Acute Effects of *Cannabis sativa* on Ischaemia/Reperfusion Injury in the Rat Brain

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Abstract: We investigated the effect of *Cannabis sativa* extract on brain damage, oxidative stress and inflammation in rats with transient global cerebral ischaemia. Rats were subjected to bilateral common carotid artery (CCA) occlusion for 45 minutes followed by 4 h of reperfusion. Rats were treated with cannabis at a dose of 20 mg/kg (expressed as Δ^9-THC) intraperitoneally (i.p) either before CCA, at time of reperfusion or after reperfusion. Alternatively, cannabis was given i.p. daily for 2 days before surgery. Markers of oxidative stress (malondialdehyde, reduced glutathione, nitric oxide) and the proinflammatory cytokine tumour necrosis factor-alpha (TNF-α) were determined in brain tissue. Histopathological evaluation was also done. Compared with the sham-treatment group, CCA occlusion resulted in increased brain malondialdehyde (54.5 ± 2.0 *vs*. 26.0 ± 1.45 nmol/g. tissue; $p<0.05$) and nitric oxide (75.2 ± 3.2 *vs*. 31.3 ± 3.0 μmol/g. tissue; $p<0.05$) contents along with decreased brain reduced glutathione (6.6 ± 0.14 vs. 8.28 ± 0.31μmol/g. tissue; $p<0.05$). There was also a pronounced rise in brain TNF-α concentrations (2248 ± 105 *vs*. 51.42 ± 3.21 pg/g. tissue; $p<0.05$). *Cannabis sativa* significantly increased reduced glutathione (by 30.3%-60.6%; $p<0.05$) and alleviated the increase in nitric oxide levels (by 51.5%- 58.5%; $p<0.05$) in the ischaemic brain tissue. Cannabis given before or at time of CCA occlusion significantly reduced brain TNF-α by 24.4% and 26.7%, respectively (1699 ± 80 and 1647 ± 54 *vs*. 2248 ± 105 pg/g. tissue; $p<0.05$). Histopathological examination of the cerebral cortex from rats subjected to CCA occlusion revealed gliosis, vacuolation and widespread neuronal degeneration. Cannabis given as a single dose 1h prior to CCA ligation or as 2 days pretreatment conferred protection against the ischaemic neuronal injury. It is concluded that in cerebral ischaemia the prior administration of cannabis exerted neuroprotective effects which could be accounted for by a decrease in nitric oxide and in the inflammatory response.

Keywords: Cannabis, brain ischemia and reperfusion, oxidative stress, neuroinflammation.

INTRODUCTION

Cannabis sativa L. (Family *Cannabinaceae*) has long been known for its psychotropic effects and is considered the most widely abused substance worldwide [1]. Cannabis preparations mostly abused are marijuana which is the dried leaves and flowering tops or hashish, the compressed resin of the female plant [2]. Cannabis exerts a wide spectrum of acute central nervous system effects including euphoria or "high", short-term memory impairment, distorted time perception, increased sensory awareness, tachycardia, increased appetite, psychomotor and locomotor impairments [3]. Chronic and heavy users of cannabis exhibit cognitive decline, impaired memory processing and attention [4], structural brain changes [5] and are liable to develop psychosis [6]. These central effects of cannabis are mediated through interaction with CB1 cannabinoid receptor, G-protein coupled receptors, expressed in neurons in brain and spinal cord [7]. The main active psychotropic ingredient in herbal cannabis is Δ^9-tetrahydrocannabinol (Δ^9-THC) [8] but other phytocannabinoids eg., cannabigerol (CBG), cannabichromene (CBC), cannabidiol (CBD), cannabidivarin (CBDV), Δ^9-tetrahydrocannabivarin (THCV) are found and these might enhance or antagonize some of the Δ^9-THC effects [9].

Ischaemic stroke has been reported in heavy users of marijuana or hashish [10-14]. In these case reports a temporal association has been found between cannabis use and the development of stroke in that cerebral or cerebellar infarcts occurred during or shortly after consuming cannabis [10-12]. Moreover, recurrent stroke developed upon re-exposure to cannabis [12]. Users of cannabis who developed ischaemic stroke were more frequently young men and consumed tobacco and alcohol compared with non-users [13, 14]. Cannabis might cause ischaemic stroke by inducing cerebral vasospasm, cerebral hypoperfusion secondary to systemic hypotension or *via* cardiac embolization [14].

In cerebral ischaemia, strong evidence indicates an important role for increased oxidative/nitrosative stress and neuroinflammation that act to exacerbate the inflammatory milieu and the initial ischemic injury through the release of reactive oxygen metabolites and inflammatory mediators [151]. Reactive oxygen metabolites are produced by the cell's metabolic

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E-ISSN: 2309-6179/17 © 2017 Savvy Science Publisher

machinery. The mitochondrial complexes I and II leak electrons to molecular oxygen forming superoxide anion which in turn could result in the formation of hydrogen peroxide (H_2O_2), hydroxyl radical (·OH) or react with nitric oxide forming peroxynitrite. The brain is susceptible to free radical-mediated oxidative damage due to high rate of oxygen consumption, its rich content of polyunsaturated fatty acids, the presence of redox-active transition metals, namely iron and copper combined with paucity of antioxidant mechanisms compared to other tissues [16]. Significant increase in reactive oxygen species occurs early in ischaemia while the increase in cerebral blood flow during re-perfusion results in robust reactive oxygen species generation with damage to the cell macromolecules causing protein and DNA oxidation, lipid peroxidation and membrane damage [17]. Cerebral ischaemia also results in the expression of inflammatory genes and the release of several inflammatory mediators eg., interleukin-6 (IL-6), IL-1β, tumour necrosis factor-alpha (TNF- α) and inducible nitric oxide synthase (iNOS) and the influx of neutrophils into the injured tissue. It was suggested that this post-ischaemic local inflammatory response might contribute to the secondary brain damage [18, 19].

In this study, we aimed to investigate the effect of acute cannabis treatment on cerebral ischemia/reperfusion (I/R) injury induced in the rat by bilateral CCA occlusion, a well-recognized model of transient global cerebral ischaemia [20, 21].

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 230-250 g were obtained from the Animal House of the National Research Centre, Cairo. Rats were kept under temperature- and light-controlled conditions and given standard laboratory rodent chow and water *ad libitum*. The experimental procedures followed the recommendations of the institutional Ethics Committee of the National Research Centre and that of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Plant Material and Extraction

Cannabis sativa L. resin (Hashish) was a gift from the Ministry of Justice- Egypt. Extract of *Cannabis sativa* L. was obtained by chloroform treatment and contained ~ 20% Δ^9-THC as determined by "GC mass spec". The dry extract was prepared according to the method of Turner and Mahlberg [22] with modification. In brief, 10 g of hashish was divided into small pieces, well grounded and heated in a glass baker in boiling water at 100 ºC for two hours for decarboxylation of the acidic cannabinoids [22]. Cannabis was then placed in chloroform overnight, extracted three times with chloroform and fractions were combined, filtered over filter paper and finally collected in a 100 mL volumetric flask. The filtrate was then evaporated under a gentle stream of nitrogen. The dry extract was protected from light and stored at 4ºC. For use in experiments, the extract was suspended in 2 mL of 96 % ethanol and the total volume in the volumetric flask increased to 100 mL by distilled water. The extract was injected at the dose of 20 mg/kg (expressed as Δ^9-THC). The injection volume was 0.3 ml/rat.

Induction of Transient Global Cerebral Ischaemia

Cerebral ischaemia was induced by ligation of both carotid arteries for 45 min followed by 4 h reperfusion. Briefly, animals were fasted 12 hours before surgery and then anesthetized with thiopental (20 mg/kg; i.p.). A longitudinal cervical incision (2 cm) was made lateral to the midline and the common carotid artery (CCA) was carefully dissected. Ischemia was induced by placing non traumatic micro-vascular clip on either CCA just prior to its bifurcation. During ischemia, body temperature was maintained at 36.5 ± 0.5˚C using heating pad and respiration pattern monitored. The vascular occlusion was maintained for 45 minutes, and then the clips were removed to resume blood flow to the ischemic region. Finally, the incisions were sutured, the animal was allowed to recover from anesthesia, and returned to a warm cage during the 4 h reperfusion period.

Experimental Design

The effect of *Cannabis sativa* extract on the rat cerebral ischaemia/reperfusion injury was studied. Cannabis was given at the dose of 20 mg/kg (expressed as Δ^9-THC).

Rats were randomly allocated into 7 groups (8 rats each):

Group I: Non-operated rats.

Group II: Sham-operated rats.

Group III: Untreated rats received i.p. saline and underwent cerebral ischemia- reperfusion injury (I/R).

Group IV: Rats treated with a single dose of cannabis extract (20 mg/kg; i.p.) 1h before CCA ligation (i.e. before ischemia).

Group V: Rats treated with a single dose of cannabis extract (20 mg/kg; i.p.) at time of CCA occlusion.

Group VI: Rats treated with a single dose of cannabis extract (20 mg/kg; i.p.) 1 h after reperfusion.

Group VII: Rats treated for two days with cannabis extract (20 mg/kg/day; i.p.) followed by cerebral I/R injury.

Brain Homogenate Preparation

After 4 h of reperfusion, rats were decapitated under light diethyl ether anesthesia and their brain were carefully isolated and dissected through the midline into two hemispheres. 0.5 g of the affected hemisphere was homogenized (using MPW–120 homogenizer, Med instruments, Poland); the homogenate was then centrifuged using a cooling centrifuge (Laborezentrifugen, 2k15, Sigma, Germany) at 3000 r.p.m for 10 min and the supernatant was used for the determination of brain level of MDA, nitric oxide metabolites, and reduced glutathione.

Measurements

I. Lipid Peroxidation Products

Lipid peroxidation was assessed by measuring the level of malondialdehyde (MDA) according to the method of Ruiz-Larrea *et al*. [23]. The thiobarbituric acid reactive substances react with thiobarbituric acid to produce a red colored complex that exhibits a peak absorbance at 532 nm.

II. Reduced Glutathione

Brain reduced glutathione (GSH) was determined spectrophotometrically by the Ellman's method [24]. The procedure is based on the reduction of the Ellman's reagent by –SH groups of GSH to form 2-nitro-5-mercaptobenzoic acid, which is determined at 412 nm.

III. Nitric Oxide

Nitrite, the stable end product of nitric oxide, is mostly used as an indicator for the production of nitric oxide. Nitric oxide measured as nitrite was determined in brain homogenates using the Griess reagent, according to the method described by Moshage *et al*. [25].

IV. Tumour Necrosis Factor Alpha

TNF-α was measured in serum using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) Kit purchased from Glory Science Co., Ltd. (Del Rio, TX, USA) according to the manufacture instructions.

Histopathological Examination

The brain of different groups was removed immediately and fixed in 10% formol saline. Paraffin sections (5μm thick) were stained with haematoxlin and eosin and investigated by light microscope.

RESULTS

Biochemical Results

Data are presented in Table **1**.

I. Carotid Artery Ligation Only

Rats subjected to bilateral CCA ligation for 45 min exhibited a significant increase in brain lipid peroxidation as indicated by malondialdehyde level which rose to 109.6% of its sham control value (54.5 ± 2.0 *vs*. 26.0 ± 1.45 nmol/g. tissue; $p<0.05$). Meanwhile, there was marked and significant increase in brain nitric oxide by 140.2% (75.2 ± 3.2 *vs*. 31.3 ± 3.0 μmol/g. tissue; $p<0.05$) and a decrease in reduced glutathione by 20.3% (6.6 ± 0.14 *vs*. 8.28 ± 0.31μmol/g. tissue; $p<0.05$) of their corresponding sham values. On the other hand, the level of the proinflammatory cytokine TNF-α showed pronounced increase by 4271.8% compared with that in the sham control group (2248 ± 105 *vs*. 51.42 ± 3.21 pg/g. tissue; $p<0.05$).

II. Cannabis Given Before CCA Occlusion

There was no significant difference in brain malondialdehyde levels between the ischaemic control group and that treated with cannabis prior to CCA occlusion (54.5 ± 3.0 *vs*. 54.5 ± 2.0 nmol/g. tissue; $p>0.05$). Nitric oxide, however, decreased by 41.5 % (44.0 ±1.8 *vs*. 75.2 ± 3.2 μmol/g. tissue; $p<0.05$) and reduced glutathione increased by 48.5 % (9.8 ± 0.34 *vs*. 6.6 ± 0.14 μmol/g. tissue; $p<0.05$). Cannabis treatment also resulted in significant decrease in brain TNF-α by -24.4 % (1699 ± 80 *vs*. 2248 ± 105 pg/g. tissue; $p<0.05$) compared with the ischaemic untreated group.

III. Cannabis Given at Time of CCA Occlusion

No significant difference in brain malondialdehyde was observed between rats treated with cannabis at

Table 1: The effect of cannabis on reduced glutathione (GSH), malondialdehyde (MDA), nitric oxide and tumour necrosis factor-alpha (TNF-α) in the brain of rats subjected to iscahemia/reperfusion (I/R) injury

Groups	GSH (μmole/g. tissue)	MDA (nmol/g. tissue)	Nitric oxide (μmole/g. tissue)	TNF-α (Pg/g. tissue)
Control	8.43 ±0.26	23.4 ± 0.95	30.32 ± 1.92	44.6 ±1.80
Sham	8.28 ± 0.31	26.0 ± 1.45	31.30 ± 3.0	51.42 ± 3.21
Cerebral I/R control	6.6 ± 0.14*	54.5 ± 2.0*	75.2 ± 3.2*	2248 ± 105*
Single dose cannabis before occlusion	9.8 ± 0.34*+ (48.5 %)	54.5 ± 3.0* (0.0%)	44.0 ±1.8*+ (-41.5 %)	1699 ± 80*+ (-24.4 %)
Single dose cannabis at occlusion	9.6 ± 0.38*+ (45.5 %)	48.5 ± 0.75* (-11.0%)	31.2 ± 1.7*+ (-58.5 %)	1647 ± 54*+ (-26.7 %)
Single dose cannabis after reperfusion	8.6 ± 0.10*+ (30.3 %)	55.4 ± 1.7* (1.6%)	42.4 ± 1.3*+ (-43.6 %)	2370 ± 146* (5.4%)
Cannabis pretreatment for 2 days before I/R	10.6 ± 0.74*+ (60.6 %)	59.0 ± 4.2* (8.3%)	32.2 ± 1.3*+ (-57.2%)	1970 ±102* (-12.4 %)

Results are mean ± SEM. Abbreviations: IR, ischemia/reperfusion.
*p<0.05 *vs*. Sham group. +p<0.05 *vs*. I/R control. One-way ANOVA and Duncan multiple comparison test. The percent change from the cerebral I/R control group is shown in parenthesis.

time of vessel occlusion and the control ischaemic group (48.5 ± 0.75 *vs*. 54.5 ± 2.0 nmol/g. tissue; p>0.05). Cannabis treatment, however, was associated with a decrease in nitric oxide by 58.5% (31.2 ± 1.7 *vs*. 75.2 ± 3.2 μmol/g. tissue; p<0.05) and an increase in reduced glutathione by 45.5 % (9.6 ± 0.38 *vs*. 6.6 ± 0.14 μmol/g. tissue; p<0.05). A significant decrease in brain TNF-α by 26.7% was also noted compared with the ischaemic control value (1647 ± 54 *vs*. 2248 ± 105 pg/g. tissue; p<0.05).

IV. Cannabis Given After Reperfusion

In this group, brain malondialdehyde was not significantly altered (55.4 ± 1.7 *vs*. 54.5 ± 2.0 nmol/g. tissue; p>0.05) but nitric oxide decreased by 43.6% (42.4 ± 1.3 *vs*. 75.2 ± 3.2 μmol/g. tissue; p<0.05) and reduced glutathione increased by 30.3% (8.6 ± 0.10 *vs*. 6.6 ± 0.14 μmol/g. tissue; p<0.05) compared with the ischaemic untreated group. On the other hand, cannabis administered following reperfusion did not significantly alter brain TNF-α (2370 ± 146 *vs*. 2248 ± 105 pg/g. tissue; p>0.05).

V. Cannabis Pretreatment for 2 Days Before CCA Occlusion

Pretreatment with cannabis for 2 days prior to brain ischaemia has no significant effect on the level of malondialdehyde (59.0 ± 4.2 *vs*. 54.5 ± 2.0 nmol/g. tissue; p>0.05). Cannabis treatment was effective in increasing brain reduced glutathione (60.6% increment: 10.6 ± 0.74 *vs*. 6.6 ± 0.14 μmol/g. tissue; p<0.05). There was also a significant and marked decrease in brain nitric oxide by 57.2% (32.2 ± 1.3 *vs*. 75.2 ± 3.2 μmol/g. tissue; p<0.05). Meanwhile, there was a non-significant decrease in TNF-α concentrations by 12.4% as compared to the ischaemic untreated group (1970 ±102 *vs*. 2248 ± 105 pg/g. tissue; p>0.05).

Histopathological Results

I. Carotid Artery Ligation Only

Microscopic examination of brain tissue from un-operated rats showed the normal structure of the cerebral cortex with its innermost granular layer, Purkinje cell layer, and the outermost molecular layer (Figure **1A**). No apparent morphological changes were observed in the sham group, although some congested blood vessels were seen (Figure **1B**). Sections of the cerebral cortex from rats subjected to I/R showed congestion of cerebral blood vessels, hemorrhage in meninges above the surface and thrombotic vessels. Sings of degeneration in some neurons in the form of pyknotic nuclei (anoxic neurons) with eosinophilic cytoplasm were present. Foci of empty neural cells with identifiable gliosis and vacuolation were also observed (Figures **1C** & **D**).

II. Cannabis Given Before CCA Occlusion

In rats given cannabis 1h before CCA occlusion, most of neuronal cells appeared normal and a few appeared degenerated in the form of karyorrhexis and cytoplasmic vacuolation. Some neurons were seen shrunken with esinophilic cytoplasm and pyknotic nuclei (anoxic neurons) (Figures **2A** & **B**).

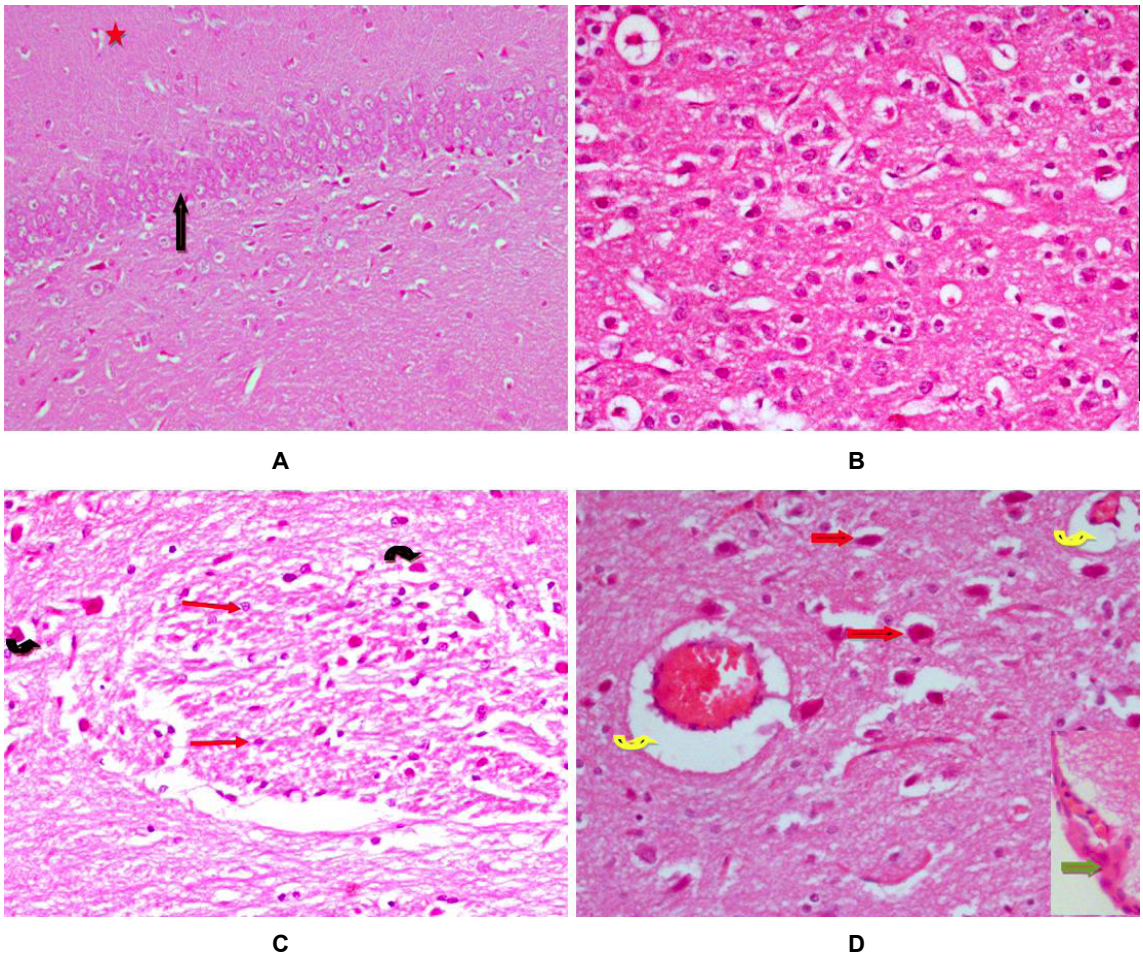
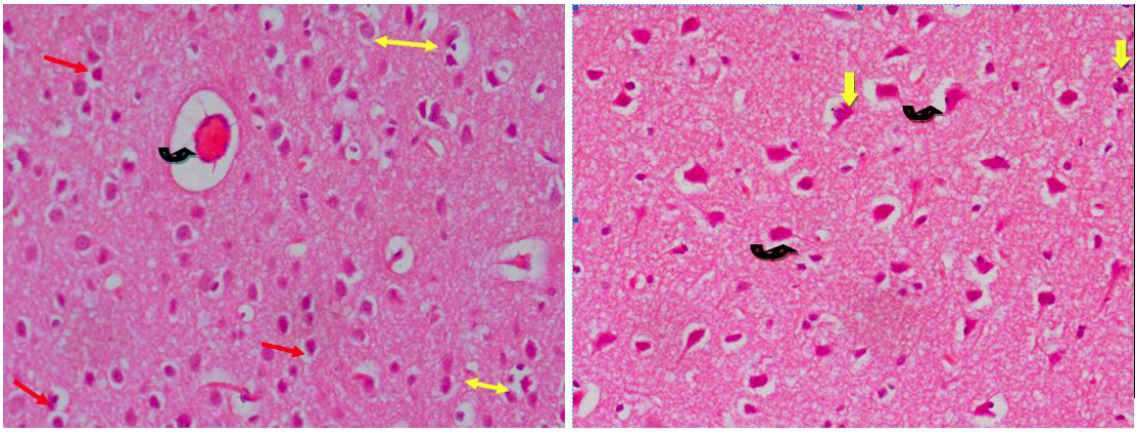
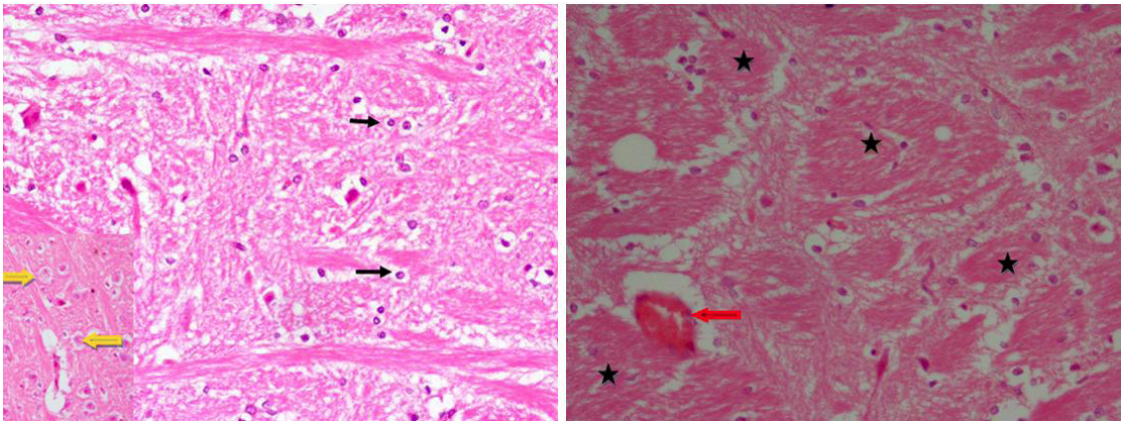


Figure 1: H & E stained brain sections. (**A**): Control non-operated rat showing normal histological structure of brain tissue (HX&E x200). (**B**) Sham-operated rat showing normal appearance (Hx&E x400). (**C**): Brain tissue after I/R showing gliosis (red arrow) and vacuolation (black curved arrow) (Hx & E x400). (**D**): Another filed of the ischemic brain showing hemorrhage in meninges above the surface and thrombotic vessels show a vessel with membrane bound vacuoles (yellow curved arrow). Sings of degeneration in some neurons and eosinophilic in others with pyknotic nuclei (anoxic neurons) (red arrow) are seen (Hx & E x400).

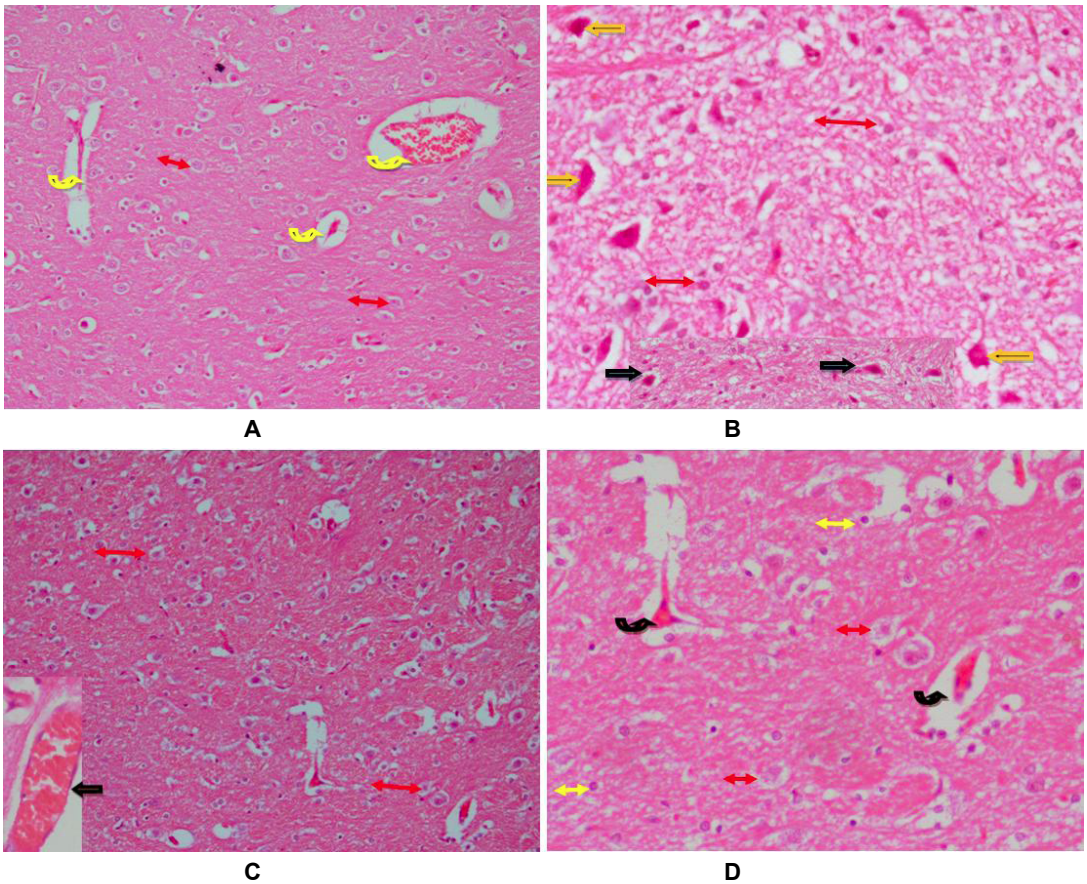


(Figure 2). Continued.



C **D**

Figure 2: H & E stained brain sections. (**A**): Cannabis given 1h before CCA occlusion: most of neuronal cells appeared normal and few degenerated in the form of karyorrhexis (yellow arrow) and cytoplasmic vacuolation (red arrow) (Hx &E x200). (**B**): Another filed showing easinophilic neuron (black arrow) (Hx &E x400). (**C**): Cannabis given at time of CCA occlusion: more pronounced pathological changes are seen in the form of widespread severe spongiform change with accompanying neuronal loss and some neuron appeared degenerated (yellow arrow) and gliosis (black arrow) (Hx & E x400). (**D**) Another filed in brain after cannabis being given after CCA occlusion showing multiple numbers of focal homogenous deeply eosinophilic plaques of various sizes and shapes (star). Within the plaque structure, nuclei of microglia cells could be seen and cytoplasmic vacuolations. Congestion of cerebral blood vessel (red arrow) and oedema are seen (Hx &E x400).



A **B**

C **D**

Figure 3: H & E stained brain sections. (**A**): Cannabis given after reperfusion showing congestion of cerebral blood vessel (yellow arrow) and degenerated neuron (red arrow) (Hx&E x400). (**B**) Another filed showing neuronal loss, eosinophilic neurons and perineuronal vacuolation develops (yellow arrow) and others with shrunken eosinophilic cytoplasm and pyknotic nuclei (anoxic neurons) (black arrow) with gliosis (red arrow) (Hx&E x400). (**C**) Cannabis given for two consecutive days prior to I/R showing some improvement in pathological changes as compared to previous group in the form of no pyknotic neurons; most neurons appeared normal but some degenerated neurons are still present (red arrow). Hemorrhage in meninges above the surface (red arrow) is seen (Hx&E x200). (**D**) Another filed showing congestion of cerebral blood vessel (black arrow) and few neurons with coagulation necrosis (red arrow) (Hx&E x400).

III. Cannabis Given at Time of CCA Occlusion

Sections of the cerebral cortex of ischemic brain of rats given cannabis after CCA occlusion showed more pronounced pathological changes in the form of multiple numbers of focal homogenous deeply eosinophilic plaques of various sizes and shapes, within the plaque structure, nuclei of microglia cells could be seen and cytoplasmic vacuolations. Congestion of cerebral blood vessels and edema were present. Widespread severe spongiform change with accompanying neuronal loss was observed (Figures **2C** & **D**).

IV. Cannabis Given After Reperfusion

Sections of the cerebral cortex of ischemic brain of rats given cannabis at time of reperfusion revealed degeneration of neurons and foci of empty neural cells. Few of neurons appeared shrunken with eosinophilic cytoplasm, pyknotic nuclei (anoxic neurons) and perineuronal vacuolation. Congestion of cerebral blood vessel and identifiable gliosis are seen (Figures **3A** & **B**).

V. Cannabis Pretreatment for 2 Days Before CCA Occlusion

In rats treated with cannabis for two consecutive days prior to I/R there was some improvement in the pathological changes as compared to previous group in the form of no pyknotic neurons. Most neurons appeared normal but some degenerated neurons were still present. Hemorrhage in meninges above the surface and congestion of cerebral blood vessels were also seen (Figures **3C** & **D**).

DISCUSSION

In this study, bilateral CCA occlusion for 4h resulted in brain oxidative stress as evidenced by the rise in the lipid peroxidation end product malondialdehyde, the increase in nitric oxide and by the depletion of the antioxidant molecule reduced glutathione. These observations are in accordance with other studies following transient or global cerebral ischaemia in rodents [20, 21]. In brain ischemia there is robust increase in reactive oxygen metabolites especially during the reperfusion period owing to the increase in O_2 delivery to brain tissue with a resultant oxidative damage to cell membrane fatty acids, proteins and DNA [17]. Our results shows that reduced glutathione, an important intracellular antioxidant and free radical scavenger [26] decreased significantly in brain tissue of rats with I/R. Glutathione (γ-glutamylcysteinylglycine) is the most abundant intracellular non-protein thiol and by switching between its reduced (GSH) and oxidized (glutathione disulfide: GSSG) forms helps to maintain the redox equilibrium of the cell [26]. The decrease in reduced glutathione following cerebral ischaemia and reperfusion is likely to reflect consumption of reduced glutathione by reactive oxygen metabolites. Thus in brain ischaemia, the antioxidant defenses are overwhelmed by the high level of reactive free radicals generated by the mitochondrial electron transport chain with consequent neuronal damage [27]. Studies indicated an important role of oxidative stress in brain ischaemic injury [28, 29]. It has been shown that mice with 50% decrease in the activity of the antioxidant enzyme superoxide dismutase exhibited higher rate of neuronal death and increased mortality after brain ischaemia [28]. Conversely, overexpression of the enzyme protected the brain in transient global cerebral ischemia and reperfusion [29].

Our results also indicated marked increase in nitric acid content in the brain of rats subjected to ischaemia/reperfusion (I/R). Increased nitric oxide level has been observed in the rat brain following middle cerebral artery ligation and bilateral CCA occlusion [20, 30]. Nitric oxide is a short-lived signaling molecule produced by nitric oxide synthase enzyme in a reaction that converts arginine to nitric oxide and citrulline. In physiologic concentrations, nitric oxide derived from the constitutive nitric oxide synthases in neuronal cells and endothelium plays an important role in maintaining vascular tone, neurotransmission and neuroplasticity. Excessive generation of nitric oxide for prolonged time, however, can be damaging to neurons [31]. Nitric oxide is an important contributor to the development of brain ischaemic injury. The latter induces the expression of the neuronal and endothelial nitric oxide synthase (NOS) isoforms [32]. The increase in brain nitric oxide during ischaemia also stems from the inducible isoform of NOS (iNOS) in neutrophils infiltrating the ischaemic brain and from the resident brain immune cells microglia [33]. This increase in nitric oxide can cause neuronal damage by inactivation of mitochondrial electron transport complexes and cellular energy depletion. Nitric oxide can also be cytotoxic *via* the formation of more reactive nitrogen species like peroxynitrite (ONOO-) generated by the reaction with superoxide anion radical. Peroxynitrite can also result in the formation of hydroxyl free radicals and nitrogen dioxide and result in nitrosylation of protein tyrosine residues [31]. Studies indicated a decrease in brain infarct volume after iNOS inhibition in rats subjected to middle cerebral artery occlusion [34]. Inhibition of nitric oxide would therefore be of benefit in reducing neuronal loss in acute cerebral ischaemia.

In this study, a pronounced increase in brain TNF-α concentration after I/R was observed. The neuroinflammatory response that accompany acute cerebral ischaemia is mediated by cytokines eg., such as TNF-α and interleukin-1β released by ischaemic neurons and glia and results in the increased endothelial adhesion molecules, breakdown of the blood brain barrier and recruitment of immune cells into the damaged tissue, thereby, exacerbating inflammation and brain damage [18, 19]. The pleotrophic cytokine TNF-α is synthesized by macrophages and monocytes and TNF-α receptors are expressed on neurons and glia cells [35]. When released in low concentrations, TNF-α could be neuroprotective *via* TNF-α receptor 2 and this has been shown in hippocampal slice culture during excitotoxicity. Higher concentration, however, increased neuronal damage [36].

Cannabis has been shown to cause neurodegenerative changes in the rat brain in the form of dark and small-sized neurons, cellular infiltration, and gliosis [37]. Cannabis also intensified neuroinflammation due to systemic lipopolysaccharide injection in mice with the development of neuronal atrophy and shrinkage, pyknosis and cell necrosis. Cannabis increased the expression of the apoptotic marker caspase-3 in rodent brain [37, 38]. *In vitro*, cannabis caused oxidative stress and impaired brain mitochondrial respiratory chain function [39, 40]. Hippocampal neuronal cell body and nuclear shrinkage also occurred after cannabis application [41] while THC given to rats impaired hippocampal neurogenesis [42]. Yet, other studies have shown neuroprotective effects for Δ^9-THC against excitotoxic brain injury, most likely via cannabinoid receptor-independent antioxidant mechanism [43, 44]. Chen *et al*. [43] showed that N-methyl-D-aspartate (NMDA) neuronal toxicity *in vitro* was reduced by Δ^9-THC. Higher concentrations, however, were toxic, via CB1 receptor stimulation. Glutamate-mediated death of rat hippocampal neurons in culture was also reduced by Δ^9-THC [44].

The findings of this study indicates that the administration of an extract of *Cannabis sativa* rich in Δ^9-THC shortly before the development of brain ischaemia is associated with neuroprotection in a rat model of transient global cerebral I/R injury. In contrast, no protection or even increased neuronal damage was observed upon later administration of the herbal cannabis. These observations are intriguing in view of the reports associating cannabis intake with the development of ischaemic brain injury in man [10-14]. In this study, cannabis was found to result in recovery of brain reduced glutathione and nitric oxide levels. Moreover, cannabis given before ischaemia significantly reduced the brain concentrations of the proinflammatory cytokine TNF-α. These findings are in accordance with our previous observations in which cannabis decreased brain oxidative stress, nitric oxide and TNF-α [38, 45, 46]. Other studies have also shown a modulatory effect for cannabinoids Δ^9-THC on the release of inflammatory cytokines (IL-6, IL-1β, IL-12 and IL-23 and TNF-α) by macrophages and brain microglia in culture [47-49]. The mechanisms by which acute cannabis given prior to brain ischaemia prevents neuronal damage might thus involve an antioxidant action and decreased release of TNF-α.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest

ACKNOWLEDGEMENTS

This works is was not supported by research grants

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Received on 15-06-2017 Accepted on 15-08-2017 Published on 11-10-2017

DOI: https://doi.org/10.12974/2309-6179.2017.05.06

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