [sperm concentration ($10^6$/mL), total sperm number ($10^6$/ejaculate), progressive motility (WHO grades a+b combined, %) defined according to the World Health Organization criteria (World Health Organisation, 2010) and morphology (normally formed spermatozoa, %) (Auger et al., 2000)]. The semen parameter alterations were confirmed using two samples collected at 3-month intervals. No fever occurred during the three months preceding each semen analysis. The clinical and biological data reported in the study were collected during routine clinical and biological procedures to explore male infertility. A physical examination was performed by the same andrologist and was concluded with a scrotal ultrasound examination. All infertile males included in the study had a normal blood karyotype. Concerning the evaluation of lifestyle parameters, all information regarding tobacco, alcohol or cannabis consumption was obtained during an interview systematically carried out before semen collection. The patients were considered regular tobacco smokers if they had a history of smoking for at least one year before semen collection. The smoking habit duration was measured using the total number of cigarettes smoked per day. The patients were considered regular alcohol consumers if they reported a daily alcohol intake of at least two drinks per day (≥20 g of
pure alcohol) with a history of at least one year before semen collection. The patients were considered regular cannabis users if they reported the consumption of at least one cannabis cigarette per week with a history of at least one year before semen collection (Lotti et al., 2015).

The males included in the current study were selected from our database of 333 infertile males who benefited from specific assessments of sperm nuclear quality due to at least one altered semen parameter from July 2003 to December 2013. When the semen volume and total sperm number per ejaculate were sufficient, these assessments consist, in our laboratory, of FISH, TUNEL assay, aniline blue staining and MSOME. The financial support for these assessments is provided by the national health insurance system for aniline blue staining and by the Health Ministry for FISH, TUNEL assay and MSOME, within the context of innovative biological acts undergoing scientific evaluation. We selected infertile males for whom the results of at least 3 of the 4 sperm nuclear assessments mentioned above were available. First, we selected the index group (exposed group: cannabis users), which comprised 27 infertile males who consumed cannabis as exclusively by smoking; other forms of cannabis consumption were excluded. Second, we established the comparison group (unexposed group: cannabis non-users), which consisted of 27 infertile males who were cannabis non-users and were matched to the exposed infertile males who were cannabis users. The strategy used for matching was individual matching with one comparison subject selected separately for each index subject recruited during the same years and was based on matching four potential confounder variables known to impair sperm nuclear quality: age (from 25 to 40 years old), tobacco and alcohol consumption.

Sperm collection was performed in the laboratory after a 3- to 5-day period of sexual abstinence. A liquefaction time of 20 to 30 minutes was allowed before semen analysis. The MSOME analysis was preceded by a sperm selection procedure performed after gradient density
centrifugation. A 2-layer gradient was prepared using the 70% and 90% fractions of Puresperm® (J.C.D. International Laboratory, Lyon, France) diluted in IVF medium® (Origio, Limonest, France). The semen sample (1 mL) was layered on top of the gradient before centrifugation at 150×g for 20 minutes. Then, the 90% fraction was washed with IVF medium® (Origio) by centrifugation at 350×g for 10 minutes. The final pellet was resuspended in 0.5 mL of IVF medium® and incubated at 37°C with 5% CO₂. The sperm concentration (10⁶/mL), rapid and slow progressive motility (%), morphology (% of normally formed spermatozoa), and survival after 17 hours (% of rapid and slow progressive motile spermatozoa after 17 hours) were assessed.

The conventional semen parameters and the sperm nuclear alterations, including the MSOME, were assessed blindly by the technicians during the study without knowledge of the cannabis exposure status of the infertile males. More precisely, the sperm nuclear alterations were assessed by the same technician during the period of recruitment. Internal and external quality controls for the conventional semen parameters were performed in the laboratory according to the ISO 15089 quality standard for medical laboratories. An internal quality control for the specific analyses of sperm nuclear damage was performed according to the abovementioned quality standard. However, no external quality control standard exists for these specific analyses.

**Fluorescence in situ hybridization (FISH)**

A three-colour FISH analysis was performed to evaluate the frequency of numerical chromosome abnormalities in spermatozoa obtained from all semen samples. Alpha-satellite centromeric probes specific for chromosomes 18, in blue (CEP 18 Spectrum Aqua Blue™,
Abbott, Rungis, France); X, in green (CEP X Spectrum Green™, Abbott); and Y, in red (CEP Y Sat III Spectrum Orange™, Abbott); were used. After hybridization, the slides were counterstained with 4’,6-diamidino-2-phenylindole (Counterstain I®, Adgenix) diluted 1:5 in antifade mounting medium. A minimum of 5000 sperm nuclei were examined at 1000× magnification under an epifluorescence microscope equipped with a triple bandpass filter set to capture DAPI/FITC/TexasRed (DMRD®, Leica, Solms, Allemagne) fluorescence as previously described (Perdrix et al., 2011). Two fluorescent spots of comparable size and intensity and separated by at least one spot diameter were considered two copies of the corresponding chromosome. Spermatozoa with diffuse fluorescent signals and overlapping nuclei were classified as ambiguous and were not included in the count. The rate of aneuploidy was calculated as the sum of XX, YY, 1818 disomic and XY hyperhaploid spermatozoa. The rate of total chromosome abnormalities was considered the sum of the disomy and diploidy rates. The rates of meiosis I and meiosis II non-disjunctions were calculated as the sum of XY hyperhaploid and diploid spermatozoa and the sum of XX and YY disomic or diploid spermatozoa, respectively.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay**

DNA fragmentation was characterized in spermatozoa from all semen samples, which were fixed with methanol and smeared onto glass slides using an In situ Cell Death Detection Kit POD® (Roche, Mannheim, Germany) according to the manufacturer's instructions. The slides were counterstained with propidium iodide diluted to 1:1000 in antifade mounting medium (Antifade®, MP-QbioGene, Illkirch, France). DNA fragmentation was characterized in 500 spermatozoa at 1000× magnification using a digital imaging system (MacProbe® version 3.3,
Perspective Scientific International LTD, Chester, England). Spermatozoa were considered to have fragmented DNA when the green fluorescence signal occupied more than 50% of the sperm head area.

**Aniline blue staining**

Sperm chromatin condensation was examined in spermatozoa from all semen samples, which were fixed with 3% glutaraldehyde (Glutaraldéhyde® Sigma, St Louis, MO, USA). The slides were stained with 5% aniline blue (Gurr®, BDH Laboratory Supplies, England) at pH 3.5 for 5 minutes. Five hundred spermatozoa from the native semen samples were analysed on each slide under a light microscope (Leitz DMRD®, Leica, Solms, Germany). Sperm nuclei were considered abnormal with chromatin condensation defects when the blue staining occupied more than 50% of the sperm head area.

**Motile sperm organelle morphology evaluation (MSOME) analysis**

The motile spermatozoa obtained after 2-layer gradient density centrifugation were examined under an inverted microscope equipped with Nomarski differential interference contrast optics (6600×) (Leica DMI 6000B, Leica, Solms, Germany). For each sample, twenty-five spermatozoa were randomly photographed and separately analysed using digital imaging system software (Leica Application Suite Interactive Measurement version 3.4.0, Leica, Solms, Germany).

Thus, the following sperm head and vacuole parameters were assessed: length (µm), width (µm) and head area (µm²), vacuole size (µm) and number, sperm head relative vacuolar
area (RVA (%)=\frac{\text{vacuole area (µm}^2\text{)/head area (µm}^2\text{)}}{100}) and type 3 spermatozoa (%) with large vacuoles occupying more than 12.4% of the sperm head area (Perdrix et al., 2012).

**Plasma hormone level measurement**

Plasma FSH, LH and sex hormone binding globulin (SHBG) levels were measured by chemiluminescence immunoassay (Immulite 2500, Siemens Healthcare Diagnostics), and the testosterone level was measured by radioimmunoassay (in duplicate) (Immunotech Beckman Coulter, Roissy, France). The lower limit of sensitivity for LH and FSH was 0.1 IU/L; for testosterone, 0.04 ng/mL.

**Statistical analysis**

The data were collected in Excel, and the statistical analyses were performed using SAS/STAT® (Statistical Analysis System Software version 9.2, SAS Institute, Inc., SAS Campus Drive, Cary, NC, USA). A conditional logistic regression test was performed to verify the comparability of the two matched groups in terms of age, tobacco consumption, alcohol consumption and considering the conventional semen parameters, possible confounding factors. The plasma hormone levels were compared between the two groups using the Mann-Whitney test. Conventional semen parameters and sperm nuclear abnormalities were compared between cannabis users and cannabis non-users by ANCOVA.

The data are expressed as the means±standard deviations or as percentages for categorical variables. A P value of < 0.05 was considered significant.


**Results**

*Description of the two groups of infertile males*

The socio-demographic, lifestyle, clinical parameters and plasma hormone levels of the two populations of infertile males (i.e., cannabis users vs cannabis non-users) are reported in Table 1. The 54 infertile males were between 25 and 40 years of age (mean age: 31.6 years). The age did not vary significantly between the two groups. The cannabis users declared a mean consumption of 10.04 (± 12.09) cannabis cigarettes per week, ranging from 1 to 6 cigarettes per week (less than 1 cigarette per day) in 14 patients (52%) to 7-42 cigarettes per week (more than 1 cigarette per day) in 13 patients (48%). No significant differences in the pairing criteria (i.e., age, tobacco and alcohol consumption, and total sperm number per ejaculate) were found between cannabis users and cannabis non-users. Mumps occurred in both cannabis users and cannabis non-users before puberty without any testicular involvement.

The baseline semen parameters are reported in Table 2. Except for sexual abstinence, which was significantly lower in the group of cannabis users, the conventional semen parameters did not vary significantly between the two groups. Leucocytospermia was not detected in the semen samples of cannabis users and cannabis non-users. Oligozoospermia was observed in 44% (12 of 27) of the cannabis users and 41% (11 of 27) of the cannabis non-users. Asthenozoospermia was present in 74% (20 of 27) of the cannabis users and 63% (17 of 27) of the cannabis non-users. Teratozoospermia was observed in 59% (16 of 27) of the cannabis users and 48% (13 of 27) of the cannabis non-users.

Plasma hormone assay data were not available for all patients enrolled in the study (n = 15) (Table 1). However, a comparative analysis of the hormone assays in 16 infertile males
in the cannabis user group and 23 infertile males in the cannabis non-user group did not reveal any significant differences between these two populations.

**Sperm nuclear abnormalities**

The data concerning sperm nuclear abnormalities are reported in Table 3 and Fig. 1. FISH, TUNEL assay and aniline blue staining were performed in 24 cannabis users and 27 cannabis non-users. MSOME analysis was conducted in 25 cannabis users and 27 cannabis non-users.

A significant increase in the rates of XY hyperhaploid and 18 disomic spermatozoa was found in cannabis users compared to these rates in cannabis non-users ($P = 0.0009$ and $P = 0.0121$, respectively). The aneuploid, diploid and total chromosome abnormality rates were higher in the spermatozoa from cannabis users than in the spermatozoa from cannabis non-users ($P = 0.044$, $P = 0.037$ and $P = 0.0027$, respectively). Meiotic non-disjunctions occurred preferentially during meiosis I in both cannabis users and cannabis non-users ($P = 0.0002$ and $P = 0.0126$, respectively). However, the rate of meiosis I non-disjunctions was significantly higher in cannabis users than in cannabis non-users ($P = 0.0005$). The rate of sperm DNA fragmentation was significantly higher in cannabis users than in cannabis non-users ($P = 0.027$).

Chromatin condensation defects did not vary significantly between the two groups. Furthermore, the sperm head length, width and area did not differ significantly between cannabis users and cannabis non-users. The sperm head RVA and the percentage of type 3 spermatozoa were equally distributed and did not vary significantly between the two groups (Table 3).

Due to the low number of cannabis smokers, the analysis of sperm nuclear abnormalities in the cannabis smokers according to the level of cannabis consumption was limited to two
qualitative subgroups (consumption of less than 1 cigarette per day and consumption of more than 1 cigarette per day). No significant differences were found between the two groups stratified by the level of cannabis consumption in any of the different sperm nuclear quality markers assessed.
Discussion

The current study revealed a potential association between cannabis consumption and sperm nuclear damage in infertile males with semen parameter alterations. One of the main results of our study was the positive association between current cannabis smoking and an increase in numerical chromosome abnormalities as well as DNA fragmentation in the spermatozoa of infertile males who regularly smoked cannabis.

Most studies performed in human males to assess the impact of cannabis consumption on sperm production and quality have only explored standard semen parameters, such as semen volume and sperm motility, concentration or morphology (Close et al., 1990; El-Gothamy and El-Samahy, 1992; Gundersen et al., 2015; Hembree et al., 1978; Lotti et al., 2015; Pacey et al., 2014; Singer et al., 1986). To our knowledge, no study has assessed the potential impact of cannabis consumption specifically on sperm nuclear quality in infertile males. Indeed, sperm chromatin maturity and DNA integrity are well established as necessary prerequisites for normal fertilization and embryo development (Irez et al., 2015; Sakkas and Alvarez, 2010).

Numerical chromosome abnormalities (i.e., aneuploidy, diploidy and total chromosome abnormalities) were more frequently observed in the spermatozoa of infertile males who were cannabis users than in the spermatozoa of those who were cannabis non-users. Cannabinoids are known to negatively affect cytoskeletal components and to reduce the mRNA levels of cytoskeletal proteins, such as tubulin and actin, in cultured Chinese hamster ovary (CHO) cells (Tahir and Zimmerman, 1991; Zimmerman and Zimmerman, 1990). Consequently, the microtubules become fragmented in CHO cells treated with Δ⁹-THC, with impairment of the microtubule assembly and disassembly process (Tahir and Zimmerman, 1991). Indeed, the cytoskeleton is indispensable for the formation of the mitotic apparatus and plays a predominant role in cell division, cell motility, intracellular transport and the chromosome movement.
Cannabinoids can consequently interact with the mitotic apparatus responsible for chromosomal segregation errors (Dalterio et al., 1987a; Henrich et al., 1980; Stenchever and Allen, 1972). \( \Delta^9 \)-THC treatment is known to induce chromosome segregation errors in human lymphocytes in vitro, with anaphase lags and unequal segregations in bipolar divisions. Furthermore, it has been proposed that \( \Delta^9 \)-THC, by affecting the formation of microtubules and spindles, may be considered a mitotic poison (Henrich et al., 1980). In mice, \( \Delta^9 \)-THC can also modify the permeability of the membrane to ions such as calcium, which is a known inhibitor of the microtubule polymerization and disrupts actin microfilament assembly (Dalterio et al., 1987a). In addition, CB2 plays a pivotal role in meiotic entry and is a potential key inducer of the mitosis-meiosis switch in male germ cells. Hyperstimulation of CB2 disrupts the temporal dynamics of the spermatogonial cycle in mice (Di Giacomo et al., 2016). The higher levels of numerical chromosome abnormalities observed in the spermatozoa of infertile males who were cannabis users may be the consequence of the clastogenic action of the exocannabinoids or the cannabinoid-induced disruption of mitotic/meiotic events or both. Exocannabinoids may be responsible for chromosome non-disjunction or segregation errors during meiotic divisions, as has been described in human lymphocytes in vitro (Henrich et al., 1980); considering our data, these effects occur preferentially during the first meiotic division.

The current study showed that DNA fragmentation was significantly increased in the spermatozoa of cannabis users compared to that in the spermatozoa of cannabis non-users. \( \Delta^9 \)-THC and AEA are inhibitors of cell proliferation and inducers of apoptosis (Di Marzo et al., 2000; Maccarrone and Finazzi-Agro, 2003). In mice, intraperitoneal injections of cannabis extract adversely affect the testes via oxidative stress through a significant increase in lipid peroxidation and a decrease in testicular lipid content (Mandal and Das, 2010). More recently, a positive relationship was established in rats between cannabis-induced sperm abnormalities and
the activity of plasma lactate dehydrogenase (LDH), which is a key regulator of nicotinamide adenine dinucleotide phosphate (NADPH) synthesis, a very powerful inducer of ROS (Alagbonsi et al., 2016). CB1 activity is known to preserve the DNA integrity of spermatozoa during epididymal transit. Indeed, in the caput epididymis of CB1−/− mice, the percentage of spermatozoa with DNA damage was higher than in that of normal mice, and DNA damage increased during epididymal transit from caput to cauda (Chioccarelli et al., 2010). Therefore, the deregulation of CB1 activity by exocannabinoids might induce sperm DNA damage during epididymal transit. In cannabis users, oxidative stress might also be mediated by tobacco consumption, because tobacco smoke contains high concentrations of ROS that participate in Fenton reactions to produce H₂O₂ and cause DNA damage such as DNA strand breaks (Elshal et al., 2009; Valavanidis et al., 2009). However, since the tobacco consumption was comparable between the cannabis users and non-users, this parameter cannot explain the increase in the DNA fragmentation rate in the spermatozoa of cannabis users compared to that in the spermatozoa of cannabis non-users. A positive correlation was found in cannabis users between the rate of sperm DNA fragmentation and the rates of aneuploidy and total chromosome abnormalities (P < 0.0013 and P < 0.0015, respectively). Moreover, other published data in infertile males have highlighted an increase in the aneuploidy rate in spermatozoa with fragmented DNA under other conditions (Muriel et al., 2006; Tang et al., 2009). Several studies have proposed that types of DNA damage, including DNA fragmentation and uncondensed chromatin, are related to each other and are secondary to disrupted histone displacement (Carell et al., 2007). In addition, CB1 activity is linked to the regulation of post-meiotic stages; in particular, CB1 activity controls the differentiation steps of spermiogenesis and is involved in chromatin packaging in the spermatids of rats (Cacciola et al., 2008), boars (Maccarone et al., 2003) and mice (Cacciola et al., 2008), as well as in the acrosome and head shape configuration in mice (Cacciola et al., 2008). Furthermore, the activation of the
CB2 receptor in mouse spermatogonia has been shown to increase the expression of Prdm9 gene, which is a gene that encodes a meiosis-specific histone, histone H3 lysine 4 trimethylation (H3K9me3) methyltransferase, which marks hotspots of recombination in prophase I (Di Giacomo et al., 2016). Exogenous cannabinoids might induce basic nuclear protein and epigenetic modifications in post-meiotic male germ cells, consequently altering sperm DNA condensation. However, in our study, cannabis consumption was not associated with abnormal sperm chromatin condensation in infertile males, a finding confirmed by both aniline blue staining and MSOME. Indeed, spermatozoa with large vacuoles corresponding to a high RVA presented a higher prevalence of sperm chromatin compaction abnormalities (Perdrix et al., 2011). Furthermore, mouse spermatozoa with uncondensed chromatin had a greater nuclear length and, consequently, a greater sperm head length (Cacciola et al., 2013). The RVA and sperm head length, width and area did not vary significantly between cannabis users and cannabis non-users and were within the normal range previously reported by our research team in a population of 109 males with normal conventional semen parameters (Perdrix et al., 2012), confirming the normal nuclear DNA packaging in most spermatozoa of cannabis users. Therefore, our data did not support the hypothesis that deregulation of the ECS by exogenous cannabis consumption alters the normal process of chromatin compaction during spermiogenesis. In addition, we can suggest that aniline blue staining is not the most suitable test to detect chromatin condensation defects in the spermatozoa of cannabis users.

Several limitations of this study need to be mentioned. First, our study was a retrospective analysis with a small sample subset size. Second, details concerning characteristics related to the socio-demographic and clinical parameters and the plasma hormone levels were not available for all patients. However, we tried to carefully exclude confounding factors such as age, leucocytospermia and other potential sources of DNA damage. The standard semen parameters
did not differ significantly between the two groups. Indeed, a greater alteration of these parameters in one of the groups might have increased the prevalence of sperm nuclear abnormalities (Calogero et al., 2001; Evgeni et al., 2014; Manochantr et al., 2012; Rives et al., 1999; Vegetti et al., 2000). In addition, oxidative stress is a major factor in the pathophysiology of varicocele, and high levels of sperm DNA fragmentation have been demonstrated in several studies (Wright et al., 2014). However, we were not able to evaluate this condition because the number of patients with varicocele was too low. In addition, no leucocytospermia was detected in the semen samples of infertile males who were cannabis users. Although age correlates positively with sperm DNA fragmentation (Das et al., 2013) and aneuploidy (Rives et al., 2002), the patients’ ages were comparable in the two groups.

Several hypotheses have been proposed to explain the mechanisms involved in the detrimental effect of regular cannabis consumption on sperm production and quality: (i) deregulation of the ECS by indirect action on the hypothalamus-pituitary axis (Alagbonsi et al., 2016; Cohen, 1976; Kolodny et al., 1974), (ii) deregulation of the ECS by direct action on the intratubular and interstitial cells of the testis, and (iii) direct action at the epididymal level by increasing the local production of ROS, leading to an adverse post-testicular environment (Alagbonsi et al., 2016; Mandal and Das, 2010). Regarding the evaluation of endocrine function in our cannabis users, the number of patients included was too low to identify a significant difference between the two cohorts. Nevertheless, our data are unsurprising and might confirm the hypothesis regarding the development of tolerance in chronic cannabis users, because the plasma hormone levels of LH and FSH normalize with a chronic intake of cannabis (Brown and Dobs, 2002; Mendelson and Mello, 1983; Thistle et al., 2017), but did not confirm that deregulation of the hypothalamus-pituitary axis is responsible for altered sperm quality. The positive correlation observed between the rate of sperm DNA fragmentation and the rate of
aneuploidy in cannabis users can support the hypothesis of direct toxicity of exocannabinoids on the intratubular cells of the testis, but we can also consider that cannabis can induce a detrimental effect in the post-testicular environment at the epididymal level by increasing the local production of ROS. However, ROS and markers of oxidative stress were not assessed in our study.

In conclusion, competition between the exocannabinoids present in cannabis and eCBs for binding to cannabinoid receptors leads to the disruption of ECS homeostasis, which could impair spermatogenesis and sperm maturation during epididymal transit and, consequently, lead not only to alterations in standard semen parameters but also to sperm nuclear damage. However, this effect was not dose-dependent, in contrast with the results of studies involving tobacco users (Wright et al., 2014). However, any potential findings of our study need to be validated with larger sample sizes and our data has to be considered as exploratory findings. To address the limitations of our retrospective study and to confirm the increase in sperm nuclear alterations in infertile male cannabis users, a prospective case-control study should be performed in a larger population of infertile male cannabis users, including a specific assessment of cannabis consumption using a standardized questionnaire and the measurement of Δ⁹-THC (THC-COOH) in the urine, blood and seminal fluid, if possible. In addition, evaluating epigenetic modifications to sperm chromatin, oxidative stress damage and the reversibility of sperm nuclear alterations after the discontinuation of cannabis consumption would be of interest.
Vitae

Doctor France Verhaeghe obtained a Medical Doctor degree at Normandy Rouen University in 2014 and also holds a specialization in Reproductive Biology. Doctor Verhaeghe currently works at the Reproductive Biology Laboratory-CECOS of Rouen University Hospital with a main focus on male infertility and lifestyle factors.

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Declarations of interest

None declared.

Author contributions

France Verhaeghe performed data analysis and interpretation, statistical analysis and manuscript writing; Pierre Di Pizio was responsible for data analysis and manuscript revision; Véronique Sétif performed experiment; Cynthia Bichara, Benoit Berby, Aurélie Rives and Fanny Jumeau: performed biological and clinical data collection; Louis Sibert was involved in patient recruitment and clinical examination; Christine Rondanino was involved in data analysis and manuscript writing; Nathalie Rives was responsible for study design and supervision, patient
recruitment, clinical data collection and manuscript writing. All authors approved the final version of the manuscript.
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smoking is associated with lower seminal vesicles and ejaculate volume, despite higher

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Biography

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Figure captions

**Fig. 1.** Distribution of DNA fragmentation (A) and total chromosome abnormalities (B) in the spermatozoa of cannabis users and cannabis non-users.

The horizontal bar represents the median value of each parameter in cannabis users and cannabis non-users.
Tables

Table 1. Socio-demographic, lifestyle, clinical parameters as well as plasma hormone levels in the two populations of infertile males, i.e., cannabis users and cannabis non-users.

The values are expressed as the means±standard deviations or as percentages for categorical variables. All comparisons were performed between cannabis users and cannabis non-users. A $P$-value of $<0.05$ is considered significant.

**FSH:** Follicle stimulating hormone  
**LH:** Luteinizing hormone  
**MAI:** Multiple anomaly index  
**n:** Number of patients  
**sd:** Standard deviation  
**SHBG:** Sex hormone binding globulin
### Parameters

<table>
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<tr>
<th>Parameters</th>
<th>Cannabis users (n = 27)</th>
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<td>BMI (kg/m²)</td>
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<tr>
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<td>29.7</td>
<td>55.5</td>
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<td>40.7</td>
<td>14.8</td>
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<td>Plasma hormone levels</td>
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<tr>
<td>FSH (mU/mL)</td>
<td>5.0 ± 3.1</td>
<td>5.5 ± 5.1</td>
<td>0.7128</td>
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<tr>
<td>LH (mU/mL)</td>
<td>4.5 ± 2.6</td>
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<td>Testosterone (µg/mL)</td>
<td>5.8 ± 2.0</td>
<td>5.2 ± 1.8</td>
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<td>SHBG (nmol/L)</td>
<td>35.4 ± 15.8</td>
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<td>40.7</td>
<td>14.8</td>
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Table 2. Sperm baseline parameters in the two populations of infertile males, i.e., cannabis users and cannabis non-users.

The values are expressed as the means±standard deviations or as percentages for categorical variables. All comparisons were performed between cannabis users and cannabis non-users. A $P$-value of < 0.05 is considered significant.

**MAI**: Multiple anomaly index

**n**: Number of patients

**sd**: Standard deviation
### Parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cannabis users (n = 27)</th>
<th>Cannabis non-users (n = 27)</th>
<th>P</th>
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<tr>
<td></td>
<td>mean±sd / n %</td>
<td>mean±sd / n %</td>
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<td><strong>Semen parameters</strong></td>
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<tr>
<td>Sexual abstinence (days)</td>
<td>3.9 ± 1.3</td>
<td>4.8 ± 1.5</td>
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<td>pH</td>
<td>8.1 ± 0.3</td>
<td>7.9 ± 0.3</td>
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<tr>
<td>Volume (mL)</td>
<td>3.52 ± 1.6</td>
<td>3.84 ± 1.3</td>
<td>0.4311</td>
</tr>
<tr>
<td>Concentration (×10^9/mL)</td>
<td>23.8 ± 25.7</td>
<td>19.76 ± 17.5</td>
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</tr>
<tr>
<td>Total sperm number (×10^9/ejaculate)</td>
<td>77.5 ± 75.16</td>
<td>78.26 ± 81.5</td>
<td>0.5741</td>
</tr>
<tr>
<td>Sperm progressive motility (a+b, %)</td>
<td>33.2 ± 16.0</td>
<td>25.9 ± 11</td>
<td>0.6634</td>
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<tr>
<td>Vitality (%)</td>
<td>77.3 ± 11.3</td>
<td>72.4 ± 10.8</td>
<td>0.7663</td>
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<tr>
<td>Sperm normal forms (%)</td>
<td>28.9 ± 17.5</td>
<td>30.6 ± 16.8</td>
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<tr>
<td>MAI</td>
<td>1.55 ± 0.21</td>
<td>1.55 ± 0.24</td>
<td>0.9586</td>
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<tr>
<td>Oligozoospermia</td>
<td>12 44</td>
<td>11 41</td>
<td></td>
</tr>
<tr>
<td>Asthenozoospermia</td>
<td>20 74</td>
<td>17 63</td>
<td></td>
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<tr>
<td>Teratozoospermia</td>
<td>16 59</td>
<td>13 48</td>
<td></td>
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</table>
**Table 3.** Comparison of sperm nuclear alterations in cannabis smokers and cannabis non-smokers.

The values are expressed as the means±standard deviations. A $P$-value of < 0.05 is considered significant.

**Aneuploidy:** sum of the frequencies of presumed disomic X, Y, 18 and hyperhaploid XY spermatozoa

**Diploidy:** sum of the frequencies of presumed XX, YY and XY diploid spermatozoa

**Total chromosome abnormalities:** sum of the diploidy and aneuploidy

**Meiosis I:** sum of the frequencies of presumed hyperhaploid XY and XY diploid spermatozoa

**Meiosis II:** sum of the frequencies of presumed disomic X and Y, and XX and YY diploid spermatozoa

$n$: Number of patients  
$sd$: Standard deviation

* $P = 0.0002$  
** $P = 0.0126$
<table>
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<tr>
<th>Sperm nuclear alterations</th>
<th>Cannabis users mean±sd (n = 24(^a) and 25(^b))</th>
<th>Cannabis non-users mean±sd (n = 27)</th>
<th>P</th>
</tr>
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<tr>
<td>Hyperhaploidy XY (%) (^a)</td>
<td>0.36 ± 0.18</td>
<td>0.20 ± 0.17</td>
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<td>Disomy YY (%) (^a)</td>
<td>0.04 ± 0.03</td>
<td>0.06 ± 0.06</td>
<td>0.10</td>
</tr>
<tr>
<td>Disomy XX (%) (^a)</td>
<td>0.03 ± 0.03</td>
<td>0.05 ± 0.05</td>
<td>0.19</td>
</tr>
<tr>
<td>Disomy 18 (%) (^a)</td>
<td>0.07 ± 0.08</td>
<td>0.03 ± 0.03</td>
<td>0.0121</td>
</tr>
<tr>
<td>Aneuploidy (%) (^a)</td>
<td>0.50 ± 0.29</td>
<td>0.35 ± 0.16</td>
<td>0.0044</td>
</tr>
<tr>
<td>Diploidy (%) (^a)</td>
<td>0.34 ± 0.31</td>
<td>0.17 ± 0.12</td>
<td>0.037</td>
</tr>
<tr>
<td>Total chromosome abnormality (%) (^a)</td>
<td>0.84 ± 0.43</td>
<td>0.51 ± 0.20</td>
<td>0.0027</td>
</tr>
<tr>
<td>Meiosis I (%) (^a)</td>
<td>0.57 ± 0.29 *</td>
<td>0.30 ± 0.16 **</td>
<td>0.0005</td>
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<tr>
<td>Meiosis II (%) (^a)</td>
<td>0.20 ± 0.12 *</td>
<td>0.18 ± 0.11 **</td>
<td>0.6449</td>
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<td>DNA fragmentation (%) (^a)</td>
<td>10.65 ± 8.63</td>
<td>6.42 ± 5.19</td>
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<td>Abnormal chromatin condensation (%) (^a)</td>
<td>16.40 ± 12.15</td>
<td>16.81 ± 10.70</td>
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<td>Sperm head length (µm) (^b)</td>
<td>4.98 ± 0.41</td>
<td>5.07 ± 0.37</td>
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<td>Sperm head width (µm) (^b)</td>
<td>3.26 ± 0.24</td>
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<td>Sperm head area (µm²) (^b)</td>
<td>13.68 ± 1.60</td>
<td>13.57 ± 1.29</td>
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<td>Mean relative vacuolar area (%) (^b)</td>
<td>6.5 ± 2.3</td>
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<td>Type 3 spermatozoa (%) (^b)</td>
<td>9.5 ± 11.0</td>
<td>16.6 ± 18.7</td>
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