

Cannabinoids inhibit the respiration of human sperm

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Objective: To investigate the effects of the psychotropic compounds Δ^9 -tetrahydrocannabinol (Δ^9 -THC) and Δ^8 -tetrahydrocannabinol (Δ^8 -THC) on sperm mitochondrial O₂ consumption (respiration).

Setting: State University of New York Upstate Medical University, Syracuse, New York.

Patient(s): Forty-one men who visited the andrology laboratory for fertility evaluation.

Intervention(s): None.

Main Outcome Measure(s): A phosphorescence analyzer that measures O₂ concentration in sperm suspensions as a function of time was used to measure respiration.

Result(s): An immediate decline in the rate of respiration was observed when Δ^9 -THC or Δ^8 -THC was added to washed sperm. The inhibition was concentration dependent, and Δ^9 -THC was the more potent of the two compounds. Respiration was much less affected when Δ^9 -THC or Δ^8 -THC was added to neat semen, suggesting the presence of protective factors in seminal plasma. Both compounds inhibited the respiration of isolated mitochondria, illustrating that direct mitochondrial damage is likely the primary mechanism of action.

Conclusion(s): The two main active cannabinoids of the marijuana plant, Δ^9 - and Δ^8 -THC, are potent inhibitors of mitochondrial O₂ consumption in human sperm. These findings emphasize the adverse effects of these toxins on male fertility. The cytoprotective capacity of seminal plasma deserves further investigation. (Fertil Steril® 2009; 91:2471–6. ©2009 by American Society for Reproductive Medicine.)

Key Words: Cannabinoids, respiration, mitochondria, sperm

In 1964, Gaoni and Mechoulam isolated the primary cannabinoid, Δ^9 -tetrahydrocannabinol (Δ^9 -THC) from the marijuana plant, *Cannabis sativa* (1). Shortly thereafter, the less abundant cannabinoid, Δ^8 -tetrahydrocannabinol (Δ^8 -THC) was isolated (2). These two psychotropic ingredients are presumably responsible for altering cognition, decreasing memory, and invoking euphoria. Additional effects of cannabinoids include sedation, analgesia, attenuation of nausea and vomiting, reduction of intraocular pressure, stimulation of appetite, relief of muscle spasticity, decreased intestinal motility, and antitumor activity by inducing apoptosis (3).

The smoking of marijuana is also shown to reduce sperm count, sperm function, and overall male fertility (4–6). Other adverse effects of cannabinoids on the fertilizing capacity of sperm have been reported (7–9). Both Δ^8 -THC and Δ^9 -THC are known to bind to the cannabinoid receptors on the mid-piece and head regions of human sperm (10). The binding

of anandamide (an endogenous cannabinoid) to sperm cannabinoid receptors impairs sperm motility, acrosome reaction, and mitochondrial function (11, 12).

Mitochondria are the principal suppliers of sperm energy. Toxins may directly damage sperm mitochondria, producing rapid adenosine 5'-triphosphate depletion. Toxins may also initiate apoptosis, which leads to mitochondrial dysfunction (13).

Apoptosis is the principal mechanism of cell death. This biological pathway is initiated by cell surface receptors (e.g., Fas/APO-1) or intracellular targets (e.g., the pro-apoptotic Bcl-2 family member Bid). The resulting signals permeabilize the outer mitochondrial membrane, releasing soluble proteins (e.g., cytochrome c) from the mitochondrial intermembrane space. Released cytochrome c binds to apoptotic protease-activating factor 1, forming an oligomer (apoptosome) that activates caspases (cysteine aspartate-directed proteases). Caspase activation leads to opening of the permeability transition pores (located at contact sites between the inner and outer mitochondrial membranes), collapse of the mitochondrial membrane potential, and impairment of cellular respiration (14). Such findings have been reported in sperm and other cells treated with Δ^9 -THC or Δ^8 -THC (15–18).

The evolving role of mitochondria as targets of toxins and of active caspases prompted us to investigate the effects of cannabinoids on mitochondrial O₂ consumption in human sperm. For this purpose, we used a phosphorescence analyzer that accurately measures O₂ concentration over long periods of time in sperm suspensions (19–21).

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MATERIALS AND METHODS

Chemicals and Solutions

The chemicals Δ^9 -THC (63.7 mM = 20 mg/mL of 95% EtOH; molecular weight 314.47; stored at -4°C under argon) and Δ^8 -THC (127.4 mM = 40 mg/mL of 99% EtOH; molecular weight 314.47; stored at -4°C under argon) were provided by the National Institute of Drug Abuse (NIDA). Laboratory standards of Δ^9 -THC (1.0 mg/mL in methanol) and Δ^8 -THC (1.0 mg/mL in methanol) were prepared by Cerilliant Corporation (Round Rock, TX) and purchased from Cambridge Isotope Laboratories (Andover, MA). Anandamide (*N*-arachidonoyl-ethanolamine, molecular weight 347.6) was purchased from Biomol International, LP (Plymouth Meeting, PA).

The Pd (II) complex of *meso*-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin (Pd phosphor sodium salt) was purchased from Porphyrin Products (Logan, UT). Modified human tubal fluid (mHTF, containing 97.8 mM NaCl, 4.69 mM KCl, 0.2 mM MgSO_4 , 0.37 mM KH_2PO_4 , 2.04 mM CaCl_2 , 4.0 mM NaHCO_3 , 21 mM HEPES, 2.78 mM glucose, 0.33 mM Na pyruvate, 21.4 mM Na lactate, 10 $\mu\text{g/mL}$ gentamicin sulfate, 5 mg/L phenol red, and 0.5% human serum albumin; pH 7.2–7.4) was purchased from Irvine Scientific (St. Louis, MO). The remaining reagents were purchased from Sigma-Aldrich (St. Louis, MO).

The Pd phosphor solution (2 mM) was made by dissolving the powder at 2.5 mg/mL in dH_2O and stored at -20°C in small aliquots. Anandamide (25 mg) was suspended in 1.0 mL of 100% EtOH (final concentration, 71.9 mM) and stored at -80°C under argon. NaCN (1.0 M) was made fresh in dH_2O , and the pH was adjusted to ~ 7.0 with 12N HCl.

High-Performance Liquid Chromatography (HPLC)

Samples of the NIDA Δ^9 -THC and Δ^8 -THC solutions were run on HPLC and compared to runs of the laboratory standard Cerilliant Δ^9 -THC and Δ^8 -THC on the same system (Beckman reversed-phase HPLC with wavelength at 228 nm, 4.6 \times 250 mm Beckman Ultrasphere IP column at 25°C). The running solvent was acetonitrile:0.1% phosphoric acid [75:25], isocratic at a flow rate of 1.5 mL/minute. In all cases, the resulting chromatograms for NIDA Δ^9 -THC and Cerilliant Δ^9 -THC were identical to one another, as were the resulting chromatograms for NIDA Δ^8 -THC and Cerilliant Δ^8 -THC.

Study Population

Semen samples from 41 patients attending the andrology laboratory at the State University of New York Upstate Medical University were studied. The World Health Organization reference limits and methods (1999) were used for the sperm analysis (22). The study was approved by the Institutional Review Board for protection of human subjects at State University of New York Upstate Medical University. Informed consent was obtained from each patient.

The samples were allowed to liquefy at 37°C for 30 minutes. Only semen samples with a negligible number of cells were selected for the analysis; this approach minimized the

contribution of other cells to respiration. Each sample was mixed well, divided into equal aliquots, and processed at 25°C . To measure respiration, an aliquot was diluted (two-fold) in mHTF and centrifuged at 25°C ($300 \times g$) for 10 minutes. The pellet was suspended in 1.0 mL mHTF, 2 μM Pd phosphor, 0.5% fat-free bovine serum albumin and Δ^8 -THC, Δ^9 -THC, or EtOH (control). After additions, the pH was tested using pH paper, and it ranged between 7.2 and 7.4, similar to that of mHTF. The sample was immediately placed in a 1-mL glass vial, sealed, and placed in the instrument for O_2 measurement. For each sample, respiration was compared between treated and untreated equal aliquots containing the same number of sperm. Statistical significance between the *k*-values in treated and untreated samples was determined by paired Student's *t*-test analyses. $P < .05$ was considered statistically significant.

Cellular Respiration

The $[\text{O}_2]$ in the sperm suspensions was determined as function of time, using the Pd phosphor. The phosphorescence decay ($1/\tau$) of the probe was exponential, with τ being linear in $[\text{O}_2]$, according to $\tau^\circ/\tau = 1 + \tau^\circ k_q [\text{O}_2]$; τ , lifetime in the presence of O_2 ; τ° , lifetime in the absence of O_2 ; and k_q , second-order O_2 -quenching constant. Samples were exposed to light flashes (10/second) from a pulsed light-emitting diode array with peak output at 625 nm. Emitted light was detected by a Hamamatsu (Opto Technology, Hamamatsu City, Japan) photomultiplier tube after passing through a wide-band interference filter centered at 800 nm. The amplified phosphorescence decay was digitized at a rate of 1 MHz by an A/D converter (19). The values of τ were determined in a series of ascorbate plus ascorbate oxidase solutions simultaneously with electrochemical measurements of $[\text{O}_2]$. A plot of $1/\tau$ versus $[\text{O}_2]$ was linear; the value of the quenching constant k_q (the slope) was $96.1 \pm 1.2/\mu\text{M/second}$ and $1/\tau^\circ$ (the intercept) was $10,087 \pm 156/\text{second}$ (21).

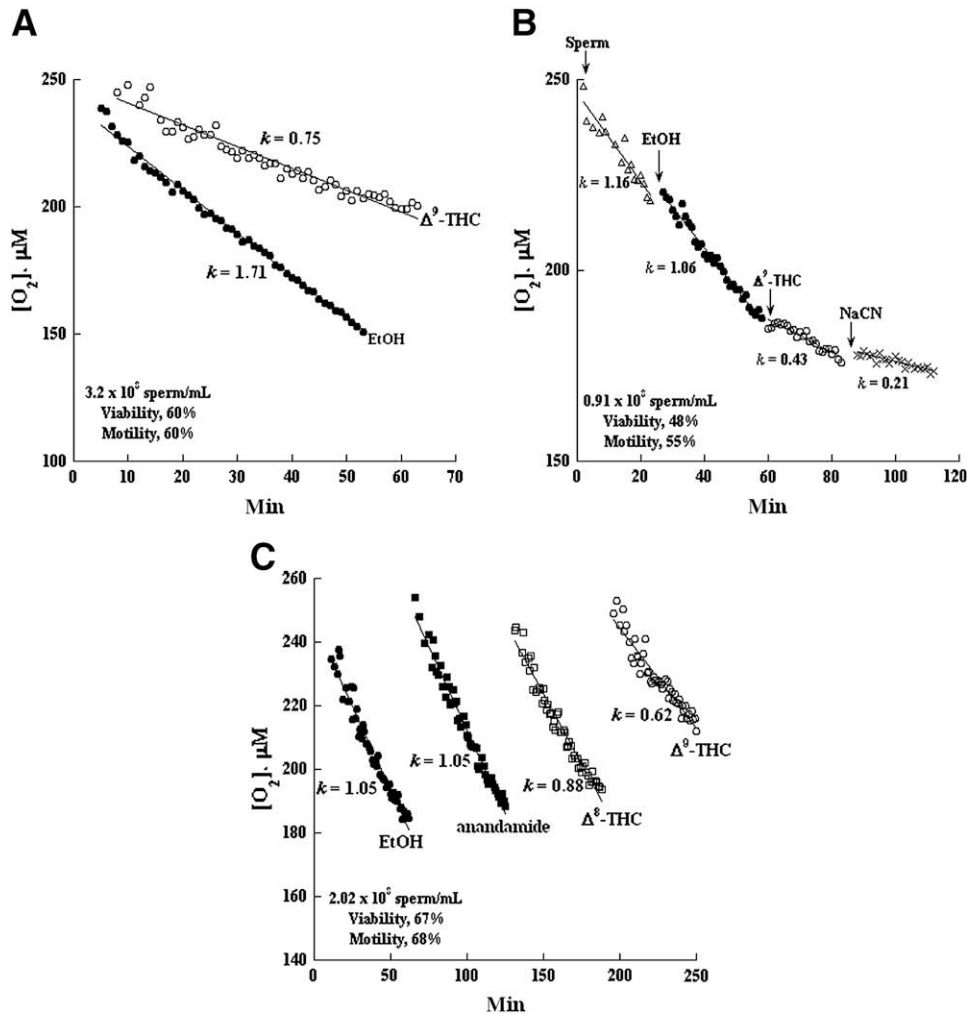
For each run, 1.0 mL of the sperm suspension was placed in a 1-mL sealed glass vial. The changes in $[\text{O}_2]$ with time were measured at 37°C (20). Mixing was accomplished with parylene-coated stirring bar (V&P Scientific Inc., San Diego). Rates of respiration were the negative of the slopes of $[\text{O}_2]$ versus *t* (zero-order rate constant, *k*, in $\mu\text{M O}_2/\text{minute}$). In most experiments, the O_2 measurements were made on suspensions containing 10^8 sperm per condition; otherwise, the values of *k* were adjusted to 10^8 sperm. The addition of 10 mM NaCN resulted in the inhibition of O_2 uptake, indicating that the decline in $[\text{O}_2]$ with time was mainly due to mitochondrial O_2 consumption.

Mitochondrial Respiration

Measurements with mitochondria were included to demonstrate a direct effect of Δ^9 -THC or Δ^8 -THC on the mitochondrial respiratory chain. Mitochondria were prepared from beef heart as described elsewhere (23). Mitochondria were suspended in 1.0 mL of 10 mM Tris-Cl (pH 8.2), 250 mM

FIGURE 1

Both Δ^9 -THC and Δ^8 -THC inhibited respiration when added to washed sperm. (A) Washed sperm plus 2 μ L EtOH (closed circles) or 120 μ M Δ^9 -THC (open circles). (B) Washed sperm with shown additions of 2 μ L EtOH, 120 μ M Δ^9 -THC, and 10 mM NaCN. (C) Washed sperm plus 2 μ L EtOH (closed circles), 120 μ M anandamide (closed squares), 120 μ M Δ^8 -THC (open squares), or 120 μ M Δ^9 -THC (open circles).



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sucrose, 2 μ M Pd phosphor, and 0.5% fat-free albumin. The mixture was transferred to a 1-mL glass vial and placed in the instrument for O_2 measurement. O_2 consumption was initiated by the addition of 50 mM succinate (data not shown). Other additions included 2 μ L EtOH (control), 120 μ M Δ^9 -THC or Δ^8 -THC, 10 mM NaCN, and glucose (1.0 mM) plus glucose oxidase (7.0 units, to deplete remaining O_2).

RESULTS

Addition of Δ^9 -THC and Δ^8 -THC to Washed Sperm

The effect of Δ^9 -THC on respiration was first tested in washed sperm. Representative experiments are shown in Figure 1A–1C. The results are summarized in Table 1. The values of k (rate of respiration in μ M O_2 /minute per 10^8

sperm; mean \pm SD, $n = 10$) in the presence of EtOH were 1.0 ± 0.30 and in the presence 120 μ M Δ^9 -THC, 0.52 ± 0.27 (48% inhibition; $P = .002$; Table 1). The values of k decreased by 44% in the presence of 60 μ M Δ^9 -THC and by 22% in the presence of 30 μ M Δ^9 -THC; concentrations ≤ 20 μ M were less effective. Thus, Δ^9 -THC caused a dose-dependent inhibition of sperm respiration. As demonstrated in Figure 1B, injection of 120 μ M Δ^9 -THC immediately inhibited respiration by about 58%. Further inhibition (80%) followed the injection of 10 mM NaCN.

The effect of Δ^8 -THC was studied (Table 1 and Fig. 1C). The values of k (rate of respiration in μ M O_2 /minute per 10^8 sperm; mean \pm SD, $n = 10$) in the presence of EtOH were 0.93 ± 0.34 and in the presence 120 μ M Δ^8 -THC,

TABLE 1

Effects of Δ^9 -THC and Δ^8 -THC on O_2 consumption when added to washed sperm and neat semen.

Samples	EtOH	Δ^9 -THC ^a	Δ^8 -THC ^a	Inhibition, %	P
Washed ^b (n = 10)	1.0 ± 0.30	0.52 ± 0.27	—	48	.002
Washed ^b (n = 10)	0.93 ± 0.34	—	0.79 ± 0.37	15	.005
Semen ^c (n = 20)	0.97 ± 0.34	0.92 ± 0.32	—	5	.26
Semen ^c (n = 20)	0.90 ± 0.36	—	0.83 ± 0.46	8	.18

Note: Forty-one samples from 41 patients were analyzed; n equals number of samples. The values (mean ± SD) are rates of respiration (*k*) in $\mu\text{M } O_2/\text{minute per } 10^8$ sperm.

^a Addition concentration of 120 μM .

^b EtOH, Δ^9 -THC, or Δ^8 -THC was added to washed sperm immediately before O_2 measurement.

^c Neat semen samples were incubated at 25°C with EtOH, Δ^9 -THC, or Δ^8 -THC for 3 hours. The sperm were then washed and analyzed for O_2 consumption.

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0.79 ± 0.37 (15% inhibition; $P=.005$; Table 1). The value of *k* decreased by 25% in the presence of 240 $\mu\text{M } \Delta^8$ -THC. Thus, although Δ^8 -THC inhibited sperm respiration, it was less effective than Δ^9 -THC. In contrast, anandamide (120 μM) had no effect on sperm respiration (Fig. 1C).

Addition of Δ^9 -THC and Δ^8 -THC to Semen Samples

We next evaluated the effects of Δ^9 -THC and Δ^8 -THC on sperm respiration when added to semen samples (Table 1 and Fig. 2). Each semen sample was divided into equal aliquots. Then Δ^9 -THC, Δ^8 -THC, or EtOH was added, and the incubation continued at 25°C for 3 hours. At the end of the incubation period, a sample was washed and the pellet was suspended in 1.0 mL mHTF plus 2 μM Pd phosphor and 0.5% fat-free albumin and placed in the instrument for O_2 measurement. The results are summarized in Table 1, and representative experiments are shown in Figure 2. In most patients, Δ^9 -THC had a minimum effect on respiration (Fig. 2B–2D). However, Δ^9 -THC inhibited respiration by 35% in some patients (Fig. 2A). The reason for this variability remains unknown. Rates of respiration (*k*, in $\mu\text{M } O_2/\text{minute per } 10^8$ sperm; mean ± SD, n = 20) in the presence of EtOH were 0.97 ± 0.34 and in the presence 120 $\mu\text{M } \Delta^9$ -THC, 0.92 ± 0.32 (5% inhibition; $P=.26$; Table 1). The effect of Δ^8 -THC on sperm respiration when added to neat semen was very similar to the effect of Δ^9 -THC (Table 1 and Fig. 2A).

Addition of Δ^9 -THC and Δ^8 -THC to Isolated Mitochondria

The effects of Δ^9 -THC and Δ^8 -THC on the respiration of beef heart mitochondria were studied. The value of *k* decreased by 32% in the presence of 120 $\mu\text{M } \Delta^9$ -THC and by 64% in the presence of 240 $\mu\text{M } \Delta^8$ -THC.

DISCUSSION

In this study, we investigated the effects of Δ^9 -THC and Δ^8 -THC on sperm mitochondrial O_2 consumption. These cannabinoids are known to reduce sperm count, disrupt spermatogenesis, induce aberrations in sperm morphology, decrease

sperm fertilizing capacity, inhibit sperm protein and nucleic acid synthesis, and impair sperm glycolysis (4–9). Clinically, studies are still needed to confirm whether the use of marijuana adversely affects the fertilizing capacity of human sperm (4).

Studies have shown functional receptors for cannabinoids in human sperm (10). Activation of these receptors by anandamide, an endogenous cannabinoid, reduces sperm motility and inhibits acrosome reaction (11). These effects are associated with dissipation of the electrochemical H^+ gradient ($\Delta\mu_{H^+}$) across the mitochondrial inner membrane (12).

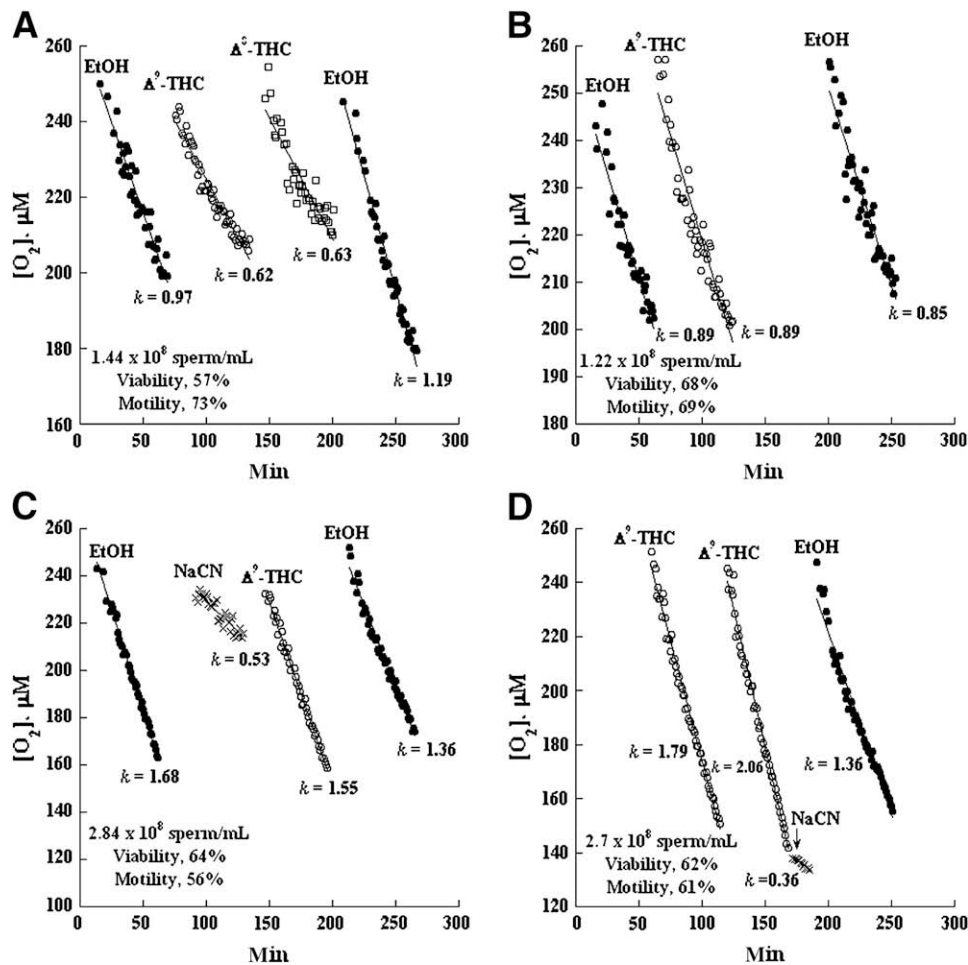
Studies have also shown that Δ^9 -THC and Δ^8 -THC inhibit cell proliferation, induce apoptosis, and collapse $\Delta\mu_{H^+}$ (15–18). These effects are functions of drug concentration and exposure time. For example, loss of $\Delta\mu_{H^+}$ was observed in lung cells exposed to 30 $\mu\text{M } \Delta^9$ -THC for 1 hour (17). In mice, in vivo treatment with Δ^9 -THC produced lymphocyte apoptosis in 6 hours (16), presumably by releasing the proapoptotic immune modulator interleukin-1 and by blocking the antiapoptotic protein Bcl-2 (18).

Our results show that the cannabinoids are potent inhibitors of sperm mitochondrial O_2 consumption (sperm respiration; Table 1 and Figs. 1–2). However, the activity profiles of the three cannabinoids are quite different. At concentrations ≥ 30 μM , Δ^9 -THC is a potent inhibitor of respiration; Δ^8 -THC is also effective, but to a lesser degree (Table 1). In contrast, anandamide is ineffective. Thus, activation of the cannabinoid receptors may not be the primary mode of action (Fig. 1C).

The mechanism of the inhibitory effect of cannabinoids on sperm respiration remains unclear. However, the fact that Δ^9 -THC and Δ^8 -THC inhibit the respiration of isolated mitochondria from beef heart (see the Results section) suggests possible direct effects of these toxins on the mitochondrial respiratory chain. Thus, Δ^9 -THC and Δ^8 -THC may exert a specific toxicity on sperm mitochondria. As shown elsewhere (24), the polycyclic structure of cannabinoids imposes a strong adverse effect on membrane-dependent processes, such as the inner mitochondrial membrane.

FIGURE 2

The chemicals Δ^9 -THC and Δ^8 -THC were less inhibitory when added to neat semen. Representative runs from four patients are shown (A–D). EtOH (closed circles), Δ^9 -THC (open circles), Δ^8 -THC (open squares), or NaCN (crosses) was added to neat semen (minute zero corresponds to the additions). The concentrations of Δ^9 -THC and Δ^8 -THC were 120 μM , except for in panel C, where the concentration was 60 μM .



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An additional finding of interest that emerged from this study is that Δ^9 -THC and Δ^8 -THC are much less poisonous to sperm respiration when added to the neat semen. This finding could reflect different activities of Δ^9 -THC and Δ^8 -THC in mHTF versus seminal plasma. It might also point to protective factors in the seminal plasma. These possibilities require further investigation.

In summary, the two main active cannabinoids of the marijuana plant are potent inhibitors of sperm respiration. The results add further emphasis to the adverse effects of these toxins on human health, including fertility.

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