



The Prophylactic Role of Chrysin against Clonazepam Induced Brain Toxicity in Male Albino Rats

Rehab M. Mosaad ^{a,b}, Marwan A. Ibrahim ^{a++*}
and Hend A. Sabry ^b

^a Department of Biology, College of Science, Majmaah University, Majmaah 11952, Saudi Arabia.

^b Department of Zoology, Women's College, Ain Shams University, Egypt.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: The bioactive substance chyrus is found in bee propolis and all plants of the species Passiflora. It is well recognized to have neuroprotective properties and a wide range of pharmacological activity.

Purpose: In this work, the consequence of Chrysin administration (50 mg/kg b.wt/day) on brain toxicity caused by Clonazepam (CZP, 2mg/kg b.wt/day) were investigated by measuring NaK ATPase, neuronal oxidative stress, neuro- inflammation, and DNA fragmentation.

Study Design: In our investigation, we used male albino rats 4 weeks old and weighing 60 ± 5 g. There were four groups of ten rats apiece: Group 1: the control group which treated a vehicle with 1% w/v Tween 80. Group 2: given 1% w/v Tween 80-suspended Clonazepam (CZP) at a dose of 2

++ Assistant Professor of Applied Physiology;

*Corresponding author: Email: m.ab.ibrahim@mu.edu.sa;

mg/kg b.wt./day. In Group 3: Chrysin suspended in 1% w/v Tween 80 was given at a rate of 50 mg/kg b.wt./day. Group 4: Clonazepam (CZP) and Chrysin were given at the same prior dosages as before (2 mg/kg b.wt./day for Clonazepam (CZP) and 50 mg/kg b.wt./day for Chrysin).

Methods: Malondialdehyde, nitric oxide, DNA fragmentation, sodium oxide dismutase, catalase, and Na-K ATPase contents were estimated.

Results: According to the biochemical analysis, after the Clonazepam therapy, the brain's contents of malondialdehyde (MDA), nitric oxide (NO), and DNA fragmentation increased, while those of superoxide dismutase (SOD), catalase (CAT), and NaK ATPase activities declined.

Conversely, the biochemical screening of animal brain tissue administered with CZP+ Chrysin revealed an improvement in the brain tissue's ability to withstand the damage caused by CZP.

Keywords: Chrysin; clonazepam; male albino rats; neurotoxicity.

1. INTRODUCTION

Drug addiction and drug misuse are major causes of brain diseases through oxidative stress, mitochondrial malfunction, apoptosis, and a reduction in neurogenesis. Drug-induced neurotoxicity and neuronal dysfunction are reflected in the neural deterioration found in drug users [1].

A long-term therapy medicine, clonazepam is a class II as maintained by the Biopharmaceutical Classification System and a derivative of nitrobenzodiazepines which are psychoactive drugs used in epilepsy control and as central nervous system depressants to decline seizure activity. It causes many side effects such as ataxia, sedation, amnesia, and myo-relaxation. Nitrobenzodiazepine works by modulating GABA receptors to produce sedative, anticonvulsant, muscle relaxant, and anxiolytic effects and regulate chloride transportation through the ion channel complex. Clonazepam has a euphoric effect that makes it a popular drug of abuse. It affects d-aminobutyric acid (GABA), causing retrograde amnesia and impairment of memory [2-6]

As an antiepileptic medication, clonazepam is frequently recommended for different neurological and psychiatric circumstances, as well as drug addiction treatment. Despite being regarded as safe; clonazepam is abused as a street drug [7-9]. Moreover, clonazepam used with another antiepileptic drug is considered an obstacle in studying its long-term effects on patients [10,11], so this study examined its daily long-term effects on rats. Clonazepam is released directly into the blood-brain barrier and distributed to many body sites which causes a lot of side effects such as anorexia and palpitation [2,12-15]. Therefore, we aimed to study some of these side effects.

The primary plant metabolites, flavonoids are polyphenolic ingredients with a wide range of biological activities, including those that are anti-inflammatory, antithrombotic, antioxidant, antiallergic, antibacterial, analgesic, and vasodilatory. These effects are mediated by numerous signaling pathways and physiological mechanisms that are involved in a number of medical disorders [16-19].

A naturally occurring flavonoid called chrysin (5,7-dihydroxyflavone) has been extracted from a variety of plants, including propolis and honey [20-22], chamomile and mushrooms [23], and a few other medicinal plants such as *Radix scutellariae* [24]. Moreover, Chrysin has been utilized in traditional medicine since ancient times. Research using various animal models examined and reported its numerous biological activities, such as anti-inflammatory, antioxidant, vasorelaxant, and neuroprotective effects [25-29]. Chrysin's solubility and bioavailability are related to its beneficial effects. Intestinal absorption of Chrysin is low, with a maximum concentration of 12 to 64 nM in serum. Among the many pharmacological properties of chrysin is antiasthmatic action. It inhibits DNA topoisomerases, histone deacetylase, nuclear factor-kB (NF-kB), and inducible nitric oxide synthase (iNOS) [12,16,17,24].

Recent research recorded that natural and synthetic flavonoids have activity on GABA type A receptors and mental disorders treatment [5]. Also, they mentioned that chrysin is a fixed ligand for benzodiazepine in which it inhibits flunitrazepam binding to the benzodiazepine binding site. Therefore, we aimed to study the effect of chrysin on Clonazepam-induced oxidative stress. As there is a high concentration of lipids in the brain, we also aimed to investigate oxidative stress, antioxidant status, and its hazard effects on DNA content and Na-K ATPase activity.

2. MATERIALS AND METHODS

2.1 The Experimental Animals

In the current investigation, forty Wister male rats at 4 weeks of age and weighing 60 ± 5 g, were used. The rats were accommodated in cages of plastic with ten animals per cage, acquired from the National Organization for Drug Control and Research's (NODCAR) animal shelter. Throughout the experiment, animals were housed at a consistent temperature of 25 ± 2 °C and subjected to a 12-hour light-and-dark cycle. For the study, a pellet diet billboard was utilized. Before the study began, the animals were allowed a week to become acclimated to the lab environment. The experimental procedures managed in agreement with the guidelines put forth by the Ain Shams University Ethics Committee of Scientific Research in Cairo, Egypt.

2.2 The drugs

Alfa Aesar Germany produced 5,7-dihydroxyflavone, or chrysin, in a 98% concentration. Generic versions of clonazepam 2 mg oral tablets are marketed under the brand name Apetryl. Multi-Apex manufactures Apetryl tablets for the pharmaceutical industry in S.A.E. Badr City, Egypt.

2.3 The Experimental Design

The animals split up into the following 4 groups, each containing ten rats: El Khashab et al. report that Group 1 is the control group that given a vehicle containing 1% w/v Tween 80. [30]. Group 2 was administered Clonazepam (CZP) [31] at a dose of 2 mg/kg b.wt./day, suspended in 1% w/v Tween 80 [32]. – Group 3 [33] was given 50 mg/kg b.wt./day of Chrysin suspended in 1% w/v Tween 80 [30]. The same prior dosages of CZP (2 mg/kg b.wt./day) and Chrysin (50 mg/kg b.wt./day) were administered to Group 4 (Clonazepam and Chrysin). daily and for six weeks, all animal groups received oral gavage treatment. Reagan-Shaw et al., noted that the chosen doses are pharmaceutical doses that are equivalent to human body weight [34].

2.4 Preparation of Tissue Homogenate

Following animal sacrifice, brain tissue was thoroughly cleaned and then rinsed with ice.

After lightly blotting each tissue using filter paper, a balance was used to weigh each one. Using a polytron homogenizer at 40 °C, we created 0.05 M phosphate buffer (pH 7) for 10% of the tissue homogenate. To exclude unbroken cells, mitochondria, erythrocytes, nuclei, and cell debris, the homogenate samples were centrifuged for 20 minutes at 10,000 rpm. The following enzymes were measured in the supernatant: catalase, sodium oxide dismutase, nitric oxide (NO), fragmentation of DNA, and malondialdehyde (MDA).

2.5 Biochemical Assays

Brain malondialdehyde was identified as thiobarbituric acid (TBA) by Ohkawa [35], and nitric oxide (NO) in brain homogenate was measured using the colorimetric method of Nims et al. [36]. DNA fragmentation in the brain was performed using Agarose Gel Electrophoresis. The colorimetric method of Biovision, USA was utilized to assess the activities of catalase (CAT), superoxide dismutase (SOD), and sodium-potassium ATPase.

3. RESULTS

Malondialdehyde contents in brain rats of different groups were measured after 6 weeks of different administrations. It recorded a significant increment (2.90 ± 0.05 nmol/mg tissue protein) after the treatment of Clonazepam, and there was a significant reduction after chrysin administration (1.71 ± 0.04 nmol/mg tissue protein). As known, the ADA did not change in the chrysin group (Table 1).

In the data illustrated in Table (1) Clonazepam induced a significant increase in nitric oxide when compared to the control group (27.08 ± 0.99 μ mol/mg tissue protein) and the association of chrysin with Clonazepam reversed this effect (18.85 ± 0.20 μ mol/mg tissue protein). The measurements of brain antioxidant status parameters are represented in Table (1). It was SOD and CAT activates in the brain tissues and were significantly increased as a result of Clonazepam administration (SOD= 0.68 ± 0.04 U/mg tissue protein & CAT= 1.35 ± 0.06 nmol/min/mg tissue protein) and reversed after the chrysin treatment (SOD= 2.05 ± 0.09 U/mg tissue protein & CAT= 3.53 ± 0.06 nmol/min/mg tissue protein).

Table 1. Malondialdehyde (MDA) content (nmol/mg tissue protein), NO content ($\mu\text{mol/mg}$ tissue protein), DNA fragmentation (OD at 260nm), CAT activity (nmol/min/mg tissue protein), SOD activity (U/mg tissue protein), and NaK ATPase (mU/mg tissue protein) are shown for the various treated groups

Parameters Groups	MDA	NO	DNA fragmentation	CAT	SOD	NaK ATPase
CONT	0.87 \pm 0.05	17.10 \pm 0.91	0.81 \pm 0.02	4.70 \pm 0.08	2.53 \pm 0.08	4.98 \pm 0.15
CHRY	0.91 \pm 0.10	16.18 \pm 0.45	0.80 \pm 0.07	4.88 \pm 0.12	2.35 \pm 0.06	5.03 \pm 0.23
CLONZ	2.90 \pm 0.05 ^a	27.08 \pm 0.99 ^a	1.70 \pm 0.04 ^a	1.35 \pm 0.06 ^a	0.68 \pm 0.04 ^a	1.89 \pm 0.10 ^a
CLONZ+ CHRY	1.71 \pm 0.04 ^{ab}	18.85 \pm 0.20 ^b	0.99 \pm 0.02 ^{ab}	3.53 \pm 0.06 ^{ab}	2.05 \pm 0.09 ^{ab}	3.90 \pm 0.11 ^{ab}

Values are the means of 8 rats \pm SE at $p < 0.05$. 'a' represents a significant change from the control group (G1), and 'b' represents a significant change from the CLONZ (G3) group

Table (1) showed the DNA fragmentation contents of all rats after the treatment of Clonazepam (1.70 \pm 0.04 OD at 260nm) and chrysin, administration revealed ameliorative effect (0.99 \pm 0.02 OD at 260nm). Also, the Na-K ATPase activity decreased after the treatment of Clonazepam (1.89 \pm 0.10 mU/mg tissue protein) and a significant increment after the chrysin (3.90 \pm 0.11 mU/mg tissue protein) treatment as compared to the control group.

4. DISCUSSION AND CONCLUSION

As an anti-anxiety drug, clonazepam influences gamma-aminobutyric acid (GABA) [37,38]. Immoderate brain activity is probable to contribute to anxiety and other psychological disorders. Clonazepam is used to treat panic disease and to prevent some forms of convulsions. On the other hand, it causes many troubles in the brain processes causing neurological impairments like in drug abusers [1].

Drug addiction induced neurotoxicity which is related to oxidative stress. In general, drug of misuse are a source of oxidative damage because they increase levels of reactive oxygen species (ROS), prompt oxidation of proteins and lipids, and depletion of the total antioxidant systems within cells [25,26].

In our study, the Malondialdehyde (MDA) content increased significantly in the clonazepam tissue homogenates. However, the SOD and CAT showed significant decreases as compared to the control rats. Sabry et al. stated that the increment in reactive species accompanied by the antioxidant defense system decreased [39].

Lakshmi et al and Nixon et al stated that the increment of MDA in nervous tissue causes a decrease in antioxidant capacity and causes apoptosis in the cerebral cortex of animals.

Moreover, the peroxidation of lipids and proteins causes neurological conditions like Alzheimer's [26,40-42].

Nitric oxide plays an important role in CNS physiology as the regulation of neurotransmitter biosynthesis. However, it reacts with the reactive oxygen species and forms peroxynitrite (ONOO.). ONOO nitrosylate protein, DNA, RNA, lipids, and other cellular molecules [43,44].

It is known that brain NO dysfunction causes many diseases as anxiety and the etiology of MDD [45]. NO can make a reaction with superoxide and produce nitrite and nitrogen dioxide which cause DNA damage [46].

A significant increment in nitric oxide as a result of clonazepam injections daily for 6 weeks. Animals treated with diazepam had increased inflammatory cellular infiltrate and mild expression of p53 identified in the nuclei of some neurons [47,48].

In the present study DNA fragmentation increased after the clonazepam treatment and our results agree with Girgis et al., who examined the clonazepam abuse and changes in the neurons and DNA in rat brains and recorded that there is a significant degradation in brain DNA as compared to control rats [49]. Moreover, Bittigau et al. recorded that there is apoptotic neurodegeneration in the clonazepam brain rat tissues [50].

Moreover, Bittigau et al. tested the effects of many antiepileptic drugs (AEDs) on 7-day-old rats, such as diazepam (30 mg/kg) and clonazepam (0.5–4 mg/kg), and recorded that diazepam exerts wide-spread apoptotic neurodegeneration and clonazepam has neurotoxic effects. They returned these data to the apoptotic death of AEDs [50].

Our study recorded a significant depletion in Na-K ATPase content as a result of clonazepam administration. Numerous studies have shown that mitochondrial dysfunction and toxic consequences are common side effects of several marketed medicines. According to Khan et al and Nadanaciva et al., mitochondria are thought to be the primary target organelle for drug-induced toxicity [51,52]. They can be injured through a variety of mechanisms, including oxidation of fatty acids, increased mitochondrial DNA damage, redox cycling, electron transport uncoupling from adenosine triphosphate (ATP) synthesis, and mitochondrial permeability transition pore opening. A variety of acute and chronic neurological disorders are influenced by mitochondrial impairment, and its involvement in epilepsy provides proof of respiratory chain action failure in the more recent sporadic forms [53].

Recent research stated that the natural flavonoids have activity on GABA type A receptors and mental disorders treatment and added that chrysin is a fixed ligand for benzodiazepine in which it inhibits flunitrazepam binding to benzodiazepine binding site [5].

Our study recorded the brain ameliorative effects of chrysin, which represented a reverse result in MDA, nitric oxide (NO), and DNA fragmentation in animals injected with clonazepam through increasing SOD and CAT. Moreover, it repaired the level of Na-K ATPase activity in the brain tissues.

In the brain tissue of mice treated with D-galactose, Anand et al found that chrysin has ameliorative effects on redox status, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione [54]. Additionally, it lessens lipid peroxidation, enhances memory impairment, and shields rats' brains from chronic cerebral hypoperfusion-induced neuronal death [27,55].

Moreover, Chrysin has many pharmacological properties including antioxidant, antidepressive, and cognitive improving effects [56,57]. It inhibits DNA topoisomerases, histone deacetylase, nuclear factor-kB (NF-kB), and inducible nitric oxide synthase (iNOS) [16,17].

Chrysin stopped the release of nitric oxide, pro-inflammatory cytokines like TNF- α and IL-1 β , and the expressions of iNOS and COX-2 by preventing signaling molecules activations linked

to neuroinflammation (c-Jun N-terminal kinase and NF-kB) [17].

Mehri et al. examined the effects of chrysin in acrylamide on increased TNF- α and stated that chrysin led to the decline of TNF- α , and this result is by the study of Gresa-Arribas et al., Also, chrysin decreases oxidative stress and inflammation, which decreases age-related cognitive decline [33,58,59].

The data of the present study illustrated that chrysin was capable of improving the hazard effects of clonazepam administration that presented as the increment in Malondialdehyde (MDA) and nitric oxide (NO). Also, chrysin was effective to diminish the DNA fragmentation and improve superoxide dismutase (SOD), catalase (CAT) brain contents by the 50 mg/kg dose.

ETHICAL APPROVAL

The protocols and investigational methods were approved by the authorities of Ain Shams University and adhere to Egyptian standards for animal protection, as per the Animals (Scientific Procedures) Act, 1986 in the United Kingdom, related guidelines, and the European Communities Council Directive of November 24, 1986 (86/609/EEC). Study Code# ASU/W/Sci-5R/23-2-27.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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