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Original Research

Flumazenil Pretreatment Reduces Mefenamic Acid-Induced Central Nervous System Toxicity in Mice

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Abstract

Background: Mefenamic acid (MFA), a common analgesic, causes central nervous system (CNS) toxicity at high doses with a proposed activity on the Gamma-aminobutyric acid (GABA) system. However, it remains unknown whether flumazenil (FMZ), a GABA type A receptor (GABAAR) antagonist, can reverse MFA toxicity. **Methods**: The behavioral and neurophysiological effects of MFA were investigated in mice with and without FMZ pre-treatment. The elevated zero maze (EZM) and marble burying tests were used to assess anxiety-like behaviors and burying activities, respectively. The standard bar test was used to evaluate catalepsy, while the actophotometer test was used to measure locomotor activity. Seizure intensity was scored, and fatalities were counted. **Results**: Without FMZ pre-treatment, MFA induced behavioral and neurophysiological effects in a dose-dependent manner as follows: At a dose of 20 mg/kg, i.p, MFA-treated mice exhibited anxiety-like behaviors, which was determined by a significant increase in the time spent in the closed areas and a significant decrease in the number of entries to the open areas of the EZM apparatus. These mice also showed a significant decrease in the burying activity, manifested as a significant decrease in the number of buried marbles. At 40 mg/kg, i.p., MFA-treated mice showed catalepsy that was associated with a significant decrease in locomotor activity. At a dose of 80 mg/kg, i.p., mice developed fatal tonic-clonic seizures (seizure score = 4). Pre-treatment with FMZ (5 mg/kg, i.p.) significantly reversed the anxiety-like behaviors and restored marble-burying activity. Additionally, FMZ prevented catalepsy, significantly restored locomotor activity, reduced seizure intensity (seizure score = 0.3) and significantly reduced mortalities. **Conclusions**: The present study's findings indicate that activation of the GABAAR is involved in the CNS toxicity of MFA, and FMZ reverses MFA toxicity by interfering with this receptor.

Keywords: neurological disease; psychiatric disease; anxiety; central nervous system; convulsions; gamma-aminobutyric acid type A receptors; GABA

1. Introduction

Flumazenil (FMZ), a specific antidote for benzodiazepines, is the first benzodiazepine receptor blocker approved for clinical use [1]. It is primarily used to reverse the sedation caused by the overdose of benzodiazepines [2,3]. The pharmacodynamics of this drug are related to its ability to block Gamma-aminobutyric acid receptor type A (GABAAR) by binding selectively at the benzodiazepine binding site [4,5].

Given that Gamma-aminobutyric acid (GABA) is a primary inhibitory neurotransmitter in the brain [6], GABAAR is thought to have constitutive activity in reducing anxiety state and inhibiting central nervous system (CNS) convulsions. Research has indicated that GABA binds to its recognition site at the postsynaptic GABAAR, a ligand-gated chloride channel, allowing chloride ions to enter neurons, causing hyperpolarization and inhibiting action potential transmission [7]. Thus, defects in GABAAR activity constitute a risk factor for developing seizures and anxiety-related disorders, which necessitate effective medical interventions [8,9]. The use of benzodiazepines, positive allosteric modulators for GABAAR, may help to reduce anxiety and inhibit CNS convulsion [10]. On the other hand, the use of beta-carbolines, which act as inverse agonists at benzodiazepine binding sites of GABAAR, is associated with a high risk of developing anxiety and CNS convulsions [11]. Interestingly, a line of studies found that FMZ can block the action of some benzodiazepines and beta-carbolines [12,13]. This may suggest, at least in part, that inhibiting the constituent activity of GABAAR is an important dynamic effect for a variety of epileptogenic and anxiogenic agents, including the analgesic drug mefenamic acid (MFA).

Previous studies have indicated that MFA, a common drug in the fenamate family of non-steroidal anti-inflammatory drugs (NSAIDs), has a modulatory effect on GABAAR that depends on the specific beta subunits present in the receptor [14]. Although MFA has been used for a variety of therapeutic benefits, particularly as an anti-inflammatory [15], analgesic [16], and antipyretic properties [17], recent studies suggest that its potential risk of CNS toxicity limits its clinical applications [18]. In several case reports and small case series, MFA overdose has been shown to cause CNS convulsions and dystonic reactions [19]. In addition, acute toxicity tests in animals, particularly rats and mice, demonstrated that intraperitoneal injection of MFA (beyond the maximum tolerated dose of 20 mg/kg body weight) could induce various adverse neurophysiological effects that occurred in a dose-dependent manner [20]. Animals received 40 mg/kg showed severe muscle spasms with a prominent decrease in locomotor activity, whereas those treated with 80 mg/kg developed fatal seizures. Our earlier study [20] demonstrated that diazepam, a benzodiazepine receptor agonist, significantly reversed CNS convulsions induced by MFA in mice. However, it is unknown whether flumazenil, a benzodiazepine receptor antagonist, can alter the intensity of MFA toxicity *in vivo*.

The present study investigated MFA toxicity with and without pre-treatment with flumazenil in mice. It was expected from this study that pre-treatment with FMZ could, at least partially, reverse the central adverse effects caused by MFA.

2. Materials and Methods

2.1 Animal Housing and Husbandry

Male Albino Swiss mice weighing 28–31 g, with an age range of 10–12 weeks, were used in this study. The mice were housed in a free-noise room under well-controlled conditions (Room temperature 25 °C $\pm$ 4, humidity 48% $\pm$ 5) and were allowed to be acclimatized to those conditions for at least 7 days before any experimental manipulation. The mice were placed in appropriate cages and submitted to free access to drinking water and standardized food pellets. All animal handling, manipulation, and treatment were conducted according to the international animal care and use guidelines and approved by Research Ethics Committee, Isra University/Jordan (2019/2018/17-174).

2.2 Animal Groups and Treatments

The reversal effects of flumazenil against CNS toxicity caused by MFA were evaluated at three dosing levels. All doses were selected based on a previous study [21]. A total of 126 mice were utilized, and they were divided into three sets, each containing seven groups. Six mice were randomly assigned to each group. The study groups included a control group that received drug vehicle without any drugs, MFA-treated groups at doses of 20, 40, and 80 mg/kg, i.p., and groups that received flumazenil (5 mg/kg, i.p.) 30 minutes before being injected with MFA at doses of 20, 40, and 80 mg/kg, i.p.

After treatment, mice were subjected to various behavioral and neurophysiological evaluations. Each evaluation was conducted in triplicate trials using three animal sets to ensure the reproducibility of the results. The experimental procedures are described in detail in the following sections.

2.3 Evaluating Flumazenil Effect against Behavioral Effects Caused by 20 mg/kg MFA

2.3.1 Elevated Zero Maze Test

The reversal effect of flumazenil on anxiety caused by MFA was investigated using the elevated zero maze test. EZM apparatus was fabricated and used as described previously [22,23]. The maze apparatus was made of a circular passage (5 cm corridor) designed at a "0" shape and elevated 50 cm from the floor. The maze was made of four quadrants: two opposite closed quadrants (enclosed by walls) and two opposite open quadrants. Mice were submitted individually to the experimental procedures 30 min after drug treatments. The experiment began when the mouse was placed in the midpoint of an open quadrant with the mouse head facing one of the closed quadrants. Anxiety behaviors were evaluated through two measuring parameters: the time spent in the closed quadrants and the total number of entries into the open quadrants.

2.3.2 Marbles Burying Test

Examination of marble-burying behavior was conducted according to a procedure described previously [24, 25]. Mice were subjected to the experiment after 30 min of drug treatment. The experiment began when each mouse was placed in a cage filled with soft bedding materials (5 cm in depth) with 20 marbles (2.5 cm in diameter) evenly arranged at the top of the bedding surface. The burying behavior was evaluated by counting the marbles buried by at least two-thirds of their original volume.

2.4 Evaluating Flumazenil Effect against Central Effects Caused by 40 mg/kg MFA

2.4.1 Evaluating Locomotor Activity

The actophotometer model (UGO Basile cage with a digital counter, photocell, and a light source) was used to measure the locomotor activity (horizontal movement) of mice, as described previously [26,27]. After 30 min of drug treatment, mice were placed individually in the activity cage while the total number of light beam interruptions (activity score) was recorded automatically over 10 min. The score of decreased activity was considered an index of CNS depression.

2.4.2 Catalepsy Evaluation

After 5 min of drug administration, mice were subjected to the standard bar test procedure described previously [28,29]. The experiment began with the mouse's

front forepaws being placed on a 5-cm elevated wooden bar above the ground, creating an unusual position for the mouse. The time it took to drop the forepaws to the ground was recorded, and the delayed onset of the forepaws dropping for more than 2 min was regarded as an index of catalepsy. The percentage of cataleptic mice was calculated in each group.

2.5 Flumazenil Effect against Central Effects Caused by 80 mg/kg MFA

2.5.1 Seizure Evaluation

After 2 min of drug administration, mice were examined individually for seizure development. The seizure intensity was scored as follows: 0 indicates that seizure was absent, 1 indicates akinesia, 2 indicates myoclonic seizure, 3 indicates rearing seizure, and 4 indicates tonic-clonic seizure.

2.5.2 Mortality Assessment

The number of deaths was counted over 14 days after the drug administration. The percentage of mortality was used as an index for death assessment.

2.6 Statistical Analysis

Data obtained from animal experimentation are presented as an average of triplicate trial ± standard deviation (SD). Using GraphPad Prism software (GraphPad version 8.0, La Jolla, CA, USA), a significant difference between groups was determined by the One-way analysis of variance (ANOVA) test followed by Tukey's test for multiple comparisons. *p*-values less than 0.05 were considered statistically significant.

3. Results

3.1 Effects on the EZM test

The findings of the EZM test showed that mice treated with MFA exhibited a significant increase in anxiety-like behaviors compared to the control group, which was determined by a significant increase in the time spent in the closed area (Fig. 1A) and a significant decrease in the number of entries to the open areas (Fig. 1B). On the other hand, pre-treatment with flumazenil significantly reversed the anxiogenic effect caused by MFA, which was evidenced by a significant decrease in the time spent in the closed area and a significant increase in the number of entries to the open areas.

3.2 Effects on Marble Burying Test

The results of the marble burying test are summarized in Fig. 2. The results showed that MFA-treated mice exhibited a significantly decreased number of buried marbles compared to the control mice. However, pre-treatment with flumazenil restored the burying activity, determined by a significant increase in the number of buried marbles.

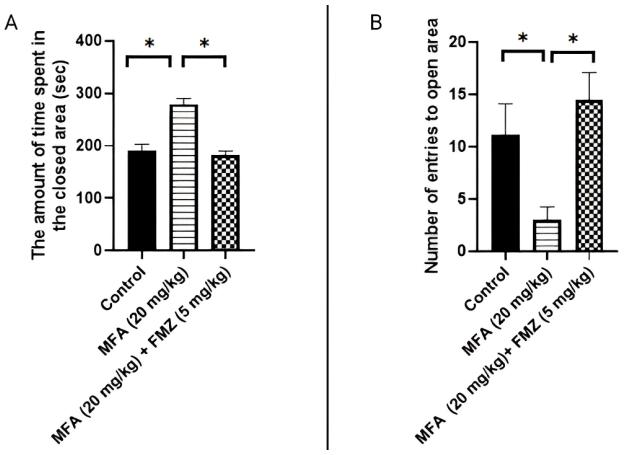

Fig. 1. Effect of MFA on the EZM test with and without FMZ pretreatment. (A) The amount of time spent in the closed area. (B) Number of entries to open area. (*) indicates a significant difference ($p < 0.0001$) between test groups using Tukey's test. MFA, Mefenamic acid; EZM, elevated zero maze; FMZ, flumazenil.

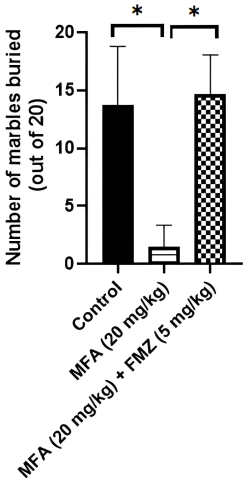

Fig. 2. Number of marbles buried in the marbles burying test. (*) indicates a significant difference between test groups using Tukey's test ($p = 0.0001$ between control and MFA treated group, $p < 0.0001$ between MFA and MFA + FMZ treated groups).

3.3 Effects on the Locomotor Activity

The results obtained by the actophotometer are shown in Fig. 3. MFA-treated mice exhibited a significant decrease in locomotor activity, which was determined by a significant decrease in the number of light beam interruptions compared to the control group. On the other hand, pre-treatment with flumazenil caused a significantly lower decrease in locomotor activity than observed after MFA treatment.

3.4 Percentage of Catalepsy

The findings of the bar test are presented in Fig. 4. The results show that treatment with MFA induced catalepsy

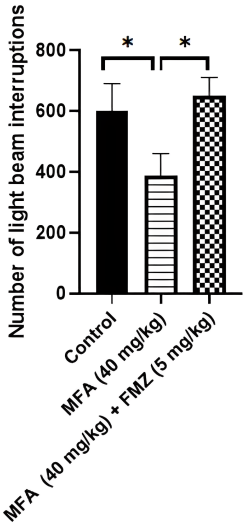

Fig. 3. Number of light beam interruptions in the actophotometer test. (*) indicates a significant difference between test groups using Tukey's test ($p = 0.0005$ between control and MFA treated group, $p < 0.0001$ between MFA and MFA + FMZ treated groups).

in 99% of treated mice. However, pre-treatment with flumazenil caused a significantly lower increase in the percentage of cataleptic mice compared to that caused by MFA treatment.

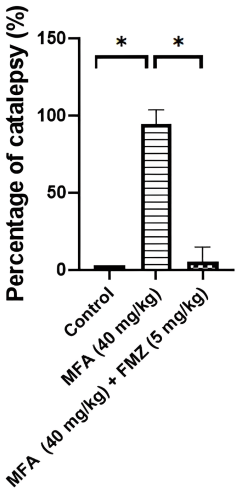

Fig. 4. Percentage of catalepsy in different animal groups. (*) indicates a significant difference ($p < 0.0001$) between test groups using Tukey's test.

3.5 Seizure Scores

Seizure scores after the administration of drugs are presented in Fig. 5A. Mice treated with MFA showed tonic-clonic seizure with an average score of 3.66. Pre-treatment with flumazenil significantly reduced the intensity of the seizure, which was manifested as recoverable akinesia with/without a myoclonic seizure.

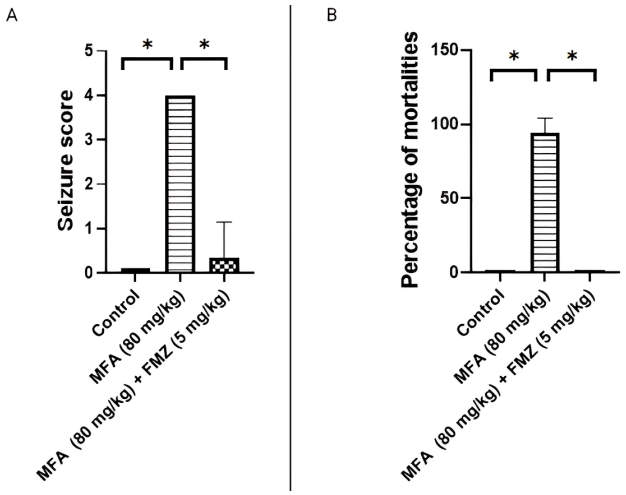

Fig. 5. Effect of FMZ pretreatment on seizure intensity and number of deaths in mice treated with MFA. (A) Seizure scores in different experimental groups. (B) Percentage of mortalities in different animal groups. (*) indicates a significant difference ($p < 0.0001$) between test groups using Tukey's test.

3.6 Percentage of Mortalities

The percentage of mortality in various experimental groups is shown in Fig. 5B. In most cases, MFA caused a rapid death that occurred 4–15 min after treatment. On the other hand, pre-treatment with flumazenil caused a significant decrease in the number of deaths caused by MFA.

4. Discussion

Central nervous system toxicity caused by drug overdose is a common reason for admission to the emergency department. The clinical manifestation of CNS toxicity may include but is not limited to, a change in mental status, disturbance of consciousness, seizures, and movement disorders. The intensity of toxicity varies among patients and depends substantially on drug dose, genetic polymorphism, or a change in drug pharmacokinetics. The pathophysiology of the central effects of drugs is attributed, at least in part, to the ability of these drugs to affect neurotransmitters via synaptic alterations in their synthesis, release, reuptake, degradation, and dynamic interactions with the receptors. In the present study, MFA-treated mice showed an increase in an anxiety state, and catalepsy, and decrease in locomotor activity and CNS convulsions in a dose-dependent manner. These findings were consistent with previous studies findings, which showed an association between MFA overdose and the risk of developing CNS toxicity [15,21].

Experimental mazes are a common method for evaluating anxiety and cognitive functions in mice [30]. The term "maze" is used to describe a human-made model that is designed in a way that can induce certain behavioral responses in animals. The elevated zero maze apparatus is a typical design for evaluating anxiety behaviors [31]. The apparatus comprises a couple of open and closed quadrants where

mice show natural aversion toward the closed quadrants [23]. In this maze, mice display typical traits of anxiety-like behaviors, such as fast resorting to closed quadrants, tendency to remain in the closed area, unwillingness to enter open quadrants, and exhibiting escape behavior manifested by repeated head dipping [32]. Since inhibitory networks of GABAergic interneurons are thought to make up the brain circuits in the amygdala [33], this neurotransmitter is essential in regulating anxiety responses in the EZM test [34]. Through GABAergic neurotransmission, the amygdala is bidirectionally connected to the medial prefrontal cortex (mPC), crucial for controlling amygdala activity associated with processing emotions and anxiety [35].

Nevertheless, numerous additional neurotransmitters, such as serotonin, opioid peptides, endocannabinoids, oxytocin, and corticotropin-releasing hormone, have been associated with modulating anxiety responses in the amygdala [33]. In this study, control mice, which received the vehicle without drugs, spent more than two-thirds of the time in the closed quadrants and showed few entries to the open quadrants. On the other hand, MFA, the test drug, resulted in a much greater amount of time spent in the closed quadrants and a significantly lower number of entries into the open quadrants, indicating that mice that received MFA were more anxious than the control group. Pre-treatment with FMZ significantly reversed the anxiogenic effect of MFA, suggesting the involvement of GABAAR in the development of anxiety associated with MFA treatment.

Behavioral studies have suggested that laboratory mice exhibit an innate burying behavior during the marble burying test [36]. This behavior is believed to reflect repetitive or stereotypic behavior that may have a compulsive nature [37]. Moreover, pharmacological studies have found that GABAergic drugs profoundly affect the marble-burying activity in mice [38]. In this study, MFA-treated mice significantly buried fewer marbles than control mice, suggesting an aberrant burying activity. Pre-treatment with FMZ significantly restored the burying activity, suggesting that GABAAR is involved, at least in part, in the regulation of burying behaviors.

Catalepsy is a neurological disorder characterized by sensory loss, muscle stiffness, postural rigidity and, in certain cases, loss of contact with the environment [39]. Although it is a rare condition, it often occurs as a symptom of other neurological disorders such as epilepsy and Parkinson's disease, as a side effect of certain medications, or as a reaction to extreme emotions or trauma [36,40]. The pathophysiology of catalepsy is not fully understood, although the loss of GABA-dopamine harmony has been identified as a causative factor [41,42]. Previous studies have indicated that harmala alkaloids and related beta-carbolines, inverse agonists in GABAAR, can induce catalepsy in rats [43]. In this study, pre-treatment with FMZ, a selective GABA receptor antagonist, significantly inhibited the development of catalepsy in MFA-treated mice, suggesting involvement of GABA signaling in MFA-induced catalepsy. Moreover, a decrease in marble-burying activity, reduced locomotion, and manifestations of catalepsy-like behavior by MFA may be attributed to the sedative-hypnotic actions associated with GABA-modulating drugs [44].

Drug-induced seizures are a common adverse effect of drug intoxication, accounting for up to 9% of all occurrences of status epilepticus [45]. Convulsive status epilepticus is a serious neurologic condition characterized by prolonged (or recurrent without recovery) tonic-clonic seizures, with an increased risk of morbidity and mortality [46]. A line of recent studies has suggested that MFA is epileptogenic when administrated at overdoses [15,18,20,47]. Although the mechanism underlying the seizures caused by MFA remains unclear, disruption of GABA signaling has been suggested as the main factor [14,48]. In this study, pre-treatment with FMZ significantly reduced seizure intensity, indicating that GABAAR may be involved in the emergence of seizures following an overdose of MFA. Given that the CNS toxicity of mefenamic acid is related to its modulatory action on beta subunits of GABAR [14], the reverse effect of flumazenil may indicate that it interferes with the dynamic action of MFA on its binding sites on GABAR. On the other hand, diazepam can reduce MFA convulsion [20] by enhancing the binding of GABA to its receptor, which increases chloride ion influx into neurons and leads to hyperpolarized postsynaptic membranes, which exacerbates CNS depression.

5. Conclusions

Given that GABAR has constitutive activity in down-regulating anxiety and CNS convulsions, the present study's findings may suggest that MFA, at high doses, acts on GABAAR, causing anxiety-like behavior and even CNS convulsions in mice. FMZ can block MFA activity, restoring GABAAR constitutive activity and preventing MFA-induced CNS toxicity. These results suggest that FMZ is a promising agent for managing MFA overdose in clinical practice. The results also suggest that MFA-induced CNS toxicity in mice is a sensitive model for screening drugs and chemicals that act on the GABAAR system.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

QJ and HAl contributed to the experimental design. QJ, RA and HAb performed the research and conducted the behavioral tests in mice. QJ, YJ, RA, KWG and SM analyzed the data. QJ wrote the manuscript. LCM, YJ, KWG, HAl and CA provided critical feedback and reviewed the manuscript. YJ, SM, LCM and CA provided input on data interpretation. All authors contributed to editorial changes in the manuscript. All authors read and approved the fi-

nal manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animal handling, manipulation and treatment were conducted according to the international guidelines for animal care and use, and approved by Research Ethics Committee, Isra University/Jordan (2019/2018/17-174).

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Conflict of Interest

The authors declare no conflict of interest.

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