Open Access Research Journal of
Life Sciences
Journals home page: https://oarjpublication/journals/oarjls/
ISSN: 2783-025X (Online)

OARJ OPEN ACCESS RESEARCH JOURNALS

(RESEARCH ARTICLE)

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Antioxidant effects of the phytocannabinoid cannabidiol (CBD) in the brain of chlorpyrifos-exposed goldfish (*Carassius auratus*)

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Open Access Research Journal of Life Sciences, 2023, 06(01), 008–016

Publication history: Received on 12 May 2023; revised on 01 July 2023; accepted on 04 July 2023

Article DOI: https://doi.org/10.53022/oarjls.2023.6.1.0044

Abstract

Pharmacological and therapeutic properties of phytocannabinoids present in *Cannabis sativa* L. (Linnaeus) are of increasing interest worldwide. In the present study, a group of goldfish (*Carassius auratus*) were used to test the antioxidant effects of cannabidiol (CBD) in the brain of *in vivo* chlorpyrifos (CPF)-exposed fish. CPF, an organophosphate insecticide, is known for its pro-oxidant effects and irreversible inhibition on cholinesterase (ChE) enzymes. The experimental fish (n=22) (22.86 ± 1.15 g body weight, 8.33 ± 0.16 cm total length) were randomly distributed in 4 different treatments: A. Control fish (n=6), B. CPF-exposed fish (n=5), C. CPF-exposed + IP injected vehicle (n=5), and D. CPF-exposed + IP-injected CBD (5 mg/Kg) (n=6). Fish were humanely sacrificed after 96 h of the experimental time and brains were processed to measure F_2-isoprostanes (F_2-IsoPs) as a biomarker of oxidative stress response (ELISA OxiSelect™ 8-iso-PGF2α). ChE activity was measured in fish blood plasma as a biomarker of CPF exposure. Mean values of F_2-IsoPs and ChE were used as statistics for comparison among treatments. Neither of the fish treatments showed signs of acute poisoning after the CPF exposure. However, plasma ChE activity showed inhibition effects in the corresponding groups after the 96-h exposure to CPF. The lowest F_2-IsoPs value (mean ± SE, pg/ml) was found in the CPF-exposed + CBD-injected fish (281 ± 45) followed by the controls (396 ± 90). The two highest values of F_2-isoprostanes were present in the CPF-exposed (728 ± 236) and the CPF-exposed + vehicle-injected fish (566 ± 90) (ANOVA, 95%, $p < 0.05$; Fisher least significant difference). Three types of functional groups within the CBD structure are considered a key factor to explain the prevention of oxidative stress: limonene, phenol and aliphatic group. These groups, particularly the phenol, transfer electrons and hydrogen atoms to avoid the lipid peroxidation in the tissues affected by pro-oxidant agents. Results in the present work showed that CBD protected of the oxidative stress caused by CPF in the brain of goldfish. This neuroprotective action of CBD against oxidative stress promotes further research to explore pharmacological and therapeutic applications.

Keywords: Cannabidiol; Neuroprotection; Oxidative Stress; Isoprostanes; Fish

1 Introduction

Oxidative stress is a disturbance in the balance between reactive oxygen species (ROS) formation and cellular antioxidant capacity [1, 2] Overproduction of free radicals can cause oxidative damage to biomolecules (lipids, proteins, DNA) that leads to various pathological conditions, such as neurodegenerative diseases. The brain is particularly vulnerable to the effects of ROS because of its high oxygen demand, its high concentration of polyunsaturated fatty acids prone to peroxidation and its rather low endogenous antioxidant defense and DNA repair systems. Previous studies have demonstrated that oxidative stress plays a central role in the pathophysiology of neurodegenerative conditions such as Alzheimer's, Parkinson's, Huntington's and amyotrophic lateral sclerosis; therefore, antioxidant therapy has been suggested for the prevention and adjuvant treatment of these conditions [3–8].

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Cannabidiol (CBD; IUPAC name: 2-[(1R,6R)- 3-methyl-6-prop-1-en-2-ylcyclohex-2-en-1-yl]-5- pentylbenzene-1,3-diol, $C_{21}H_{30}O_2$) (Figure 1), is the major non-psychoactive cannabinoid component among 150+ phytocannabinoids derived from *Cannabis sativa* L. [9, 10]. It is a 21-carbon terpenophenolic compound which is formed following decarboxylation from a cannabidiolic acid precursor [10, 11].

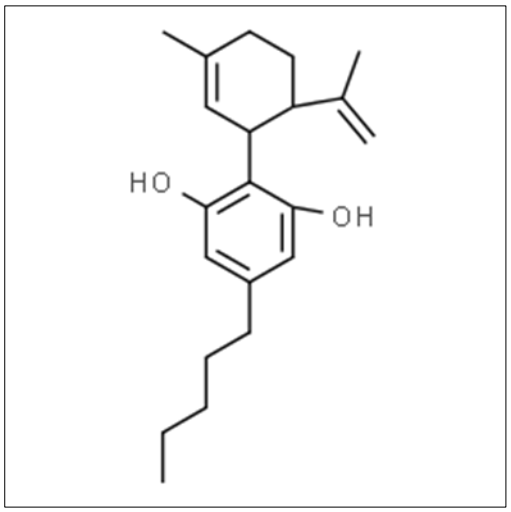

Figure 1 Chemical structure of cannabidiol (CBD) (ChemSpider ™)

Previous reports showed that CBD may have therapeutic benefits. In fact, this compound has proven to have antinflammatory [12–16], neuroprotective [17–29], anxiolytic [20, 30], analgesic [31], antidepressant [32], hepatoprotective [33] and immunomodulator effects [34-36]. In the following years, CBD has been studied to determine its antioxidant efficacy in different quantum chemical and mechanical methods [11], *in vitro* models of neurodegenerative diseases [22, 37-42], and a few *in vivo* models in CNS with promising results [33, 43-45].

However, to the best of our knowledge, the neuroprotective antioxidant effect of CBD against chlorpyrifos effects in the central nervous system (CNS), using F_2-isoprostanes (F_2-IsoPs) as biomarker, was not yet investigated. Hence, we have conducted the present study to investigate the potential neuroprotective effect of CBD acting as an antioxidant molecule in goldfish (*Carassius auratus*) exposed to chlorpyrifos as a pro-oxidant agent.

2 Material and methods

2.1 Animals

Sexually mature female and male goldfish (*Carassius auratus*, n=22, mean body weight 22.86 ± 1.15 g and mean body length 8.33 ± 0.16 cm) were obtained from a commercial farm in Bogotá, Colombia. Fish were acclimated for three weeks in the Aquatic Toxicology Laboratory of Universidad Nacional de Colombia in 80-L glass tanks with dechlorinated tap water (total hardness 34.2 mg $CaCO_3$ L^{-1}, pH 6.5-7.0, and constant aeration). Water temperature was maintained at 20.4 ± 1.2 °C. Fish were exposed to a natural 12:12 h light/dark photoperiod and the tank water was partially changed every 3 days. During acclimation, the fish were fed *ad libitum* with commercial goldfish food (Tropical Fish Pellets Tetra®). The fish were handled according to the guidelines of the *Colombian Law for Animal Health Protection and Instructions for Animal Experimentation for Scientific Purposes* [Ethics approval No. 09-2018].

2.2 Experimental treatments

Goldfish were randomly distributed into four experimental treatments: A. Control fish (n=6), B. CPF-exposed fish (n=5), C. CPF-exposed + IP injected vehicle (n=5), and D. CPF-exposed + IP-injected CBD (5 mg/Kg) (n=6). Controls were not exposed to CPF, vehicle solution or CBD and underwent anesthesia and handling as experimented by Treatments B, C and D. Treatment B were exposed to CPF. Treatment C were exposed to CPF and received i.p. injection with the vehicle solution. Treatment D were exposed to CPF and received i.p. injection with CBD at a dose of 5 mg/Kg.

2.3 Chemicals and reagents

Cannabidiol crystals were kindly supplied by Endoca ™ (Hoofddorp, Netherlands) as certified analytical grade (99.9% purity). CBD injection was given in the corresponding fish before chlorpyrifos exposure [33, 44]. CBD crystals were dissolved in a mixture (v/v/v) of 7:2:1 polyethylene glycol 400 (PEG 400) / 99.9% ethanol / phosphate buffered saline (PBS) [46, 47]. Group C received vehicle solution as a control of the CBD treatment.

2.4 Intraperitoneal (i.p.) injections

Fish received a single i.p. injection according to the modified protocol of Kinkel and col. [48]. Prior to injection, subjects were anesthetized gradually decreasing core temperature from 20°C to 4°C. Researchers chose this technique to avoid any influence or interference of chemical anesthesia in the results.

2.5 Chlorpyrifos exposure

CPF (Lorsban™ 4 EC) was purchased from Dow Agrosciences (Bogotá, Colombia). Exposure concentration of CPF in experimental treatments B, C and D was 3.5 μg L^{-1} for 96 hours. This concentration was determined as sublethal based on preliminary tests. This value corresponds to $^1/_{44}$ (2.29%) of the CPF median lethal concentration (LC_{50}) for this species, reported as 153 μg/L [49]. Fish were not fed during the experimental phase.

2.6 Plasma cholinesterase activity assay

Acetylcholinesterase (AChE) was quantified using a modification of Ellman's method [50]. This method is based on the degradation rate of acetylthiocholine by the AChE activity, into acetate and thiocholine. Thiocholine complexes with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) yielding a product that is monitored by measuring its absorbance at 405 nm. Briefly, 1.3 mL of phosphate buffer (pH 8.0, 0.1 M) were mixed with 50 μl of plasma and 50 μl solution of DTNB, followed by the addition of 10 μl of the enzyme substrate, acetylthiocholine. The change in absorbance was recorded for 2 minutes using a Stat Fax® 3300 (Awareness Technology, Inc.™). The change in absorbance was converted into enzyme activity by utilizing a derived formula. AChE activity was expressed as nanomols (nM) of complex formed per min per mL of plasma. All measurements were made in duplicate at 25°C.

2.7 Whole brain homogenization

After the experimental time, fish were anesthetized by lowering water temperature to 4°C and then euthanized by cervical dislocation. The brain of each specimen was extracted and weighed on an analytical balance (Ohaus® Adventurer®). Whole brain was homogenized in Phosphate Buffered Saline (1x PBS) at pH 7.4 using 1 mg of brain tissue and 5 μL buffer in a Potter-Elvehjem tissue homogenizer. Serine and cysteine protease inhibitor phenylmethylsulfonyl fluoride (1x PMSF) was added. After centrifugation at 12500 rpm (~ 17500 x *g*) for 30 min at 4°C, the supernatant was kept and used for successive isoprostane analysis [51].

2.8 8-iso-Prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) quantification: ELISA assay

Lipid peroxidation was determined by measuring the levels of F_2-isoprostane in the brain supernatants with a commercially available competitive enzyme immunoassay ELISA kit (OxiSelect™ 8-iso-Prostaglandin $F_{2\alpha}$ ELISA Kit; Cell Biolabs, Inc., San Diego, California, USA). This isomer (also known as 8-isoprostane; 8-epi $PGF_{2\alpha}$; $iPF_{2\alpha}$-III; 15-F_2t-isoprostane; or 15-F_2t-IsoP) has been used as a reliable biomarker of F_2-isoprostane levels. The concentration of 8-iso-$PGF_{2\alpha}$ is presented as pg/mL. The kit´s sensitivity is estimated in the range of 49 pg/mL to 200.000 pg/mL of 8-isoprostane. Samples were assayed for free (unbound) and for total (free + esterified) 8-iso-$PGF_{2\alpha}$. Total 8-iso-$PGF_{2\alpha}$ was measured after alkaline hydrolysis of tissue lysate. Briefly, 200 μL of supernatant were combined with 50 μL of 10N sodium hydroxide (NaOH) and incubated at 45°C for 2 hours. After incubation, 50 μL of 10N hydrochloric acid (HCl) were added and samples were centrifuged at 12,000 rpm (~ 16128 x *g*) for 5 min at room temperature. Finally, a 1:1 sample dilution with neutralization solution (pH= 6 ~ 8) was performed if needed. All ELISA assays were performed in duplicate following manufacturers' recommendations. The quantity of 8-iso-$PGF_{2\alpha}$ was determined by comparing the absorbance at 450 nm in the samples with those of a standard curve.

2.9 Statistical analysis

All obtained data were tested for normality (Shapiro-Wilk test) and homogeneity of variances (Bartlett's test). The statistical analysis was performed by means of one-way analysis of variance (ANOVA), followed by a Fisher's Least Significant Difference (LSD) test in order to determine significant differences among the F_2-isoprostanes values. A p value less than 0.05 was considered statistically significant. Results are expressed as mean ± standard error of the mean

(SEM). All statistical analysis were performed by using the statistical software R 3.6.0 for Windows (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

3 Results and discussion

3.1 Experimental fish

None of the fish of any of the experimental treatments showed apparent signs of distress or deleterious effects due to the CPF exposure, the I.P. injections or the handling during the procedures prior to the onset of the 96 hour-experimental time. All the fish maintained normal behavior in the experimental tanks.

3.2 Plasma acetylcholinesterase (AChE) activity

Plasma AChE activity (nM min/mL plasma, mean ± SEM) in experimental groups was as follows; A (controls): 23879 ± 4081, B (CPF-exposed): 10059 ± 1761, C (CPF-exposed + vehicle): 10956 ± 2293 and D (CPF-exposed + CBD): 11795 ± 1051. There was a significant inhibition of AChE activity in all of the CPF-exposed groups that ranged from 50.8 % to 57.9 % ($p=0.0064$; Figure 2).

3.3 Brain 8-iso-$PGF_{2\alpha}$ levels

Brain 8-iso-$PGF_{2\alpha}$ (pg/ml, mean ± SEM) had significant differences among experimental treatments. The highest value was shown by treatment C (CPF-exposed + vehicle) 728 ± 236, followed by B (CPF-exposed) 566 ± 91 and A (controls) 396 ± 91. Treatment D (CPF-exposed + CBD injected fish) had the lowest F_2-IsoPs of all the treatments, 281 ± 45, which was statistically significant different from the other two CPF-exposed groups (B and C). The fish exposed to CPF alone and CPF + vehicle had the highest levels of F_2-IsoPs compared to the control group. Brain levels of F_2-IsoPs were significantly reduced in the group treated with CBD compared to the experimental groups exposed to CPF but not treated with CBD ($p=0.0271$) (Figure 3). F_2-IsoPs values of the CBD treated-fish (Group D), which was exposed to CPF too, were even lower than those of controls (group A), not exposed to either the pro-oxidant CPF or CBD.

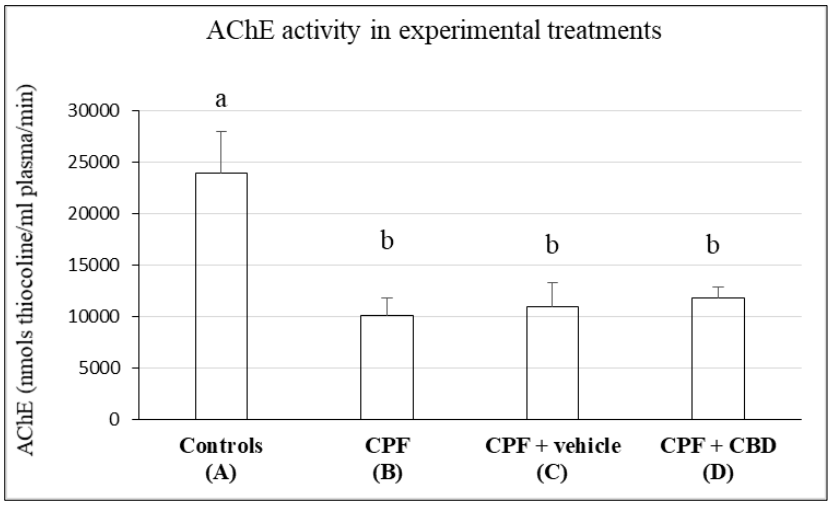

Figure 2 Plasma acetylcholinesterase (AChE) activity measured in *Carassius auratus* acutely exposed to 3.5 μg L^{-1} of chlorpyrifos. Assays were performed in duplicate. Data are presented as mean ± standard error of the mean SEM (n = 5-6). Bars with different letters from each other are statistically significant different ($p<0.05$)

Chlorpyrifos (CPF) is an organophosphate (OP) pesticide widely used in agriculture and known for its deleterious effects on the CNS [52]. Following OPs exposure, cholinergic neuronal excitotoxicity and dysfunction results from the acute and excessive stimulation of cholinergic receptors due to irreversible inhibition of acetylcholinesterase (AChE) [53, 54].

Besides the main mechanism of OPs toxicity, oxidative stress is another effect caused by these insecticides. Overstimulation of cholinergic and glutamatergic nervous system is followed by intensified generation of reactive species and oxidative damage [55]. Researchers has found in *in vivo* and *in vitro* models that CPF can significantly decrease the activity and expression of antioxidant enzymes such superoxide dismutase (SOD), catalase (CAT) [56, 57], glutathione peroxidase (GPx) and glutathione s-transferase (GST) [58, 59] in different organs, as well as an increase in

lipid peroxidation reflected in higher levels of malondialdehyde (MDA) in the thiobarbituric acid reactive substances (TBARS) test [49, 56, 58, 59].

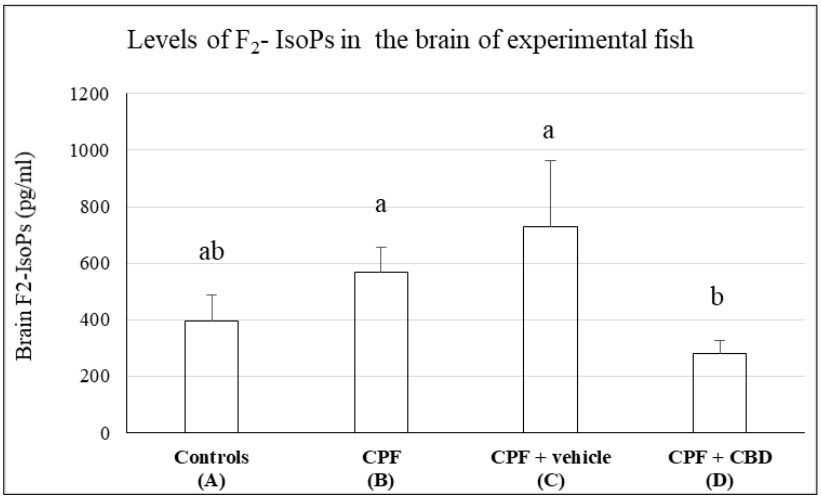

Figure 3 Brain 8-iso-Prostaglandin $F_{2\alpha}$ levels quantified by ELISA test. Assays were performed in duplicate. Data are presented as mean ± SEM (*n* = 5-6 per experimental group). Bars labeled with different letters from each other are statistically significant different ($p<0.05$)

In the present study, fish exposed to 3.5 μg/L CPF showed no symptoms of poisoning or any changes in behavior during the experimental time. However, CPF was a potent inhibitor of AChE in goldfish plasma. In all the CPF-exposed treatments, AChE activity was significantly lower than in controls (50.8 to 57.9 % inhibition). Similar results had been reported by Yen and col. [60] who used zebrafish (*Danio rerio*) exposed to CPF. As for the baseline values found in the controls (23879 ± 700 nM tiocolina/min/mL), these are very similar to the ones reported by Chuiko and col. [61] for the *Cyprinidae* (18600 ± 700 nmol/min/mL), taxonomic group to which *Carassius auratus* belongs.

Cholinesterase activity, particularly AChE, is a very sensitive biomarker that reflects the exposure to OPs at even sublethal concentrations. Results of AChE activity in CPF-exposed treatments (B, C and D) confirmed the exposure and the inhibitory catalytic effects caused by CPF despite the absence of symptoms. The main purpose of evaluating effects of CPF on AChE activity was to demonstrate that the insecticide was causing changes at the biochemical level of exposed fish, and then to investigate further correlation with brain F_2-IsoPs and likely CBD protection on the oxidative stress caused by CPF.

F_2-IsoPs are very stable molecules present in all tissues and biological fluids. Measuring of F_2-IsoPs has made more accurate the *in vivo* assessment of lipid peroxidation and oxidative stress [62]. Since 2005 the most reliable biomarker for evaluation of oxidative stress in humans and animals is the measurement of F_2-IsoPs, and therefore considered a "gold standard". The two approaches for this measurement are mass spectrophotometry and immunological-based methods. The latter are more accessible and are based on the ELISA technique [63]. Reports on the use of F_2-IsoPs in fish species are not abundant in the literature. Bulloch and col. [64] developed a methodology to determine F_2-IsoPs in fish mucus using high performance liquid chromatography in tandem with mass spectrometry. Most of the reports on the use of F_2-IsoPs in animals use urine to determine the level of oxidative stress [65]. In the present discussion, comparisons among the experimental treatments will serve for the purpose of determining the utility of F_2-IsoPs as biomarker of oxidative stress, given the scarcity of reports in piscine species.

The levels of F_2-IsoPs (free 8-iso-$PGF_{2\alpha}$) had significantly higher values in the brain tissue of the two CPF-exposed fish (treatments B and C) in the present investigation. These results complied with those reported by López-Granero and col. [66] in the brain of male Wistar rats exposed to CPF. In the present work, control fish had a lower (396 ± 91 pg/mL) concentration of F_2-IsoPs in the brain homogenates as compared to those of the CPF-exposed fish (566 ± 91 pg/ml) and the CPF-vehicle exposed (728 ± 236 pg/ml). This represents a significant difference of 1.4-fold and 1.8-fold, respectively. The lowest F_2-IsoPs level was found in the brain of CPF-exposed + CBD-injected fish (281 ± 45 pg/ml), a value even lower than the one in the controls.

Neuroprotection can be defined as any intervention that prevents death of neurons, mitigate neurodegenerative processes, and delay the progression of disease or clinical symptoms. It is considered that there are seven mechanisms

through which neuroprotection is achieved: antiapoptosis, antinflamatory responses, blocking of glutamate-mediated toxicity, trophic factors, blocking of aggregating proteins, antioxidant mechanisms and other miscellaneous [67]. Different studies offer solid proof of the neuroprotective properties of CBD [11, 17, 19, 20-23, 27]. Two main studies have evaluated the CBD neuroprotective mechanism through antioxidation in the central nervous systems *in vivo*. El-Remessy and col. [45] showed that in male Sprague-Dawley rats, glutamate caused apoptosis of the retina cells due to the formation of peroxinitrite. CBD attenuated this reaction aside from any activation of cannabinoid receptors. On the other hand, Cassol-Jr and col. [44] found that the acute (6 hours) and the prolonged (10 days) exposure to different concentrations of CBD (2.5, 5.0 and 10.0 mg/Kg) reduced significantly two biomarkers of oxidative stress (TBARS and carbonyl proteins) in lung, liver, kidney, heart, spleen and brain. In addition, CBD reduced cognitive failure and mortality in male Wistar rats that underwent sepsis due to cecum perforation. The present work shows further evidence of antioxidant benefits of CBD in a piscine animal model (*Carassius auratus*) using brain F_2-IsoPs as biomarkers of effect.

Understanding the structure and geometry of the chemical functions in the CBD molecule may help to decipher the mechanisms for its antioxidant properties. CBD has three different *moieties*: limonene, phenol and aliphatic group (Figure 1) [11]. Contrary to other cannabinoids, CBD has two hydroxyl phenolic groups. Borges and da Silva [11] propose that the direct transfer of either one of the H atoms or one of the electrons from the phenolic groups of the CBD molecule to the free oxidative radical R• is the mechanism to reduce or prevent oxidative stress. Phenolic groups are known for its antioxidant properties [68]. Phenolic acids are efficient antioxidants that quench excessive free radical-induced body damage and chronic diseases [69].

4 Conclusion

In short, results of the present investigation showed the antioxidant and hence neuroprotective effects of the cannabinoid CBD in a fish research model. Taking into consideration the key role that ROS play in the physiopathology of neurodegenerative diseases, present results could support the use of CBD as a therapeutic. Further research is needed to promote and encourage this use into clinical trials and other approval phases.

Compliance with ethical standards

Acknowledgments

Authors thank the Neuroscience Program at the School of Medicine as well as the School of Veterinary Medicine at Universidad Nacional de Colombia for their financial support. Authors appreciate scientific and technical support from Dr. Roger A. Cachope and Dr. Diana Susana Vargas. ENDOCA kindly provided the CBD crystals that helped to accomplish the present investigation.

Disclosure of conflict of interest

No competing financial interests or of any other nature exist for the present investigation.

Statement of ethical approval

The present investigation was performed based on the approval and guidelines of the *Colombian Law for Animal Health Protection and Instructions for Animal Experimentation for Scientific Purposes* [Ethics approval No. 09-2018].

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