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Research article

Monocyte chemoattractant protein-1 and cyclooxygenase enzymes in rats treated with drugs of abuse

Omar M.E. Abdel-Salam*[1], Eman R. Youness[2], Enayat A Omara[3], Amany A Sleem[4]

[1]*Department of Toxicology and Narcotics, Medical Research and Clinical Studies Institute, National Research Centre, Cairo, Egypt.*
[2]*Medical Biochemistry, Medical Research and Clinical Studies Institute, National Research Centre, Cairo, Egypt*
[3]*Pathology, Medical Research and Clinical Studies Institute, National Research Centre, Cairo, Egypt.*
[4]*Pharmacology, Medical Research and Clinical Studies Institute, National Research Centre, Cairo, Egypt.*

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***Corresponding Author: Omar M.E. Abdel-Salam**, Department of Toxicology and Narcotics[1], National Research Centre, Cairo, Egypt.
FAX: 202-33370931
Email id: omasalam@hotmail.com

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Keywords: Tramadol; cannabis; addiction; monocyte chemoattractant protein; cyclooxygenase**.**

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Abstract

The CC chemokine monocyte chemoattractant protein (MCP)-1/CCL2 and cyclo-oxygenase-2 enzyme are potent mediators of neuroinflammation. We studied the effect of treatment with *Cannabis sativa* resin extract and/or tramadol, two common drugs of abuse on the level of MCP-1 in brain and serum of rats. In addition, serum levels of cyclo-oxygenase 1 (COX-1) and cyclo-oxygenase 2 (COX-2) enzymes in rats given cannabis were also determined. *Cannabis sativa* resin at 5, 10 or 20 mg/kg (expressed as delta-9-tetrahydrocannabinol), tramadol at 5, 10 or 20 mg/kg or tramadol at 10 mg/kg combined with cannabis at 5-20 mg/kg were given once a day, subcutaneously, for six weeks. Results showed that, as compared to corresponding controls, Cannabis sativa resin produced a significant increase in MCP-1 increased in brain tissue (29.8-56.4%) and serum (14.8-31.0%). MCP-1 showed also significant increments in brain (34.6%) and serum (22.1%) by 20 mg/kg tramadol. After the concomitant treatment with both cannabis and tramadol, MCP-1 increased in brain by 88.5-145.7% and in serum by 32.6-64.8%. Moreover, cannabis given at 20 mg/kg significantly increased serum COX-1 and COX-2 by 362% and 58.8% compared to corresponding control values. The histopathological study of the striatum of cannabis-treated animals revealed the presence of apoptotic cells and pyknotic nuclei and congestion of blood vessels. The striatum from animals given tramadol showed neuronal perinuclear cytoplasmic vacuolations and apoptotic cells. Moreover, the combined administration of both cannabis and tramadol resulted in neuronal necrosis, vacuolation, apoptosis and pyknosis. Collectively, these results indicate that treatment with cannabis resin and/or tramadol is associated with a proinflammatory response that involves MCP-1 and cyclooxygenases and which could result in the development of brain injury.

Introduction

Cannabis is the most widely abused psychoactive substance worldwide. The two most commonly abused are marijuana consisting of the dried leaves and flowering tops and hashish which is the compressed resinous exudate of the flowering tops of the cannabis plant. It has been estimated in 2021, that 219 million adult subjects have used cannabis globally [1, 2]. Cannabis is abused for its recreational effects which include a sense of mild euphoria and relaxation, distortion of sensory imagery and time perception [3]. These effects of herbal cannabis are caused by its major psychoactive component i.e., delta-9-tetrahydrocannabinol (Δ^9-THC) [4] acting on cannabinoid CB1 receptors in brain in areas that control attention, learning, memory and motor function [5]. The other cannabinoid CB2 receptor is found mainly in immune cells in the periphery [5, 6]. On chronic use,

cannabis disrupts memory, impairs cognitive functions, psychomotor performance, academic performance and achievements and driving skills [3, 7, 8]. There is also the risk of developing psychotic illness e.g., schizophrenia, especially when exposure starts during adolescence and continues for years [9]. Moreover, there is evidence from brain imaging studies that suggests structural alterations in heavy and chronic cannabis users [10]. Evidence from animal and in vitro studies suggest that cannabis is toxic to neurons causing mitochondrial dysfunction, caspase-3 activation, and apoptotic cell death [11, 12]. Cannabis is thus not a benign drug though many adolescents perceive the drug of little harm.

In recent years, there has been noticeable increase in the non-medical use of tramadol in Egypt and other countries in Middle East [2]. The drug is a centrally acting analgesic. It a synthetic 4-phenylpiperidine analog of codeine and acts as an agonist on μ-opioid receptors. In addition, tramadol inhibits the reuptake of the brain neurotransmitters noradrenaline and serotonin. In clinical practice, tramadol is an effective analgesic for controlling moderate or severe pain in both chronic and acute conditions e.g., postoperative cancer pain [13]. Animal studies suggested neurotoxic effects for tramadol. Rats treated with 50 mg/kg for 4 weeks showed increased oxidative stress, neuronal cells disorganization, apoptosis and intercellular edema in cerebral cortex [14, 15]. Rats given tramadol 5-20 mg/kg for 6 weeks exhibited dose-dependent neurotxicity e.g., shrunken neurons having pyknotic nuclei besides darkened cytoplasm and irregularly damaged neuronal cells with apoptotic and pyknotic nuclei [16].

Monocyte chemoattractant protein (MCP)-1 or CCL2 is a potent proinflammatory chemokine which can attract monocytes and induce inflammation following injury in vivo. MCP-1 is produced by neurons, astrocytes, microglia and mononuclear phagocytes and regulates migration and infiltration of monocytes/macrophages into the injured and inflamed brain tissue [17]. Genetic deletion of MCP-1 resulted in defective mononuclear cell recruitment in animal models of autoimmune encephalomyelitis and stroke [18, 19]. In contrast, in mice with stroke, MCP-1 overexpression resulted in increased leukocyte local transmigration and perivascular accumulation and increased brain infarction volume [20]. Moreover, MCP-1 knockout (MCP-$1^{-/}$) mice showed reduced pro-inflammatory cytokines and chemokines, and a decrease in activated microglia in brain in response to systemic lipopolysaccharide injection compared with control (MCP-$1^{+/+}$) mice [21].

The cyclooxygenase (COX) enzymes, COX-2 and COX-2 catalyze the first step in the synthesis of prostaglandinds, prostacyclin and thromboxane A2 from arachidonic acid. Cyclooxygenase-1 (COX-1) is the isoform constitutively expressed in most tissues and is considered a cytoprotective or housekeeper. In contrast, COX-2 is induced under inflammatory conditions via inflammatory cytokines and growth factors [22] and thought to have an instrumental role in neurodegenerative diseases [23, 24].

In view of the important role for MCP-1 and COX enzymes in mediating neuroinflammation and the lack of studies on the effect of cannabis extract, tramadol or their combined administration on this chemokine, this study was designed in order to delineate the effect of these drugs of abuse on the level of MCP-1 in brain and serum of rats.

Materials and methods

Animals

Male Sprague-Dawley rats, weighing between 140-150g (from Animal House of the National Research Centre) were used. Rats were group-housed under temperature- light-, and humidity-controlled conditions and given free access to standard laboratory rodent chow and water. Rats were acclimated to the test room for one week prior to experiments. The experiments were done following the guidelines of the Institute Ethics Committee (Project number 10001004) and complied with the Health Guide for the Care and Use of Laboratory Animals by the U.S. National Institutes of Health (Publication No. 85-23, revised 1996).

Drugs and chemicals

Cannabis sativa resin or hashish and tramadol were a kind gift from the Laboratory of Forensic Sciences of the Ministry of Justice, Egypt. Other chemicals and reagents used in the study were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A) and were of analytical grade.

Preparation of cannabis resin extract

The extract of *Cannabis sativa* was prepared from hashish (the solid dried resin of the plant). The extraction was done with chloroform according to Turner and Mahlberg [25] after modification. Chloroform was shown to be a more suitable solvent compared with light petroleum or ethanol with 98-99 % THC yield upon a single extraction and almost 100% on double extraction [26]. In brief, the resin (10 g) was grounded in a mortar, heated in an oven (100°C) for 60 min with the aim to decarboxylate the cannabinolic acids. Chloroform extraction of the resin was done overnight. The extract was then filtered followed by evaporation under nitrogen gas, and storage at 4°C being protected from light with the use of aluminium cover. The residue was re-suspended in 2% ethanol-saline for injection. Delta-9-tetrahydrocannabinol (Δ^9-THC) content of the extract was quantified with the use of gas chromatography–mass spectrometry (GC-MS). The resin contained around 20% Δ^9-THC and about 3% cannabidiol.

Study design

Rats were allocated randomly into equal treatment groups (six rats each) that received subcutaneous injections of either the vehicle, *cannabis* extract, tramadol or *cannabis/tramadol* combination.

Group 1 was treated with the vehicle (0.2 ml saline).

Group 2, 3, 4 were given cannabis extract (5, 10 or 20 mg/kg).
Groups 5, 6, 7 were administered tramadol (5, 10 or 20 mg/kg).
Groups 8, 9, 10 were treated with tramadol at 10 mg/kg along with cannabis extract (5, 10 or 20 mg/kg).
The different drugs were given via the subcutaneous route, once a day, for six weeks. Thereafter, rats were euthanized by decapitation. The brain from each rat was quickly removed, dissected and snap-frozen using liquid nitrogen. Brain samples were homogenized in a glass tube using a Teflon pestle in an ice-cold phosphate buffer solution (PBS: 50 mM Tris-HCl pH 7.4), centrifuged for 5 min at 9000 g and 4°C. The supernatants were stored at -80°C till the biochemical assays.

Quantification of MCP-1
The chemokine (C-C motif) ligand 2 (CCL2; MPC-1) was quantified with an enzyme-linked immunosorbent assay (ELISA) from R&D Systems Inc. - Minneapolis, USA according to the instructions by the manufacturer.

Quantification of COX-1
The cyclooxygenase-1 (COX-1) enzyme was quantified using rat-COX-2 ELISA according to the instructions provided by the manufacturer (SunLOng Biotech Co., LTD).

Quantification of COX-2
The cyclooxygenase-2 (COX-2) enzyme was quantified using rat-COX-2 ELISA according to the instructions provided by the manufacturer (SunLOng Biotech Co., LTD).

Histopathological studies
Representative brain sections were fixed immediately in 10% neutral-buffered formalin for tissue fixation, processed, and paraffin sections (5 μm thickness) were stained with hematoxylin and eosin (Hx & E) for the histopathoplogical study. Sections were examined and photographed using digital camera (Microscope Digital Camera DP70, Tokyo, Japan).

Statistical analysis
Data in the study are expressed as mean ± SE. Data were analyzed with the use of one-way analysis of variance (ANOVA) and Duncan's multiple range test or Student's t test where appropriate. SPSS software (SPSS Inc., Chicago, IL, USA) was used. Effects having a probability of $p < 0.05$ were considered significant.

Results
Effect of cannabis, tramadol or both on brain MCP-1
After repeated treatment with 5, 10 or 20 mg/kg cannabis, MCP-1 levels in brain were significantly increased by 29.8%, 47.9% and 56.4% compared with the control group (12.2 ± 0.5, 13.9 ± 0.8, and 14.7 ± 1.0 vs. 9.4 ± 0.2 pg/ml). MCP-1 levels increased after 10-20 mg/kg tramadol by 17.5%, and 34.6% compared with the saline group (11.05 ± 0.8, 12.65 ± 0.4 vs. 9.4 ± 0.2 pg/ml). Only the latter value was statistically significant. However, the concomitant treatment with both cannabis and tramadol induced 88.5%, 101.1% and 145.7% increments in brain MCP-1 compared with the saline control value (17.72 ± 0.6, 18.9 ± 1.1, 23.1 ± 0.7 vs. 9.4 ± 0.2 pg/ml) (Figure 1).

Effect of cannabis, tramadol or both on serum MCP-1
The administration of 5-20 mg/kg of cannabis induced 14.8%, 16.7% and 31% increases in serum MCP-1 with respect to the saline group (30.3 ± 0.42, 30.8 ± 1.53, 34.58 ± 0.61 vs. 26.4 ± 0.57 pg /ml). Serum MCP-1 increased by14.4%-22.1% by treatment with 10-20 mg/kg tramadol (30.2 ± 0.78 and 32.25 ± 1.14 vs. 26.4 ± 0.57 pg) and by 32.6%, 45.9%, and 64.8% after the concomitant treatment with cannabis and tramadol (35.01 ± 0.63, 38.52 ± 0.69 and 43.5 ± 0.94 pg/ml) compared to the saline treated group (26.4 ± 0.57 pg/ml) (Figure 2).

Effect of cannabis on serum cyclooxygenases
The mean serum level of cyclooxygenase-1 enzyme in rats given cannabis at 20 mg/kg was 2.31± 0.28 ng/ml. This value was significantly higher than the mean serum level of cyclooxygenase-1 in the control group which was 0.5 ± 0.03 ng/ml (362% increase; $p<0.0001$) (Figure 3A).
Significantly higher serum level of cyclooxygense-2 by 58.8% was found in rats treated with cannabis (20 mg/kg) compared to control value (281.5 ± 10.6 8 vs. 177.3 ± 2.28 ng/ml; $p<0.0001$) (Figure 3B).

Histopathological studies
Sections from control mice showed normal appearance of neurons and basophilic cytoplasm (Figure 4A). Sections of animals that received cannabis at 5 mg/kg showed very few apoptotic cells and pyknotic nuclei (Figure 4B). However, in the group received 10 or 20 mg/kg there was mild structural damage in the form apoptotic cells and pyknotic nuclei and congestion of blood vessels (Figure 4C & D).
The striatum from animals given tramadol at doses of 5, 10 or 20 mg/kg showed degenerative changes with perinuclear cytoplasmicvacuole in neurons, apoptotic cells, pyknotic nuclei, oedema and congestion of blood vessels in a dose-dependent manner (Figure 5A, B & C).
The histopathological study of the group treated with tramadol 10 and cannabis at doses of 5 or 10 mg/kg showed smaller amount of degenerative changes, necrosis, apoptosis, and pyknosis in neuronal cells (Figure 5D & E). In contrast, the striatum of rats treated with tramadol at 10 mg/kg together with cannabis at 20 mg/kg revealed necrosis with vacuolation, apoptosis and pycknosis in neuronal cells. Congestion of blood vessels and some neuronal loss of nucleoli were also observed (Figure 5F).

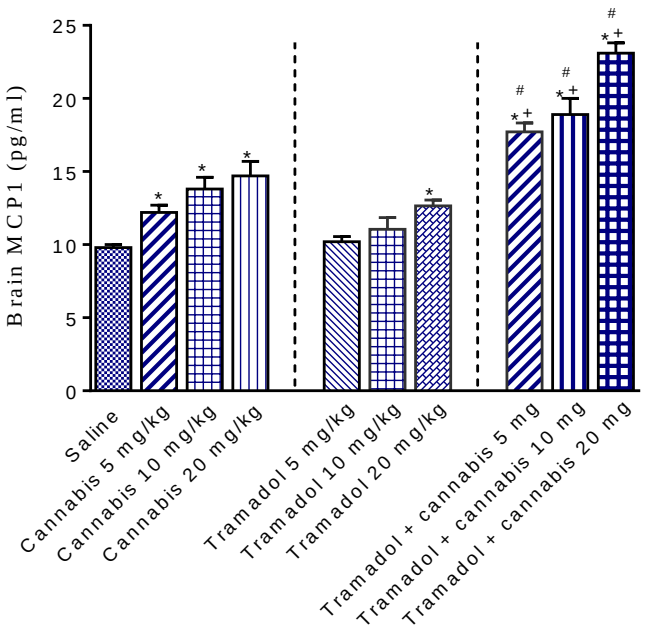

Figure 1. Monocyte chemoattractant protein-1 (MCP-1) in brain of rats given cannabis extract, tramadol or cannabis/tramadol. Values are mean ± SEM. *p< 0.05 vs. saline and between other groups as shown on the figure. +p<0.05 vs. only cannabis. # p<0.05 vs. only tramadol. One-way ANOVA and Duncan's multiple range test.

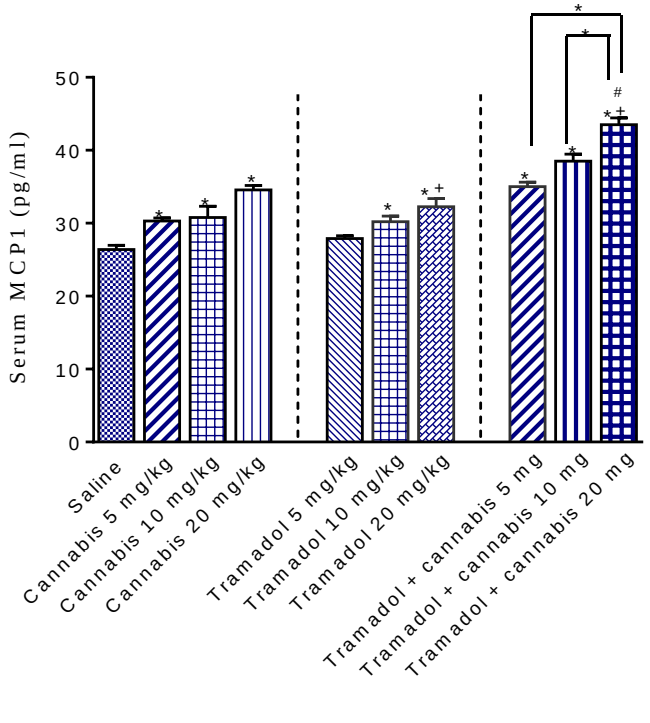

Figure 2. Serum levels of monocyte chemoattractant protein-1 (MCP-1) after treatment with cannabis, tramadol or cannabis/tramadol. Values are mean ± SEM. *p< 0.05 vs. saline and between other groups as shown on the figure. +p<0.05 vs. only cannabis. # p<0.05 vs. only tramadol. One-way ANOVA and Duncan's multiple range test.

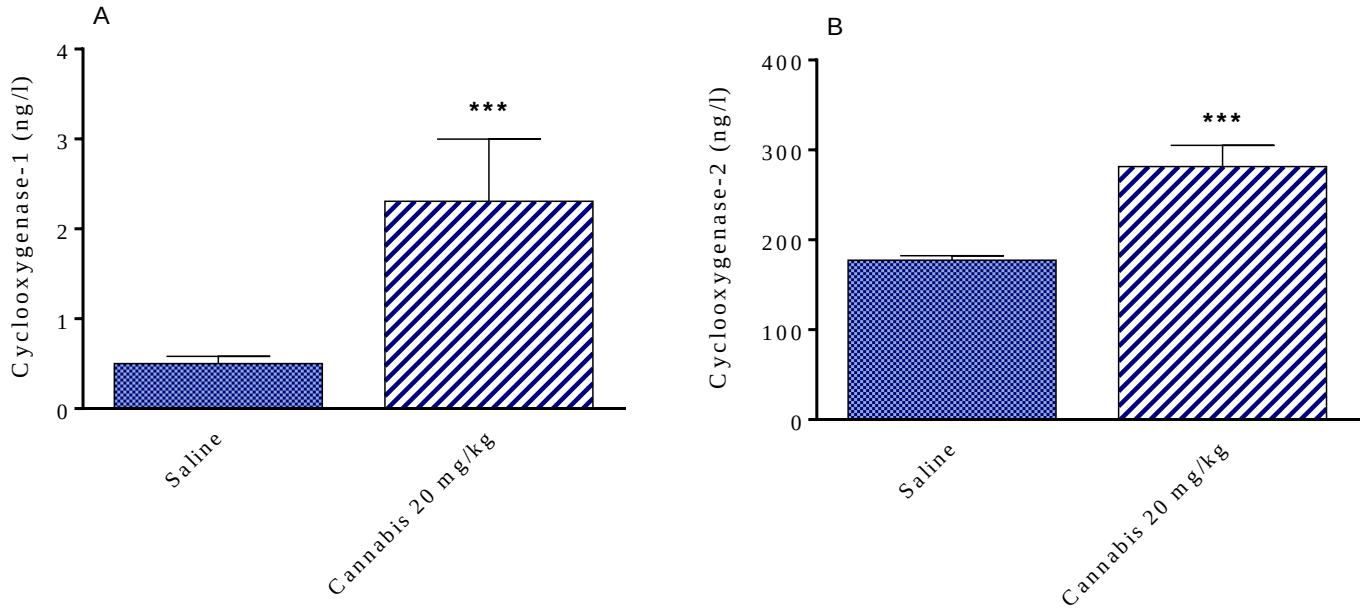

Figure 3. Serum levels of cyclooxygenase 1 & 2 in rats administered cannabis at 20 mg/kg. Values are mean ± SEM. ***p< 0.0001 vs. saline. Student's t test.

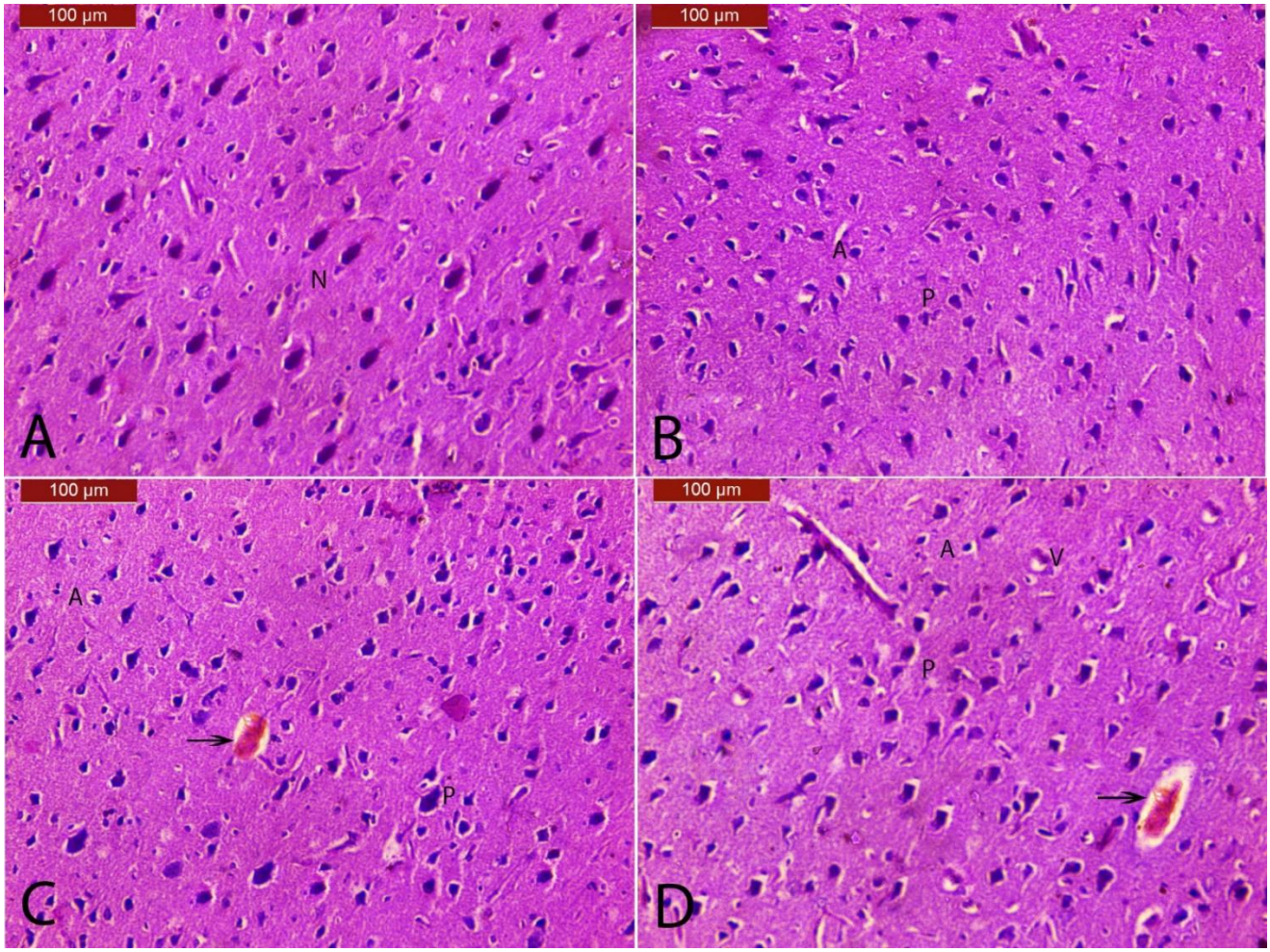

Figure 4. Representative H &E stained striatal sections from rats treated with (A) Saline: neurons with the surrounding supporting cells with normal nuclei and basophilic cytoplasm (N). (B) Cannabis 5 mg/kg: few apoptotic cells (A), darkly stained pyknotic (P) nuclei (H & E x 400). (C) Cannabis 10 mg/kg: few apoptotic cells (A) and darkly stained pyknotic nuclei (P) with congestion of blood vessels (arrow). (D) Cannabis 20 mg/kg: mild apoptotic cells (A) and darkly stained pyknotic nuclei (P) with congestion of blood vessels (arrow) (H & E x 200).

Discussion

In this study, brain and serum levels of the chemokine MCP-1 were assessed in rats treated with a cannabis resin extract rich in Δ^9-THC, tramadol or their combination for 6 weeks. We found a significant and dose-dependent increase in the level of MCP-1 in both the brain tissue and serum of cannabis treated rats. MCP-1 was also significantly increased in the brain and serum after 20 mg/kg of tramadol and by the combined treatment with both cannabis and tramadol. Moreover, the co-administration of tramadol and cannabis resulted in significantly higher level of MCP-1 in brain compared to that caused by only cannabis resin or only tramadol. Our results in addition indicate that cannabis the dose of 20 mg/kg resulted in significant increases in serum cyclooxygenases.

During the last decade, there was a growing interest in the use of cannabis or cannabis-based medicines for treating neurologic conditions eg., Parkinson's disease, epilepsy or multiple sclerosis [27]. In these disorders, the presence of neuroinflammation is considered to be an important requirement key for the development of neurodegeneration [28]. Hence the importance of delineating the effect of cannabis or cannabinoids on neuroinflammtion. MCP-1 is involved in inflammatory immune responses by regulating monocytes and macrophages recruitment to the sites of inflammation. In mouse and human brain, MCP-1 and its receptor CCR2 are expressed mainly by microglia. MCP-1 is also produced by neurons [17]. It has been shown that cerebral overexpression of MCP-1 promotes neuroinflammation and glial cell responses [29]. In contrast, in MCP-1-/- mice there was reduced microglia activation and pro-inflammatory cytokines and chemokines in brain during systemic endotoxaemia [21].

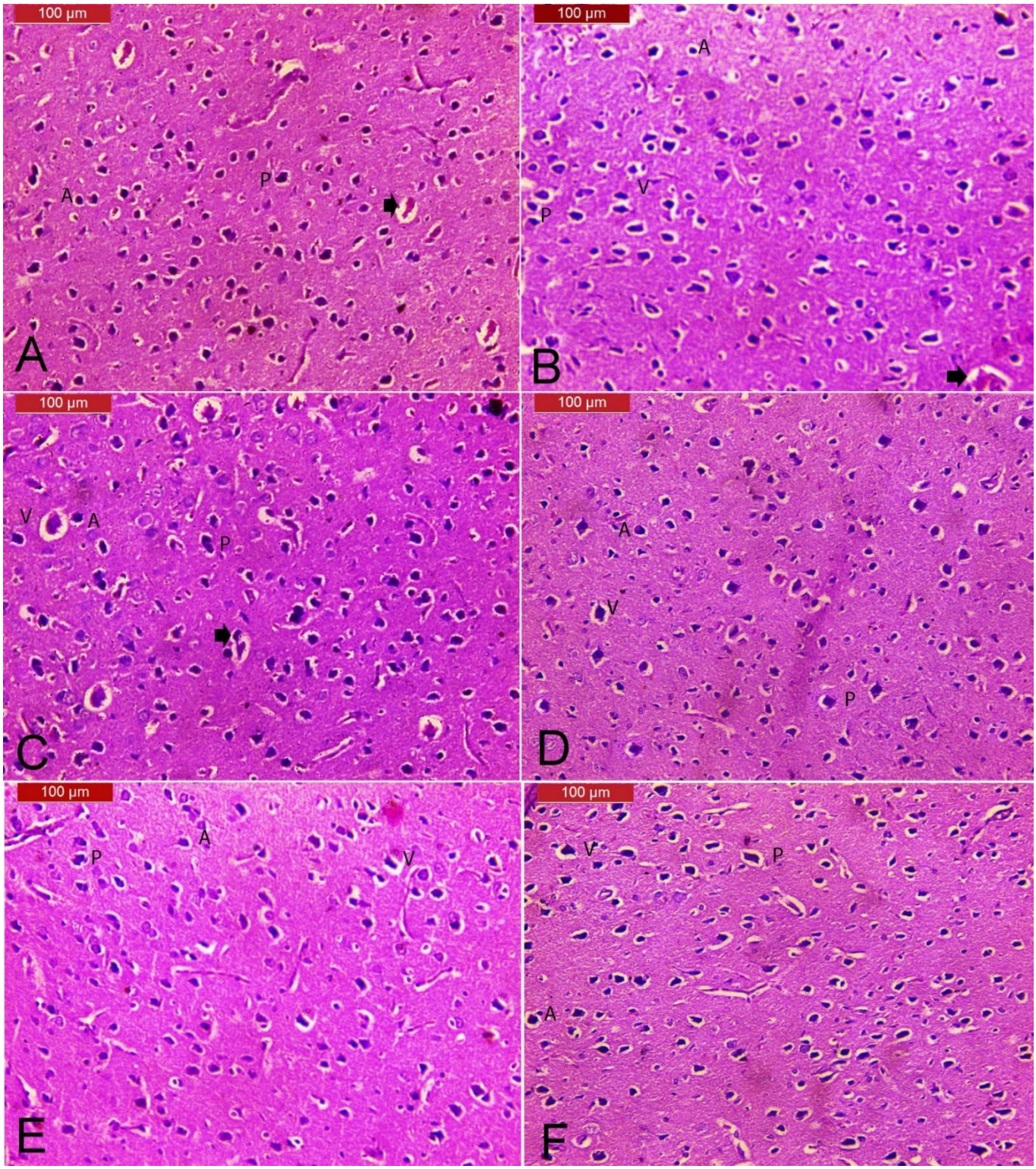

Figure 5. Representative H & E stained sections from the striatum of rats treated with (A) Tramadol 5 mg/kg: neurons few perinuclear cytoplasmic vacuoles (V), few apoptotic cells (A) and darkly stained pyknotic nuclei (P). (B) Tramadol 10 mg/kg: few perinuclear cytoplasmic vacuoles in neurons (V), few apoptotic cells (A), darkly stained pyknotic nuclei (P) and oedema (arrow). (C) Tramadol 20 mg/kg: few perinuclear cytoplasmic vacuoles in neurons (V), few apoptotic cells (A) and pyknotic darkly stained nuclei (P) and oedema (arrow). (D) Cannabis 5 mg/kg/tramadol 10 mg/kg: few perinuclear cytoplasmic vacuoles in neurons (V), few apoptotic cells (A) and pyknotic darkly stained nuclei (P). (E) Cannabis 10 mg/kg/tramadol 10 mg/kg: mild perinuclear cytoplasmic vacuole in neurons (V), few apoptotic cells (A) and pyknotic darkly stained nuclei (P) and oedema (arrow). (F) Cannabis 20 mg/kg/tramadol 10 mg/kg: moderate perinuclear cytoplasmic vacuole in neurons (V), few apoptotic cells (A) and pyknotic darkly stained nuclei (P) and oedema (arrow) (H & E x 200).

When activated by inflammatory signals, glia cells produces proinflammatory cytokines, and chemokines e.g., MCP-1, prostanoids and reactive oxygen species which contributes to neurodegeneration [30]. In patients having Alzheimer's disease, high plasma MCP-1 levels showed correlation with greater severity and more rapid cognitive decline [31]. Studies, however, have provided unclear/contrasting results as to the effect of cannabis/cannabinoids on MCP-1. In human monocyte-astrocyte co-culture, TLR7 activated monocytes stimulated astrocytes to produce MCP-1 (also IL-6) which could be inhibited by THC, possibly involving in part CBR2 receptors [32]. In lung tissue, the synthetic cannabinoid agonist Cp55,940 acting via CBR1 induced the expression of MCP-1 along with several other chemokines and proinflammatory cytokines [33]. The CBR2 agonist JWH-015 was reported to suppress the production of nitric oxide and TNF-α by microglia after their stimulation by IFN-γ or amyloid-beta peptide [34]. In synoviocytes from rheumatoid arthritic joints in vitro, the increased production of MCP-1 (also IL-6) following stimulation with TNF-α was reduced by treatment with CBR2 agonist JWA133 [35]. In a recent systematic review, Henshaw *et al.* [36] found that the non-psychotropic cannabinoids cannabidiol, cannabigerol or cannabidiol/Δ^9-THC combination were associated with anti-inflammatory effect in animal models in vivo. In contrast, Δ^9-THC showed no effect on pro-inflammatory cytokines. Chronic usage of cannabis in humans appears to affect the phenotype and count of circulating monocytes [37] and reduce their ability to respond to MCP-1 [38]. In physically active subjects, however, there was no effect for cannabis on basal levels of inflammatory markers C reactive protein and IL-6 [37]. There was also no effect for cannabis use on the serum levels of MCP-1 in chronic marijuana users [39]. Other studies found increased levels of the chemokine CCL11 in current users of cannabis as compared to past users and also non-users [40]. In the above mentioned studies, however, there was no estimation of the dose or type of cannabis consumed by the subjects, no serum THC determination and no cannabis was administered.

The cyclooxygenase enzymes COX-1 and COX-2 catalyzes the conversion of arachidonic acid into prostaglandinds and thromboxane A2 [22]. The classical view of COX-1 being responsible for the production of cytoprotective prostaglandins in contrast to the harmful COX-2 derived prostanoids has recently been challenged. Evidence supports an important role for both COX-1 and COX-2 in neuroinflammation. Mice with COX-1 genetic deletion showed attenuated inflammatory responses and reduced neuronal damage following challenge with β-amyloid injection [41]. COX-$2^{-/-}$ mice showed increased activation of glial cells, and increased mRNA and protein expression of a number of cytokines, chemokines, iNOS as well as increased neuronal damage after the intracerebroventricular injection of lipopolysaccharide [42]. In rats, intraperitoneal administration of THC at 0.5-2.0 mg/kg increased brain concentrations of prostaglandins E2 and F2 alpha [43]. In mice repeated administration of THC (10 mg/kg) resulted in increased brain COX-2 expression while genetic deletion of COX- prevented THC-induced impairments in hippocampal long-term synaptic plasticity, and fear memories [44]. In the current study, we demonstrated that cannabis was capable of increasing the serum levels of both COX-1 and COX-2, thereby, suggesting that cannabis may induce or promote brain neuroinflammation.

Cannabis varies in their composition and potency, with the latter being an expression of their content of THC which increased dramatically over the past decade [45]. Further, the biological effects of cannabis cannot be ascribed only to THC as cannabis is a complex mixture of hundreds of different chemical compounds including cannabinoids, flavenoids e.g. flavocannabiside or flavosativaside [46] Some of the non-psychotropic cannabinoids eg., exert an additive or even an antagonizing action to certain effects of THC [47]. Moreover, anti-inflammatory effects have been reported for cannabidiol [48] and cannabigerol [49].

Tramadol is an efficient analgesic which showed the liability for abuse in Egypt and other countries [2]. The drug in high doses was shown to cause oxidative stress and neurotoxicity in animals studies [14, 16, 50]. There was chromatin condensation, cytoplasmic shrinkage, neuronal necrosis with gliosis in cerebral cortex and striatum of tramadol-treated animals [50]. The proinflammatory cytokine TNF-α and the immunoreactivity of the apoptotic marker caspase-3 increased in cerebral cortex by the single administration of tramadol at 20 mg/kg [51]. Moreover, serum TNF-α and IL-1β as well as brain myeloperoxidase levels, indicative of infiltration of neutrophils were raised in rats given tramadol for 9 weeks [52].

Conclusion

The finding in this study provided the first evidence for increased MCP-1 in brain and serum of rats following repeated cannabis and/or tramadol administration. Additionally, cannabis increased serum levels of COX-1 and COX-2. Our findings suggest that neuroinflammation is an important contributor to neurodegeneration in the brain of heavy cannabis users and could also mediate neuronal injury in abusers of tramadol.

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Conflicts of interest

The authors declare no conflicts of interest

Financial support

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Consent for publication

All authors agreed with the content and that all gave explicit consent to submit and publish.

Data availability

All data generated or analyzed during this study are included in this published article.

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