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DEFICIENCY

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Original

Changes on metabolic parameters induced by acute cannabinoid administration (CBD, THC) in a rat experimental model of nutritional vitamin A deficiency

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Abstract

Introduction: Vitamin A deficiency can result from malnutrition, malabsorption of vitamin A, impaired vitamin metabolism associated with liver disease, or chronic debilitating diseases like HIV infection or cancer.

Background & aims: Cannabis administration has been described as a palliative symptom management therapy in such pathological stages. Therefore, this research aimed to study the effects of acute administration of cannabidiol (CBD) or tetrahydrocannabinol (THC) on the levels of retinol in plasma and in the liver, and biochemical parameters related to lipid and glucose metabolism (cholesterolaemia, triglyceridemia and glycemia) in a rat experimental model of vitamin A deficiency.

Methods: The experimental animal model of Vitamin A deficiency was developed during a 50-day experimental period in which rats consumed a vitamin A-free diet. Cannabidiol (10 mg/kg body weight) or tetrahydrocannabinol (5 mg/kg body weight) were administered intraperitoneally 2 hours prior to sacrifice of the animals.

Results: The nutritional deficiency caused a significant decrease in plasmatic and liver contents of retinol and biochemical parameters of glycemic, lipidic, and mineral metabolism. Acute intraperitoneal administration of Cannabidiol and tetrahydrocannabinol did not improve the indices of vitamin A status in either control or vitamin A-deficient rats. However, it had a significant effect on specific biochemical parameters such as glucose, triglycerides, and cholesterol.

Conclusion: Under our experimental conditions, the reported effects of cannabinoid administration on certain signs of nutritional vitamin A deficiency appeared to be mediated through mechanisms other than changes in retinol metabolism or its mobilization after the acute administration of such compounds.

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Key words: Vitamin A deficiency. Cannabinoids. CBD/THC. Retinol. Retinol binding protein. Lipid metabolism.

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CAMBIOS EN PARÁMETROS METABÓLICOS INDUCIDOS POR LA ADMINISTRACIÓN AGUDA DE CANABINOIDES (CBD, THC) EN UN MODELO EXPERIMENTAL DE RATA DEFICIENTE EN VITAMINA A INDUCIDO POR LA DIETA

Resumen

Introducción: La deficiencia en vitamina A está asociada a la malnutrición, malabsorción de este nutriente, metabolismo alterado de vitaminas por enfermedad hepática, o enfermedades crónicas debilitantes como VIH, cáncer o infección. La administración de cannabis ha sido descrita como una terapia eficaz en el tratamiento sintomático de determinadas manifestaciones de la deficiencia nutricional en vitamina A y de diversas enfermedades crónicas debilitantes.

Objetivos: El objetivo de este trabajo era estudiar el efecto de la administración de tetrahydrocannabinol (THC) y cannabidiol (CBD) sobre las concentraciones plasmáticas y hepáticas de retinol y sobre parámetros bioquímicos relacionados con el metabolismo glucídico y lipídico (coesterolemia, trigliceridemia, glucemia) en un modelo experimental de rata deficiente en vitamina A.

Métodos: El modelo experimental de deficiencia en vitamina A se desarrolló durante un periodo experimental de 50 días en los que las ratas consumieron una dieta libre en vitamina A. La administración de tetrahydrocannabinol (THC) (10 mg/kg peso corporal) y cannabidiol (CBD) (5 mg/kg peso corporal) se llevó a cabo por vía intraperitoneal 2 horas antes del sacrificio de los animales al final del periodo experimental.

Resultados: La deficiencia nutricional en vitamina A causó un descenso significativo en el contenido plasmático y hepático de retinol y en parámetros bioquímicos de metabolismo glucídico, lipídico y mineral. La administración intraperitoneal aguda de tetrahydrocannabinol y cannabidiol no mejoró los índices de estado nutricional de vitamina A en ratas deficientes o control. Sin embargo, tuvo un efecto significativo sobre parámetros bioquímicos específicos como la glucemia, coesterolemia y trigliceridemia.

Conclusión: Bajo nuestras condiciones experimentales, el efecto de la administración de cannabinoides sobre determinadas manifestaciones de la deficiencia en vitamina A parece estar mediada por mecanismos no relacionados con cambios en el metabolismo de retinol o su movilización tras la administración aguda de los compuestos cannabinoides ensayados.

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Palabras clave: Deficiencia en vitamina A. Cannabinoides. CBD/THC. Retinol. Proteína de unión a retinol. Metabolismo lipídico.

Abbreviations

VAD; Vitamin A deficiency.
CT; Control group.
CBD; Cannabidiol.
THC; Tetrahydrocannabinol.
GEC; Growth Efficiency Coefficient.
FTI; Food transformation index.
ADC; Apparent digestibility coefficient.
%R/A; Percent nitrogen retention/nitrogen absorption.
SEM; Standard error of the mean.

Introduction

Vitamin A is an essential micronutrient for normal bone growth, reproduction, embryonic development,¹ hematopoiesis, maintenance of the immune system,² and the differentiation and proliferation of epithelial cells.³ Hypo- and hypervitaminosis A have been known to affect many of the physiological processes in the cell. Vitamin A deficiency (VAD) can result from malnutrition, malabsorption of vitamin A, or impaired vitamin metabolism associated with liver disease. In addition, VAD is widely prevalent in debilitating diseases like HIV infections or cancer in what is known as cancer cachexia syndrome.⁴ Furthermore, VAD has been associated to more active infections, acute phase response and increased mortality of HIV patients.^{5,6} VAD is among the three main micronutrient deficiencies worldwide, together with those of Fe and I. According to UNICEF, an estimated 253 million preschool age children suffer a subclinical deficiency of this vitamin.⁷ This increases their susceptibility to infections and plays an essential role in the evolution and prognosis of infectious diseases. However, the problem is not exclusive to developing countries; in Spain, more than a third of the population consumes diets with low vitamin A content. It has been reported that 43% of men and 37% of women consume vitamin A deficient diets,⁸ although such relatively low dietary estimations do not match with serum concentrations of retinol that are within the range of adequate values reported for a healthy population.^{9,10} Strategies for the treatment of VAD status may involve the dietary supplementation of vitamin A, as well as alternative strategies that may be used together with dietary supplementation in circumstances of shortage or poor health status in chronic patients that restrict the access to dietary supplements and make the use of symptom-related therapies needed. In this regard, the administration of natural products such as cannabis and its derivatives is of great interest due to its accessibility in geographical areas where VAD is endemic and they have been successfully used to treat some of its symptoms like night blindness,¹¹ or else in chronic debilitating states where their use is acquiring special relevance to treat some of the symptoms derived from the

illness per se or from its pharmacological treatment. Cannabinoids belong to the chemical class of terpenophenolics, and include the psychoactive cannabinoid, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), as well as other non psychoactive components such as cannabidiol (CBD). These compounds act on two types of receptors: the CB1 receptor is found in the brain and peripheral tissues,^{12,13,14} while the CB2 receptor is primarily found outside the central nervous system, in tissues associated with immune function.^{15,16} Furthermore, recent studies demonstrate the presence of CB2 receptors in the central nervous system (cerebellum, brainstem, spinal nucleus, hippocampus, olfactory tubercle, cerebral cortex, amygdala, striatum, and thalamic nuclei) under basal conditions.^{17,18,19} Based on all these data, CB2 receptors emerge as an element that is likely to be involved in neuroprotection and could be a potential therapeutic target for the treatment of neurodegenerative disorders.²⁰ Cannabis and its derivatives can be excellent symptom management alternatives in HIV infection, cancer, and their treatments, since they exhibit promising beneficial orexigenic and anorexic effects on appetite,²¹ muscle pain, nausea, anxiety, nerve pain, and depression.^{22,23,24} However, no information is available with regard to their influence on retinol status or lipid or glucose metabolism, which may be negatively affected by nutritional VAD and chronic illness where VAD is widespread. Moreover, such potential effects could differ after acute or long-term drug administration. In order to study the mechanisms and potential benefits of cannabinoid administration on vitamin A deficiency, an experimental model of such nutritional deficiency must be developed in which the effects of acute administration of the test compounds studied have been sufficiently validated. Therefore, the aims of this study were: 1) To study how nutritional vitamin A deficiency could affect the plasma and hepatic content of well known markers of vitamin A status like retinol and retinol binding protein (RBP), markers of lipid and glucose metabolism like plasma total-cholesterol, triglycerides or glucose, markers of mineral metabolism like plasma Ca, P, or Mg content, as well as the nutritive utilization of protein and phosphorus; 2) To test whether the acute intraperitoneal administration of cannabinoids THC and CBD could affect some of the above mentioned parameters and compensate the effects exerted by VAD on lipid and glycemic metabolism.

Materials and methods

Drugs

Δ^9 -Tetrahydrocannabinol (THC) was purchased from Lipomed AG (Switzerland). 2-[(1R, 6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol (Cannabidiol, [CBD]) was purchased from Tocris Bioscience (UK). Drugs were dissolved in

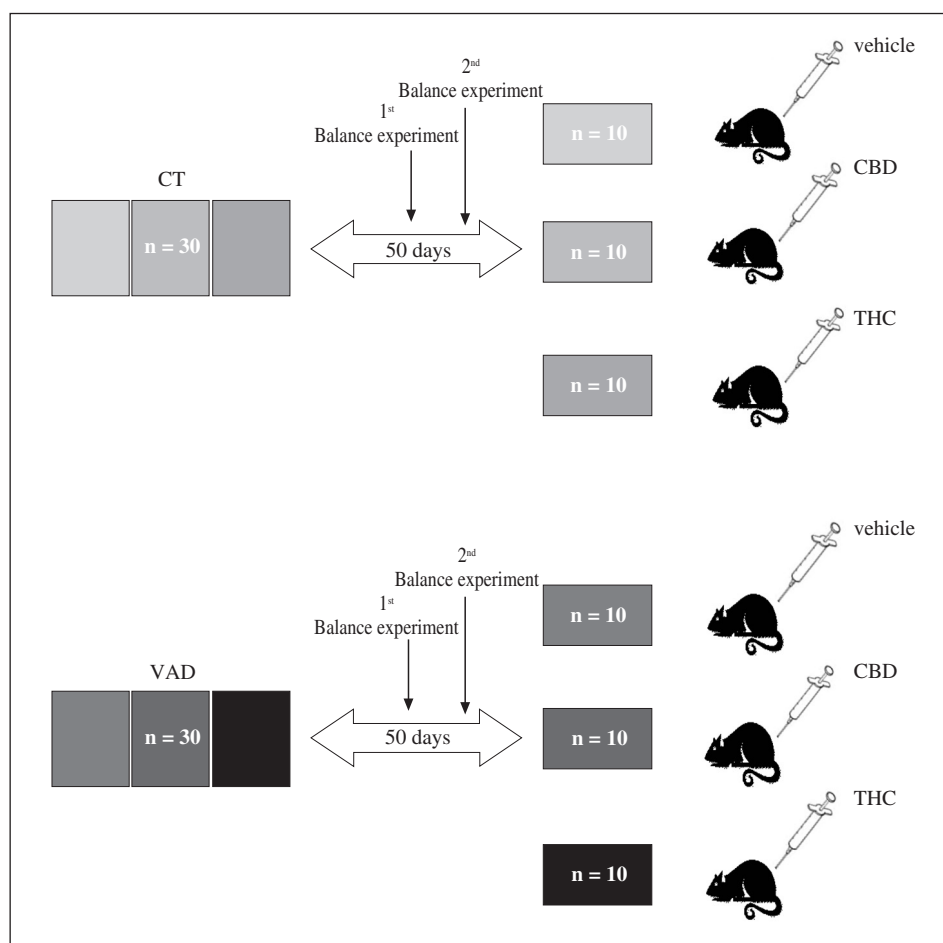


Fig. 1.—Experimental design.

ethanol: tween-80: 1% methyl cellulose: 0.9% NaCl (1p:2p:2p:40p).

Animals and experimental design

60 male Wistar rats with an average initial body weight of 56 ± 0.6 g were allocated in two experimental groups (n = 30) (fig. 1). One group consumed a vitamin A-deficient diet, whereas the other group was fed a control diet that supplied enough quantities of vitamin A to meet the nutrient requirements of a growing rat.²⁵ The experiment lasted for 50 days during which animals were housed in individual stainless-steel metabolic cages designed for the separate collection of feces and urine. The cages were located in a well-ventilated thermostatically controlled room ($21 \pm 2^\circ$ C), with relative humidity ranging from 40 to 60% and 12 h light/dark cycle.

Throughout the experimental period all rats had free access to double-distilled water and consumed the two different diets *ad libitum*. Trying to adapt protein content of the diet to the nutrient requirements of the laboratory rat,²⁵ two different protein levels were used along the experimental period. The animals consumed 15% protein diet level until an average body weight of

150 grams was reached. From that point ahead, a 12% protein level was used. The final body weight of the animals averaged 291 ± 7.0 and 223 ± 7.4 grams in Control and Vitamin A-deficient experimental groups, respectively. Food intake was recorded daily and body weight was measured every four days from the first week of experimental period. On weeks 5 (days 33-37) and 7 (days 45-49) of the experimental period, 2 balance experiments were carried out during which feces and urinary output were collected daily and separately for each rat and frozen at -20° C. The frozen rat feces were freeze-dried, weighed and ground for analysis of protein.

On day 28 of the experimental period, blood samples (0.5 mL) were taken from the tail vein for retinol analysis. After completion of the 50-days feeding experiment, the rats were deprived of food for 12 h, and 10 different animals of each experimental group were injected intraperitoneally with vehicle (ethanol: tween-80: 1% methyl cellulose: 0.9% NaCl [1p:2p:2p:40p]), THC (5 mg/kg body weight) or CBD (10 mg/kg body weight), respectively (fig. 1), two hours prior to being sacrificed. The doses administered were selected based on a careful review of the literature regarding the medicinal use of cannabinoids on several different experi-

mental rodent models.^{26,27} Sacrifice took place at intervals of 15 min by CO₂ inhalation. Blood was collected (with heparin as anticoagulant) and centrifuged at 1,500 × g for 15 min to separate plasma that was frozen in liquid N₂ and stored at -80° C. Liver was extracted, weighed, and immediately frozen in liquid N₂ and stored at -80° C. Femurs, brain, kidneys, testes, and spleen were extracted and stored at -20° C. To minimize the photoisomerization of vitamin A, plasma and liver samples were taken under reduced yellow light. All experiments were undertaken according to Directional Guides Related to Animal Housing and Care²⁸ and all procedures were approved by the Animal Experimentation Ethics Committee of the University of Granada.

Experimental diets

All diets were formulated to meet nutrient requirements of rats following the recommendations of the American Institute of Nutrition,²⁹ with slight modifications. AIN-93G diets were mixed using Vitamin A-free casein prepared after incubation of the protein powder at 105° C for one week as the sole source of protein in both Control and Vitamin A-deficient diets. AIN-93 vitamin mix with or without vitamin A (retinol palmitate 500,000 UI/g) was used in the preparation of the control and vitamin A-deficient diets, and peanut oil was used as a source of vitamin A-free dietary fat. The composition of the semisynthetic diet per 100 g of dry matter was: 17.1 or 13.6 g vitamin A-free casein for a 15% or 12% protein level, respectively, 0.5 g methionine, 10 g sucrose, 65 g wheat starch, 5 g cellulose, 7 g peanut oil, 3.5 g mineral mix (AIN-93G-MX), 1 g vitamin mix (AIN-93G-VX) with or without vitamin A (Control or vitamin A-deficient diet, respectively), and 0.25 g choline bitartrate.

Chemical analysis of diets, feces, urine and tissues

Moisture content was determined by drying to constant weight in an oven at 105 ± 1° C. Ash was measured by calcination at 500° C to a constant weight. Total nitrogen was determined according to Kjeldahl's method. Crude protein was calculated as N × 6.25. Total phosphorus was measured spectrophotometrically using the technique described by Chen et al.³⁰. Analytical results were validated by standard references CRM-383 (Harricot beans), and CRM-709 (Pig feed) (Community Bureau of Reference, Commission of the European Communities). Mean ± SEM of five independent values for N, ash and P were as follows: N, CRM-383 = 1.03 ± 0.01% vs certified value of 1.05 ± 0.02%, Ash, CRM-383 = 2.48 ± 0.006% vs certified value of 2.4 ± 0.1%; Protein, CRM-709 = 196.6 ± 3.2 g/kg vs certified value of 199 ± 5 g/kg, Ash, CRM-709 = 4.29 ± 0.03% vs certified value of 4.2 ± 0.4%, P,

CRM-709 = 5.42 ± 0.006 mg/g vs certified value of 5.4 ± 0.7 mg/g.

The concentration of Ca, Mg, glucose, triglycerides, total- and HDL-cholesterol in plasma samples was measured using analytical kits designed for colorimetric measurement of the above mentioned parameters (Spin-react, S.A., Girona, Spain). Quantification of blood parameters was done using a KX-21 Automated Hematology Analyzer (Sysmex Corporation, Kobe, Japan).

Plasma and liver retinol analyses

Plasma and liver retinol concentration was measured by reversed phase HPLC. Retinol was extracted from plasma (200 µL) with a mixture of absolute ethanol containing 0.01% butylated hydroxytoluene (BHT) (200 µL) and hexane (2,000 µL). Liver aliquots (1 g) were carefully minced on a refrigerated plate and homogenized in 2000 µL of ethanol containing 0.01% BHT with the use of a sonicator. The liver homogenate was saponified in 1 mL of 11N KOH for 40 min at 60° C and extracted with 2,000 µL hexane for analysis. 2 mL of the organic phase extracted from plasma or liver were dried under a stream of N and redissolved in either 500 µL of methanol in case of plasma or 500 µL of methanol: chloroform (4:1, v/v) in case of liver. The samples were filtered prior to being analyzed by HPLC.

HPLC analysis

The analytical HPLC system consisted of a pump (model LC6A, Shimadzu, Japan), analytical reverse phase C-18 column (150 × 4.6 mm) and fluorimetric detector (EX = 330 nm, EM = 480 nm).³¹ The chromatograms were collected, stored and processed with a computerized integrator (model Shimadzu, CR4A, Japan). Retinol was used as external standard with concentrations ranging from 0.25 to 1 µg/mL. In that range of concentrations, the response of the detector proved to be linear, the height and the surface of the peaks being proportional to the concentration of compound injected. Chromatography was performed at 35° C, and the optimal composition of the mobile phase was: methanol-H₂O (95:5, v/v). Freshly prepared mobile phase was filtered (0.22 µM, Millipore, USA) and degassed under vacuum (Branson 2200, UK) for 10 min. The flow rate was 1 mL/min, and 20 µL of each sample were injected.

Plasma retinol binding protein

Analysis was performed by ELISA using a dual mouse/rat commercial kit (ALPCO diagnostics, Salem, NH, USA) for quantitative detection of RBP4 in mouse or rat serum.

Biological Indices

The following indices and parameters were determined for each group according to the formulas given below: Growth Efficiency Coefficient (GEC; weight gain in grams per day/protein intake in grams per day); food transformation index (FTI; total intake in grams of dry matter per day/increase in body weight in grams per day); apparent digestibility coefficient (ADC) (i) for nitrogen; nitrogen retention (balance) (ii), and percent nitrogen retention/ nitrogen absorption (%R/A) (iii):

$$\text{ADC} = [(I - F) / I] \times 100 \quad (\text{i})$$

$$\text{Balance} = I - (F + U) \quad (\text{ii})$$

$$\% \text{ R/A} = \{ [I - (F + U)] / (I - F) \} \times 100 \quad (\text{iii})$$

Where I = Intake, F = Fecal excretion, and U = Urinary excretion.

Statistical analysis

The Statistical analysis was applied with the use of SAS, version 8.02 (SAS, 1999). Results are given as mean values and standard errors of the mean. Time-repeated measurement analysis was applied to food intake and weight gain data in order to analyze within subject effects (time) or within group effects (dietary treatment) on the above mentioned parameters. The effect of dietary treatment and developmental stage of experimental period on the nutritive utilization of protein was analyzed by 2×2 factorial ANOVA with dietary treatment and developmental stage of the experimental period as the main treatments. The effect of dietary treatment and drug administration on retinol concentration in liver, levels of P, Ca, Mg, glucose, triglycerides, total- and HDL-cholesterol, retinol, and RBP in plasma, weight of different tissues, and hematic parameters, was analyzed by 2×3 factorial ANOVA with dietary treatment and drug administration as the main treatments. Tukey's test was used to detect differences between treatment means. The level of significance was set at $P < 0.05$.

Results

Development of the experimental VAD model, food intake and body weight gain

The changes observed in daily food intake and body weight gain of control (CT) and vitamin A-deficient (VADt) rats (4 weeks old with an average body weight of 56 ± 0.6 grams at the start of the experiment) during the 50-day experimental period are presented in figures 2A and 2B. Time-repeated measurement analysis reveals a significant time effect, dietary treatment effect, and time \times treatment interaction on daily food

intake. The effect of VAD on the above parameters was clearly appreciable from day 33 of the experimental period. From this point, daily food intake (in grams per day) remained stable in the control group, whereas it decreased steadily in the VADt group until the end of the experiment (day 50). Daily weight gain was also significantly affected by time and dietary treatment, with clear differences between the two experimental groups being observed from day 28.

The effects of VAD on daily food intake and body weight gain were matched by significantly lower levels of plasmatic and hepatic retinol, compared to control rats, after the 50-day experimental period (tables I and II), which corresponded to the end of the second balance experiment. Such retinol levels satisfied the established criteria for vitamin A deficiency used in the present study that corresponded to plasma and liver retinol concentrations below 50% of the non-deficient experimental group. However, no significant differences in plasma retinol concentrations were observed after 28 days (0.25 ± 0.02 and 0.23 ± 0.03 $\mu\text{g/mL}$ for CT and VAD, respectively); at this time-point, differences in body weight gain resulting from the dietary treatment were already noticeable.

Digestive and metabolic utilization of protein

The daily food intake of the animals fed the control diet was similar during the two balance experiments (table I), and significantly higher than that of the animals fed the VAD diet. Furthermore, the daily food intake in the latter group was lower during the second balance experiment than in the first one ($P < 0.05$). The body weight gain and growth efficiency indices of the animals that consumed the control diet were superior to that of the VADt animals during both balance experiments. Moreover, in the latter experimental group, body weight gain was null and even some weight loss was observed during the second balance experiment.

Digestive utilization of N, expressed as Apparent Digestibility Coefficient (ADC), was high and similar in both experimental groups during the first balance experiment (days 33-37) (table I). Nevertheless, the net absorption of N was higher in the control animals due to their higher daily intake of this nutrient. On the other hand, during the second balance experiment (days 45-49), a significant reduction in N digestibility was observed in the VADt animals compared to the control group and compared to the values obtained during the first balance experiment. This lower digestibility can be attributed to a higher level of fecal N excretion associated with a significantly lower N intake by the VADt animals during the second balance experiment, and resulted in a lesser amount of N being absorbed by this experimental group ($P < 0.05$).

The metabolic utilization of N, expressed as %R/A, underwent a considerable reduction during the second balance experiment compared to the first one, in both

Table I
Influence of vitamin A deficiency and developmental stage of the experimental period on the nutritive utilization of protein*

	Food intake (g/d)	Body weight gain (g/d)	N intake (mg/d)	Fecal N (mg/d)	Urinary N (mg/d)	Absorbed N (mg/d)	ADC (%)	Balance (mg/d)	R/A (%)	GEC	FTI
CT Period 1 (33 d-37 d)	19.6 ^a	4.87 ^a	398.2 ^a	34.8 ^a	112.5 ^a	363.4 ^a	91.3 ^a	250.9 ^a	69.0 ^a	1.96 ^a	4.06 ^a
CT Period 2 (45 d-49 d)	18.7 ^a	2.19 ^b	374.2 ^{ab}	36.1 ^a	143.7 ^b	338.1 ^{ab}	90.3 ^a	194.3 ^b	57.1 ^b	0.94 ^b	9.22 ^a
VAD Period 1 (33 d-37 d)	17.2 ^b	3.77 ^a	338.8 ^b	31.3 ^a	126.7 ^{ab}	307.5 ^b	90.8 ^a	180.8 ^b	58.8 ^b	1.78 ^a	4.71 ^a
VAD Period 2 (45 d-49 d)	13.4 ^c	-1.11 ^c	265.7 ^c	57.7 ^b	138.8 ^b	208.0 ^c	78.3 ^b	69.2 ^c	33.1 ^c	–	–
SEM	0.48	0.41	9.56	2.30	6.39	8.64	0.72	8.69	2.57	0.2278	22.3
Diet Effect	P<0.0001	P<0.0001	P<0.0001	P=0.0003	P=0.2559	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P=0.0001	P=0.3233
Period Effect	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P=0.0038	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P=0.2619
Diet × Period Interaction	P=0.0040	P=0.0106	P=0.0151	P<0.0001	P=0.1457	P=0.0001	P<0.0001	P=0.0032	P=0.0117	P=0.0036	P=0.3080

*Results are Means of 10 independent animals. SEM, pooled standard error of the mean.

^{ab,c}Different superscripts within the same column indicate significant differences (P<0.05).

Table II
Effect of vitamin A deficiency and cannabinoids administration after a 50-day experimental period on RBP levels of plasma and retinol content of plasma and liver*

	CT		VAD		SEM	Diet Effect	Drug Effect	Diet × Drug Interaction
	Vehicle	CBD	THC	THC				
Plasma RBP (µg/mL)	5.41 ^a	9.31 ^b	6.07 ^a	1.53 ^c	1.13 ^c	1.34 ^c	0.79	P<0.0001 P=0.0809 P=0.0143
Plasma retinol (µg/mL)	0.11 ^a	0.12 ^a	0.08 ^b	0.002 ^c	0.002 ^c	0.003 ^c	0.005	P<0.0001 P=0.0015 P=0.0006
Liver retinol (nmol/g)	0.96 ^b	1.99 ^c	1.10 ^{bc}	0.01 ^a	0.02 ^a	0.02 ^a	0.22	P<0.0001 P=0.0503 P=0.0536

*Results (expressed in fresh sample) are Means of 10 independent animals. SEM, pooled standard error of the mean.

^{ab,c}Different superscripts within the same row indicate significant differences (P<0.05).

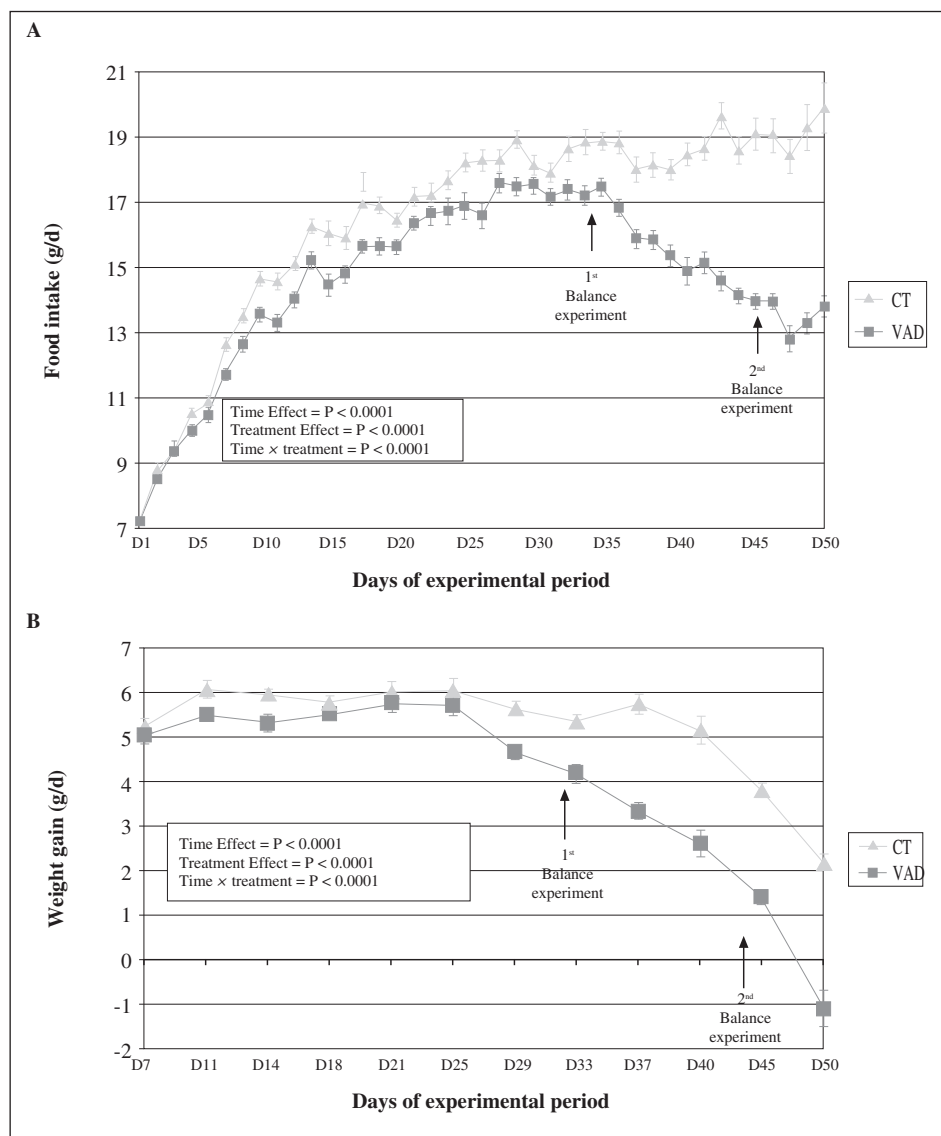


Fig. 2.—Effect of vitamin A deficiency on daily food intake by rats over a 50-day experimental period (grams/day). The vertical arrow indicates the start of first and second balance experiment. B) Effect of vitamin A deficiency on daily weight gain of rats over a 50-day experimental period (grams/day). The vertical arrows indicate the start of first and second balance experiment.

experimental groups. In addition, VAD contributed to a further decrease in this metabolic index compared to the control group in both balance experiments.

Effect of vitamin A deficiency on P metabolism

The VADt animals exhibited a lower dietary intake and urinary excretion of P compared to the control rats during the second balance experiment, a finding that reflects the lower daily food intake by the former experimental group during this period. Due to the lower amount of P ingested by the VADt animals (51.8 ± 1.56 vs 37.1 ± 0.85 mg/dL in CT and VADt animals, respectively), their plasmatic levels of this mineral were significantly lower than in the control animals (8.44 ± 0.17 vs 6.81 ± 0.23 mg/dL in CT and VADt animals, respectively). However, no significant differences were apparent in femur P (104.8 ± 0.76 vs 100.5

± 1.29 mg/g DM in the CT and VADt animals, respectively).

Despite lower plasmatic levels of P in the VADt animals, the nutritive utilization of this mineral did not seem to be affected under our experimental conditions by the nutritional deficiency, given that the relationship between urinary excretion and daily food intake of the mineral was similar in both experimental groups (11.6 ± 1.10 vs $11.3 \pm 0.97\%$ in CT and VADt animals, respectively).

Influence of vitamin A deficiency and cannabinoid administration on plasmatic retinol and RBP and hepatic retinol levels

VAD caused a significant decrease in retinol content in plasma and the liver, which was associated with a concomitant decrease in plasma RBP levels (table II),

whereas no effect on the above mentioned plasmatic parameters was observed after the intraperitoneal administration of THC or CBD. In contrast, the intraperitoneal administration of CBD to the control animals led to a considerable increase in plasmatic RBP and hepatic retinol levels, whereas similar plasmatic levels of RBP were found after vehicle or THC administration.

Effect of vitamin A deficiency and cannabinoid administration on biochemical parameters

VAD had a significant effect on several biochemical parameters, causing a considerable decrease ($P < 0.05$) in glucose, total- and HDL-cholesterol content of plasma, as well as significantly increasing the levels of triglycerides in vehicle-administered animals. These findings were reflected in a significant diet effect on the above mentioned parameters (table III). Upon administration of CBD, glycaemia was not affected, whereas the content of HDL-cholesterol in plasma was significantly reduced in comparison with vehicle-injected animals in both the CT and VADt experimental groups. Plasmatic T-cholesterol was only significantly reduced in the VADt animals. Plasma triglycerides were not affected by CBD administration in the control rats, whereas a significant reduction was observed in the VADt experimental group. Intraperitoneal administration of THC led to a significant increase in glycaemia, and a significant decrease in the plasmatic levels of total- and HDL-cholesterol in both CT and VADt rats. In contrast, plasma triglycerides were significantly increased by THC administration in control rats; a finding that was not observed in the VADt group.

Plasmatic levels of Ca, P and Mg were significantly affected by the dietary treatment, and lower amounts of these minerals in plasma were associated with consumption of the VADt diet. The effect of cannabinoid administration varied among the different minerals studied, but tended to decrease their plasmatic levels in comparison with vehicle administration.

Effect of vitamin A deficiency and cannabinoid administration on hematic parameters

VAD led to a significant increase in leukocyte and erythrocyte counts, hemoglobin content and hematocrit of vehicle-injected animals by the end of the 50-day experimental period, but did not affect MCV, MCH or MCHC (Table III). Intraperitoneal administration of CBD or THC did not cause any significant modification in the different hematic parameters studied with respect to erythrocyte metabolism in comparison with vehicle administration in either CT or VADt animals. On the other hand, the administration of CBD caused a significant decrease in leukocyte count of the VADt animals without modifying this

parameter in the control group. The intraperitoneal administration of THC led to a significant decrease in leukocyte count in both CT and VADt animals.

Discussion

Validity of the experimental model

Under our experimental conditions, the validity of the VAD model is demonstrated by the significant reduction in retinol content in the liver and plasma, the significant reduction in plasmatic RBP levels, and changes in food intake, weight gain, biochemical and hematic parameters. These results are in agreement with those reported in several other studies.^{32,33,34,35} Furthermore, and in accordance with our results, Barber et al.³⁶ located the start of VAD-derived changes in food intake and weight gain at 32-34 days of consumption of a VADt diet, whereas Carney et al.³⁷ did not find any modification in the above mentioned parameters as a result of VADt consumption for 28-30 days.

Changes in protein metabolism and hepatic urea cycle enzymes have been reported following VAD, although such changes were not reflected in any significant alteration in the nutritive utilization of protein.^{38,39} In this regard, we sought to clarify the relationship between alterations in nutritive utilization of protein and developmental stage of vitamin A deficiency. Therefore, two different balance experiments were planned. The first one corresponded with the start of VAD (days 33-37 of the experimental period), whereas the second corresponded with the final stages of our experimental period (days 45-49) in which VAD was completely developed. In the latter period, the N balance of the VADt animals was so low that protein malnutrition status would have been reached if the nutritional VAD had continued.

Regarding P metabolism, Grases et al.⁴⁰ described an increase in urinary P excretion and alterations in renal histology as a result of VAD, although no data were presented by those authors concerning the amount of P ingested by the CT or VADt animals. Under our experimental conditions, the lower indices of P status represented by plasmatic P were more closely related to a lower dietary intake of this mineral than to any alterations in its metabolism.

Taken together, our data seem to show that under the experimental conditions described, the reduction in body weight suffered by VADt animals could be a consequence of their lower food intake associated with impaired bioavailability of certain nutrients such as protein.

Influence of vitamin A deficiency and cannabinoid administration on retinol status

As reported by other authors,^{32,41} VAD caused the depletion of retinol stores from the liver and a significant

Table III
Effect of vitamin A deficiency and cannabinoids administration on different biochemical and hematic parameters*

	CT		VAD		SEM	Diet Effect	Drug Effect	Diet × Drug Interaction
	Vehicle	CBD	THC	THC				
Biochemical parameters (plasma)								
Triglycerides (mg/dL)	44.8 ^a	46.0 ^a	209.3 ^b	75.6 ^c	7.27	P < 0.0001	P < 0.0001	P < 0.0001
T-Cholesterol (mg/dL)	83.8 ^a	69.4 ^{ab}	72.9 ^{ac}	67.7 ^{bc}	3.59	P < 0.0001	P < 0.0001	P = 0.6340
HDL-Cholesterol (mg/dL)	42.7 ^a	10.5 ^b	34.1 ^c	14.3 ^b	1.10	P < 0.0001	P < 0.0001	P < 0.0001
Glucose (mg/dL)	103.0 ^a	112.4 ^a	136.9 ^b	73.8 ^c	4.14	P < 0.0001	P < 0.0001	P = 0.8580
P (mg/dL)	8.3 ^{ab}	8.4 ^a	7.3 ^{bc}	7.7 ^{ab}	0.25	P = 0.0007	P = 0.0277	P = 0.0063
Ca (mg/dL)	9.8 ^a	8.6 ^b	8.0 ^b	8.3 ^b	0.24	P < 0.0001	P < 0.0001	P = 0.0542
Mg (mg/dL)	2.1 ^a	1.7 ^{bc}	2.1 ^a	1.8 ^{bc}	0.06	P = 0.0021	P < 0.0001	P = 0.0468
Hematic parameters								
RBC (× 10 ⁶ /μL)	7.5 ^a	7.7 ^a	8.0 ^{ab}	8.6 ^b	0.17	P < 0.001	P = 0.5849	P = 0.1995
HGB (g/dL)	13.7 ^a	13.8 ^a	14.4 ^{ab}	15.7 ^c	0.26	P < 0.001	P = 0.5799	P = 0.0254
HCT (%)	41.9 ^a	42.7 ^a	44.9 ^{ab}	47.5 ^b	0.82	P < 0.001	P = 0.410	P = 0.0483
MCV (fL)	55.8 ^a	55.4 ^a	56.3 ^a	55.1 ^a	0.44	P = 0.0244	P = 0.9615	P = 0.2703
MCH (pg)	18.3 ^a	17.9 ^a	18.1 ^a	18.3 ^a	0.21	P = 0.4219	P = 0.1017	P = 0.4376
MCHC (g/dL)	32.7 ^a	32.3 ^a	32.1 ^a	33.1 ^a	0.22	P = 0.1912	P = 0.0073	P = 0.7839
WBC (× 10 ³ /μL)	6.4 ^{ab}	7.3 ^b	4.3 ^c	12.9 ^d	0.45	P < 0.0001	P < 0.0001	P < 0.0001

*Results are Means of 10 independent animals. SEM, pooled standard error of the mean.

^{ab,cd} Different superscripts within the same row indicate significant differences (P<0.05).

drop in plasma retinol. Such depletion was evident at the end of second balance experiment, and probably had been initiated by the end of the first one, given that other manifestations of VAD such as lower food intake or weight gain were taking place at that time. Although RBP synthesis is not diminished by VAD, this transport protein accumulates in the liver under conditions of poor retinol status,³³ and thus its levels in plasma are then dramatically reduced, as was the case with our VADt animals. As a matter of fact, our findings related to the mobilization of RBP as a result of CBD administration in CT rats could illustrate a potential interaction between the liver metabolism of this transport protein and the peripheral cannabinoid receptors present in the liver.⁴² Nevertheless, this interaction was only observable in CT and not in VADt animals. On the other hand, and independently of the specific drug administration, higher RBP levels in plasma were closely related to higher levels of retinol in the liver among the control rats.

Biochemical parameters

Kang et al.⁴³ have reported the development of altered lipid catabolism in the VADt liver. These authors found a decrease in the expression of genes encoding enzymes of mitochondrial fatty acid oxidation, together with increased hepatic microcytic lipid accumulation and triglyceride levels. The nuclear receptor genes PPAR and PPAR were down regulated in the VADt liver, whereas leptin receptor gene expression was induced. An alteration in lipid metabolism is a possible explanation for the significant changes in the levels of plasma triglycerides, total- and HDL-cholesterol, and glucose found in rats fed the VADt diet under our experimental conditions. With decreased lipid oxidation, fuel partitioning may shift to glucose catabolism as seen by alteration in the expression of certain glucose transporters and pyruvate dehydrogenase kinase 4 in the VADt liver.⁴⁴

Plasma levels of lipids were also affected by cannabinoid administration in both CT and VADt animals under our experimental conditions. This effect could be mediated by the PPAR nuclear receptor, which plays an important role in regulating lipid metabolism and glucose homeostasis.⁴⁴ The CB₁ receptor is also involved in adipocyte differentiation, stimulated lipogenesis, and reduced adiponectin expression.^{43,45}

Hematology

The increase in red blood cell count, hemoglobin, and hematocrit of the VADt rats is in agreement with the findings of other authors^{35,46,47,48} who attribute such hemoconcentration to an effect of VAD on rat growth and the volume of fluid compartment of blood rather to any direct effect on hematopoiesis. On the other hand, no effect of VAD was observed concerning red blood cell volume or hemoglobin content, thus excluding the possibility of

severe alterations in Fe metabolism at this stage, although VAD has been commonly associated with altered Fe status.^{35,46,47,48} With regard to the increased leukocyte count, a possible role of hemoconcentration caused by VAD cannot be ruled out. However, under our experimental conditions, a cannabinoid-derived effect was also evident and this was more pronounced in the VADt than in the control animals. Bouaboula et al.⁴⁹ have reported the presence of cannabinoid receptors in leukocytes, although we are not aware of the potential mechanisms through which cannabinoid administration may have affected leukocyte count.

In conclusion, the 50-day experimental period of VAD depleted the hepatic retinol stores, caused a significant decrease in plasma retinol and retinol binding protein (RBP), and affected specific markers of lipid and glucose metabolism. Acute intraperitoneal administration of cannabinoids did not induce any significant modification of retinol content in plasma and liver of VADt animals. However, it did significantly affect the plasmatic levels of cholesterol and triglycerides in both control and VADt rats.

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