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Evaluation of protective effects of non-selective cannabinoid receptor agonist WIN 55,212-2 against the nitroglycerine-induced acute and chronic animal models of migraine: A mechanistic study

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ABSTRACT

Aim: Migraine is a neurological debilitating disorder. Previous studies have shown that cannabinoid receptor agonists have analgesic effects in various models of pain. In this study, therefore, we investigated anti-nociceptive effects of WIN 55,212-2, and the role of either CB_1 or CB_2 receptors in nitroglycerine (NTG)-induced animal model of migraine.

Methods: The present study was conducted on both male and female rats receiving NTG (10 mg/kg, i.p.) to induce acute (single dose of NTG) and chronic (repetitive doses of NTG) models of migraine. Additionally, three groups received WIN 55,212-2 (0.33, 1, 3 mg/kg, i.p.) 45 min before behavioral tests. Additionally, AM251 and AM630 (CB_1 and CB_2 receptor antagonist, respectively, 1 mg/kg, i.p.) were used to evaluate the possible involvement of CB1 and CB2 receptors during the protective effects of WIN 55,212-2.

Key findings: We found that NTG (10 mg/kg, i.p.) in both acute and chronic models increased sensitivity to pain. In acute model, we found that WIN 55,212-2 (almost high doses) decreases the level of pain mainly through CB_1 receptor due to CB_1 antagonist abrogates its protective effects, however, in formalin test CB_2 receptors also had crucial roles in both phases at 3 mg/kg of WIN 55,212-2. In chronic model, WIN 55,212-2 (0.33, 1 and 3 mg/kg) significantly attenuated NTG-induced hyperalgesia through both CB_1 and CB_2 receptors.

Significance: Our data supported the argument that activation of CB_1 and CB_2 receptors by WIN 55,212-2 may be considered a new medication for migraine, however in lack of each receptor leads to different responses from deletion to the reduction of analgesic effects.

1. Introduction

Migraine is considered one of the most common disorders seen in people with chronic disability [1]. Despite dramatic increases in the incidence of migraine, our knowledge about its pathophysiology is incomplete and in many patients, despite the presence of multiple medicines, there is found no adequate healing response [1]. Although the pathophysiology of migraine is difficult to describe, there is a general

Abbreviations: CGRP, Calcitonin gene-related peptide; CB, cannabinoid; CSD, cortical spreading depression; DMSO, dimethylsulfoxide; FAAH, fatty acid amide hydrolase; i.p., intraperitoneally; saline, NaCl solution; NO, nitric oxide; NTG, nitroglycerin; NTC, nucleus trigeminalis caudalis; CB1, type one cannabinoid receptor; CB2, type two cannabinoid receptor

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consensus that the main underlying mechanism of migraine attacks are neurogenic inflammation in dura matter due to activation of the trigeminovascular system [2].

One of the most studied human's models of a migraine is designed by administration of the excessive levels of nitroglycerin (NTG) [3]. In experimental models, it has been shown that NTG is able to induce inflammation and also activate the trigeminovascular system [4,5], which anti-migraine medicines such as sumatriptan can attenuate NTG-induced allodynia [6]. Therefore, NTG-induced allodynia could be considered a reliable animal model for investigation of proper treatments for migraine [1,7].

Today, it is believed that cannabinoid (CB) receptors play important roles in regulating the nociception, control of psychomotor, memory function, neuroendocrine regulation, motion control, appetite regulation, and vomiting [8–11]. Moreover, several studies reported the serious role of the endocannabinoid system in the regulation of migraine pathophysiological mechanisms, that any disruption in this system may develop the incidence and pain intensity of migraine [11]. Furthermore, several lines of evidences have documented that either the activation of each endocannabinoids system by either cannabinoid type one or two receptors (CB_1 or CB_2, respectively) agonist, or the inhibition of enzymes involving in degradations of endocannabinoids such as fatty acid amide hydrolase (FAAH), contribute to an anti-nociception effect in the NTG-induced pain model of migraine at the periphery, spinal, and supra-spinal levels, as well as in the processing of pain information [12–15].

On this basis, the present study was designed to examine the therapeutic effects of WIN 55,212-2, a non-selective cannabinoid receptor agonist, and the contribution of either CB_1 or CB_2 receptors in both NTG-induced acute and chronic models of migraine in rat.

2. Materials and methods

2.1. Experimental animal

In the present study, we used both male and female rats weighing 220–260 g that purchased from Razi Institute, Iran; although previous studies have reported that there were no differences between male and female in NTG-induced animal model of migraine [16–18]. Rats were randomly divided into groups by six animals of each gender ($n = 12$/group). Animals were housed in a 12/12 h light/dark cycle. Food and water were available ad libitum. Experiments were conducted between 09.00 and 14.00 at ambient temperature (22–25 °C). Noteworthy, all rats were acclimatized to the test chamber before starting [19]. All experiments were approved by the ethical committee of Kashan University of Medical Sciences (Grant No. 94101).

2.2. Drugs administration

A stock of 5 mg/ml NTG (dissolved in 30% alcohol, 30% propylene glycol, and water) was purchased from Caspian Tamin®, Tehran, Iran, which freshly diluted in 0.9% *w/v* NaCl solution (saline) to a concentration of 1 m/ml [20,21]. NTG (10 mg/kg) was administered intraperitoneally (i.p.) 2 h before nociceptive tests. WIN 55,212-2, AM251, and AM630 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and dissolved in a solution containing 5% *v*/v DMSO and 0.9% v/v NaCl solution. WIN 55,212-2 was administered 45 min before nociceptive tests, which in the antagonist group AM251/AM630 also injected 15 min before WIN 55,212-2 [22].

2.3. Experimental procedures

In the present study, we evaluated the effect of WIN 55,212-2 in both acute and chronic models and in the presence and absence of either CB_1 (AM-251) or CB_2 (AM-630) receptor antagonists as follows.

2.3.1. Acute model

In this set of experiments, the protective effects of WIN 55,212-2 were investigated against NTG-induced acute animal model of migraine, which summarized in Fig. 1B.

Protocol 1 was done to induce the animal model of migraine using NTG (10 mg/kg, i.p.) administration and evaluation the effects of medication WIN 55,212-2 (0.33, 1 and 3 mg/kg) on the NTG-induced hyperalgesia. Noteworthy, previous studies have indicated that there are no differences between saline (0.9% NaCl) and the solution dissolving the NTG (6% alcohol, 6% propylene glycol and 0.9% saline) [16–18].

- Control (i.p.): injection of saline 2 h before nociceptive tests [16–18].
- Vehicle (i.p.): injection of saline and DMSO 2 h before nociceptive tests
- NTG (i.p.): injection of NTG (10 mg/kg) and then the vehicle of WIN 55,212-2, 2 h and 45 min before nociceptive tests, respectively, [5,20,23]
- NTG + WIN 55,212-2 (i.p): injection of NTG (10 mg/kg) and then different doses of WIN 55,212-2 (0.33, 1 and 3 mg/kg), 2 h and 45 min before nociceptive tests, respectively, [24].

Protocol 2 was carried out to evaluate the role of CB_1 receptor in the protective effect of WIN 55,212-2 in animal model of migraine using NTG (10 mg/kg, i.p.) administration.

- NTG + AM251(i.p.): injection of NTG (10 mg/kg), and then AM251(1 mg/kg), two and 1 h before nociceptive tests, respectively, [25,26].
- NTG + AM251 + WIN 55,212-2 (i.p.): injection of NTG (10 mg/kg), AM251 (1 mg/kg), two and 1 h, respectively, as well as WIN 55,212-2 (3 mg/kg) 45 min before nociceptive tests.

Protocol 3 was also carried out to evaluate the possible role of CB_2 receptors on the protective effect of WIN 55,212-2 on the animal model of migraine using NTG (10 mg/kg, i.p.) administration.

- NTG + AM630(i.p.): injection of NTG (10 mg/kg), and then AM630(1 mg/kg), two and 1 h before nociceptive tests, respectively, [10].
- NTG + AM630 + WIN 55,212-2 (i.p.): injection of NTG (10 mg/kg), AM630 (1 mg/kg), two and 1 h, respectively, as well as WIN 55,212-2 (3 mg/kg) 45 min before nociceptive tests.

2.3.2. Chronic model

In this model, we evaluated the protective effects of WIN 55,212-2 against the chronic animal model of NTG-induced migraine. For this aim, NTG (10 mg/kg, i.p.) or vehicle was administrated every other day for 9 days. On the tenth day, all nociceptive tests were done at two steps, first before the intervention (30 min before NTG administration) and after the intervention (2 h after NTG administration). Noteworthy, previous studies have also shown that there are no differences between saline (0.9% NaCl) and the solution dissolving the NTG (6% alcohol, 6% propylene glycol and 0.9% saline) [16–18]. For formalin test, all nociceptive tests were only performed after the intervention (2 h after NTG administration). The protocol was summarized in Fig. 1C.

Protocol 1 was done to induce the animal model of migraine using NTG (10 mg/kg, i.p.) administration and also evaluate the protective effects of WIN 55,212-2 (0.33, 1 and 3 mg/kg) on NTG-induced hyperalgesia.

- Control (i.p.): injection of saline 2 h before nociceptive tests [16–18].
- Vehicle (i.p.): injection of saline and DMSO 2 h before nociceptive tests

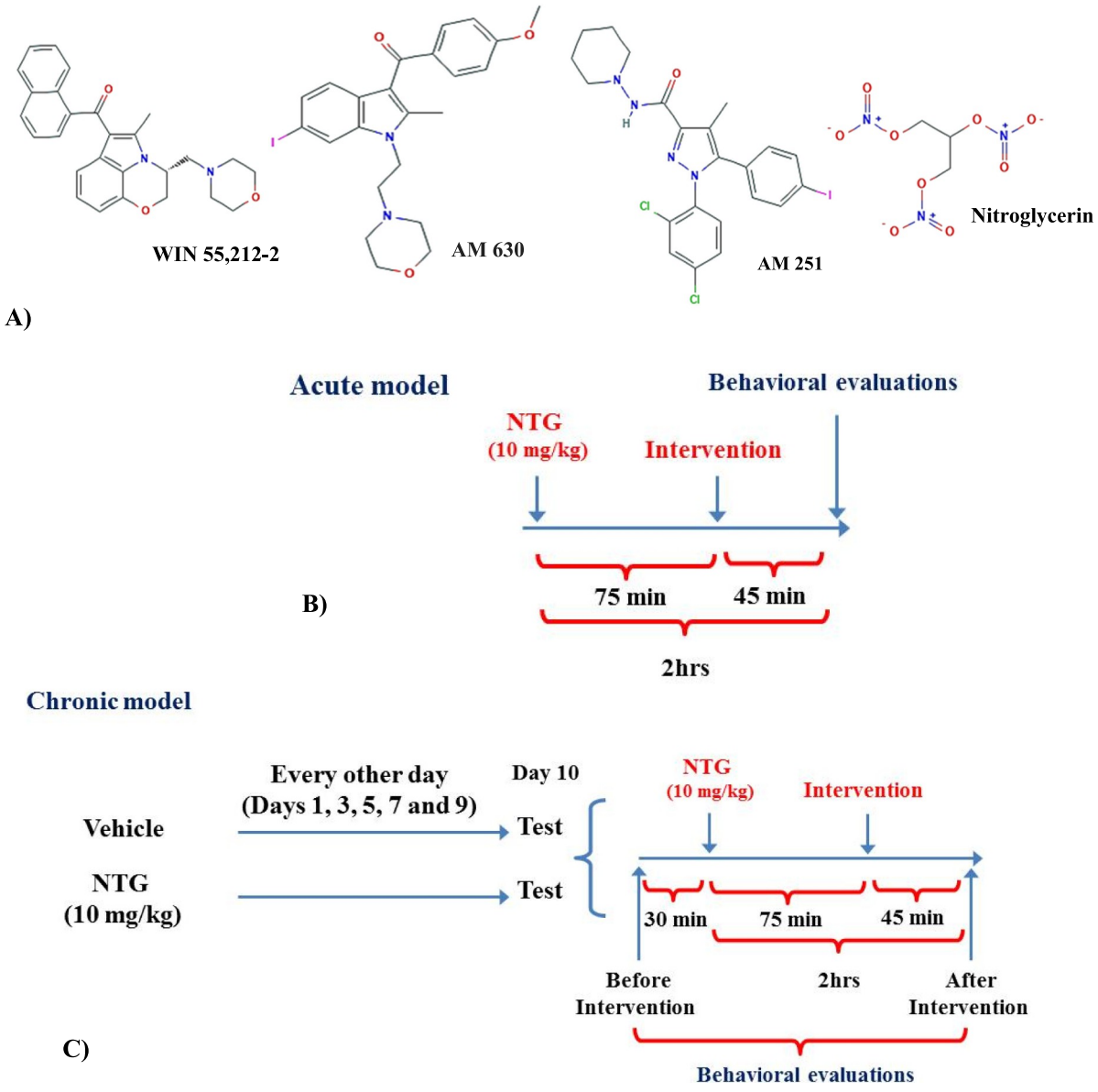


Fig. 1. Chemicals structure (A), and acute (B) and chronic (C) experimental outlines.

- NTG (i.p.): injection of NTG (10 mg/kg) and then the vehicle of WIN 55,212-2, 2 h and 45 min before nociceptive tests, respectively, [5,20,23]
- NTG + WIN 55,212-2 (i.p): injection of NTG (10 mg/kg) and then different doses of WIN 55,212-2 (0.33, 1 and 3 mg/kg), 2 h and 45 min before nociceptive tests, respectively, [24].

Protocol 2 was carried out to evaluate the role of CB_1 receptor on the protective effect of WIN 55,212-2 in the animal model of migraine using NTG (10 mg/kg, i.p.) administration.

- NTG + AM251(i.p.): injection of NTG (10 mg/kg), and then AM251(1 mg/kg), two and 1 h before nociceptive tests, respectively, [25,26].
- NTG + AM251 + WIN 55,212-2 (i.p.): injection of NTG (10 mg/kg), AM251 (1 mg/kg), two and 1 h, respectively, as well as WIN 55,212-2 (0.33, 1 and 3 mg/kg) 45 min before nociceptive tests.

Protocol 3 was also carried out to evaluate the possible role of CB_2 receptors on the protective effect of WIN 55,212-2 in the animal model of migraine using NTG (10 mg/kg, i.p.) administration.

- NTG + AM630(i.p.): injection of NTG (10 mg/kg), and then AM630(1 mg/kg), two and 1 h before nociceptive tests, respectively, [10].
- NTG + AM630 + WIN 55,212-2 (i.p.): injection of NTG (10 mg/kg), AM630(1 mg/kg), two and 1 h, respectively, as well as WIN 55,212-2 (0.33, 1 and 3 mg/kg) 45 min before nociceptive tests.

2.4. Tail flick test

To assess thermal nociceptive thresholds, the latency of reflex tail withdrawal (tail flick test) to remove the tail was carried out with a tail flick test instrument (Ugo Basile, Italy), which equipped with an automatic interrupter radiant heat. Before the assessments, the animal was gently restrained and placed on the tail-flick apparatus to be habituated for the following procedure. Radiant heat was focused on 4–7 cm from the tail distal end and latency from onset of stimulation to withdrawal of the tail was recorded. Tail flick latency for each rat was calculated as the average of three consecutive measurements in 5-min intervals. Cut-

Table 1
Summary of ANOVA test analysis.

Model	Test	Types of ANOVA test	F value (DFn, DFd)	*P* value
Acute	Tail Flick	One-way	F (9, 110) = 14.16	$P < 0.0001$
	Radiant heat plantar test	One-way	F (9, 110) = 23.35	$P < 0.0001$
	Von Frey filament test	One-way	F (9, 110) = 26.59	$P < 0.0001$
	Formalin test (1st phase)	One-way	F (9, 110) = 29.51	$P < 0.0001$
	Formalin test (2nd phase)	One-way	F (9, 110) = 18.37	$P < 0.0001$
Chronic	Tail Flick	Two-way	F (13, 308) = 22.63	$P < 0.0001$
	Radiant heat plantar test	Two-way	F (13, 308) = 22.28	$P < 0.0001$
	Von Frey filament test	Two-way	F (13, 308) = 29.31	$P < 0.0001$
	Formalin test (1st phase)	One-way	F (13, 154) = 17.66	$P < 0.0001$
	Formalin test (2nd phase)	One-way	F (13, 154) = 15.48	$P < 0.0001$

Off limit time for each measurement was set as 20 s to avoid tissue damage [27,28]. For each animal, the tail flick latency was calculated as the average of three separate determinations taken with at least 2 min between each trial.

2.5. Radiant heat plantar test (thermal allodynia)

To assess thermal nociceptive thresholds, the latency of paw withdrawal was carried out with a radiant heat plantar test instrument (Ugo Basile, Italy), which equipped with an automatic interrupter radiant heat. After the acclimatization period, the radiant heat light source was emitted on the plantar surface of the hind paw. The time required for the animal to withdraw the paw was measured as paw withdrawal latency, however, a cut-off of 25 s was considered to prevent tissue injury. For each animal, the withdrawal latency was reported as the average of three separate determinations, taken with at least 2 min between each trial [20,29].

2.6. Von Frey filament test (mechanical allodynia)

For assessing the mechanical nociceptive thresholds, the animal was placed in a comfortable position within a Plexiglas box with mesh flooring. After cessation of exploratory behavior, the plantar surface of the animal hind paw was stimulated with the series of von Frey filaments (bending force ranging from 8 to 300 g). A response was determined as lifting or shaking of the paw upon stimulation. Each filament was applied three consecutive times on the hind paw as long as either the mouse withdrew its paw or the fiber was bent. The withdrawal threshold was considered the lighter filament of evoking at least two withdrawal responses during three consecutive applications with the same filament. Each filament was applied for approximately 1 s and the interstimulus interval was also approximately 5–10 s [6,30].

2.7. Formalin test

In this experiment, rats were investigated with formalin for the evaluation of inflammatory tonic pain. Each animal was placed in a Plexiglas observation chamber (30 × 30 × 30 cm) with a mirror (angled at 45°) positioned to permit observation of the animal's paws. 50 µl of 5% formalin was injected subcutaneously into the center of the plantar surface of a hind paw with a 30-gauge needle. The length of time in which the animal flinches and shakes or licked the injected paw were considered during the first 5 min of the post-injection period (phase 1) and then 20 min after formalin injection for the 10-min period (phase 2). The first phase of the response to formalin is considered the result of chemical activation of nociceptors, whereas the second phase reflected the inflammatory reaction and central processing [14,31].

2.8. Statistical analysis

The obtained results were expressed as the mean ± SEM. Following the passing of the normal test Kolmogorov–Smirnov's, statistical analysis was performed by using one-way or two-way ANOVA test for acute or chronic models, respectively, and followed by Dunnett's post-test; using GraphPad Prism 6.0 software (San Diego, CA, USA). Data were considered statistically significant when a $p < 0.05$ was achieved. F values were reported in Table 1. The data and statistical analysis comply with the recommendations on experimental design, analysis [32] and data sharing and presentation in preclinical pharmacology [33,34].

3. Results

3.1. The effects of WIN 55,212-2 on NTG-induced acute model of migraine

As illustrated in Fig. 2, acute administration of NTG (10 mg/kg) significantly decreased the levels of tail flick latency (Fig. 2A, $p < 0.001$), and paw withdrawal latency (Fig. 2B, $p < 0.001$) and threshold (Fig. 2C, $p < 0.001$), but markedly increased the levels of paw licking time in both first (Fig. 2D, $p < 0.001$) and second (Fig. 1E, $p < 0.01$) phases of formalin test compared to the control group. In contrast, treatment with WIN 55,212-2 notably improved the levels of tail flick latency (Fig. 2A, 1 and 3 mg/kg, $p < 0.001$ for both cases), and paw withdrawal latency (Fig. 2B, 3 mg/kg, $p < 0.001$) and threshold (Fig. 2C, 0.33, 1 and 3 mg/kg, $p < 0.001$ for all cases), but markedly attenuated the levels of paw licking time in both first (Fig. 2D, 0.33, 1 and 3 mg/kg, $p < 0.001$ for all cases) and second (Fig. 2E, 3 mg/kg, $p < 0.01$) phases of formalin test compared to the NTG receiving group.

In the presence of CB_1 receptor antagonist AM251 (1 mg/kg) and NTG (10 mg/kg), all effects of WIN 55,212-2 were significantly decreased in comparison to the absence of AM251 (Fig. 2A–E, $p < 0.001$, for all cases), however, the protective and significant effects were observed for both phases of formalin test (Fig. 2A–E, $p < 0.001$ for both cases). In the other hand, our record showed that adding the CB_2 receptor antagonist AM630 (1 mg/kg) to WIN 55,212-2 (3 mg/kg) did not alter its protective effects against NTG-induced hyperalgesia on the tail flick and paw withdrawal latencies (Figs. 1, 2A and B). Although this combination significantly reduced the protective effects of WIN 55,212-2 (3 mg/kg) on tests of paw withdrawal threshold (Fig. 2C, $p < 0.001$) and both phases of formalin (Fig. 2D and E, $p < 0.001$ for both cases), the combination of AM630 (1 mg/kg) to WIN 55,212-2 (3 mg/kg) markedly enhanced the level of paw withdrawal threshold (Fig. 2C, $p < 0.001$), and reduced the levels of both first (Fig. 2D, $p < 0.001$) and second phases of formalin test (Fig. 2E, $p < 0.01$) compared to the NTG-induced migraine group.

3.2. The effects of WIN 55,212-2 on NTG-induced chronic model of migraine

Repeated administration of NTG (10 mg/kg), every other day for 9 days and day 10, notably reduced the levels of tail flick and paw

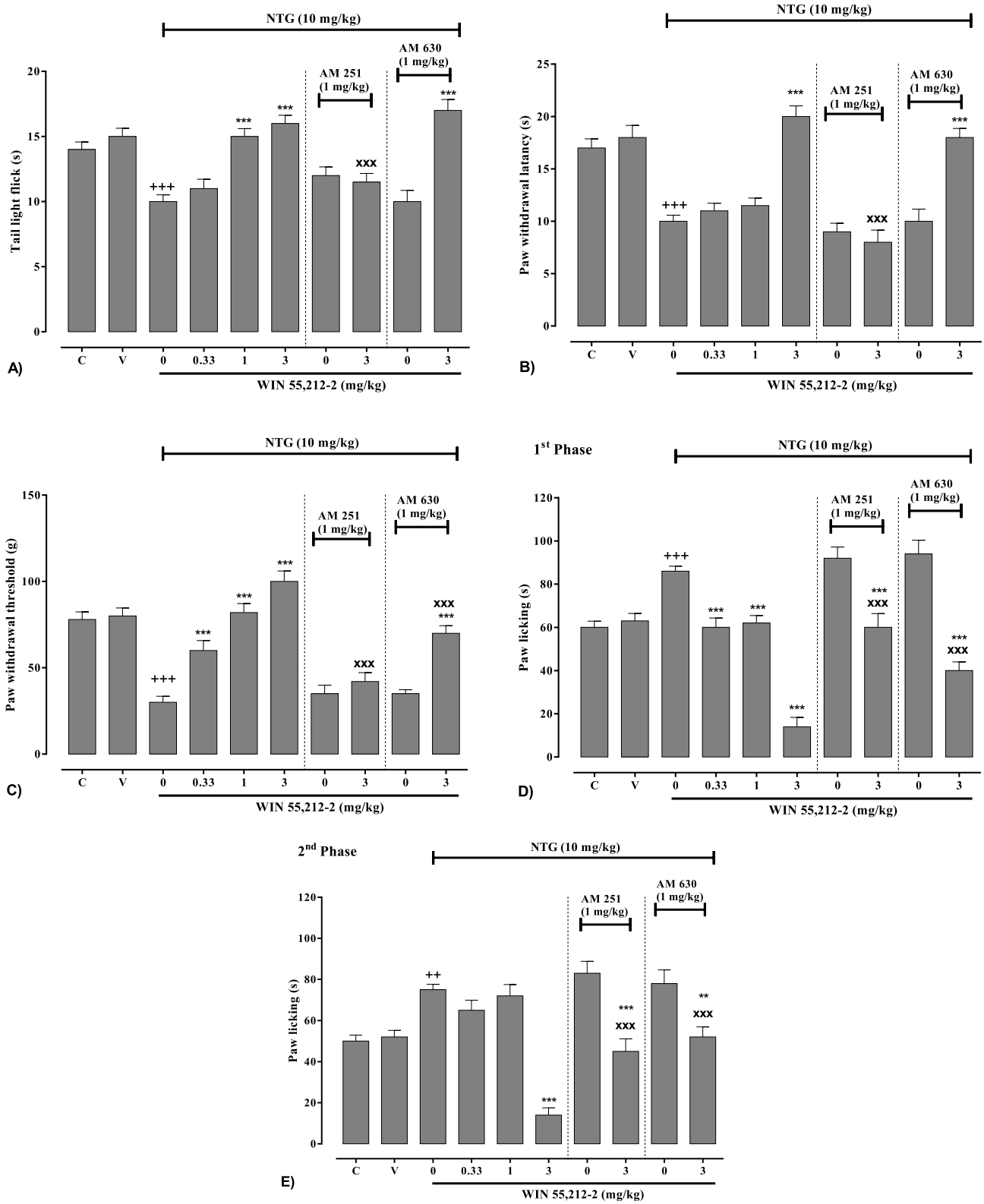


Fig. 2. The effects of different doses of WIN 55,212-2 on the acute model of nitroglycerin (NTG)-induced (10 mg/kg, i.p.) thermal allodynia [the tail flick (A) and paw withdrawal latencies (B)] and mechanical allodynia [the paw withdrawal threshold, (C)] tests as well as first (D) and second (E) phases of formalin test in the presence or absence of either CB_1 or CB_2 receptor antagonist (AM251 or AM630, respectively), ($n = 12$). Data were shown as mean ± SEM. Following the passing of the normal test Kolmogorov–Smirnov's, statistical analysis was performed by using one-way ANOVA test, and followed by Dunnett's post-test; using GraphPad Prism 6.0 software (San Diego, CA, USA). $^{+}$ shows a comparison between the control or vehicle group with the NTG group, $^{++}$: $p < 0.01$, $^{+++}$: $p < 0.001$; * shows a comparison between WIN 55,212-2 treated groups in the presence or absence of the CB_1 or CB2 receptor antagonist to the respected NTG group, **: $p < 0.01$ and ***: $p < 0.001$; ^{x} indicates a comparison between the equal doses of WIN 55,212-2 groups in the presence or absence of either CB_1 or CB2 receptor antagonist, ^{xxx}: $p < 0.001$.

withdrawal latencies as well as the level of paw withdrawal threshold compared to the control group (Fig. 3A, B and C, $p < 0.001$ for all cases). Furthermore, NTG (10 mg/kg) significantly increased the duration of paw licking time of formalin test in both first (Fig. 4A, $p < 0.001$) and second (Fig. 4B, $p < 0.001$) phases in comparison to the control group. In contrary, our result indicated that treatment with WIN 55,212-2 75 min after last dose of NTG on day 10, significantly and dose-dependently elevated the levels of tail flick (Fig. 3A, 1 and 3 mg/kg, $p < 0.001$ for both cases) and paw withdrawal (Fig. 3B, 1 and 3 mg/kg, $p < 0.001$ for both cases) latencies as well as the level of paw withdrawal threshold (Fig. 3C, 0.33, 1 and 3 mg/kg, $p < 0.001$ for all cases) in comparison to the NTG-induce migraine group. Additionally, WIN 55,212-2 (1 and 3 mg/kg) markedly abrogated the duration of formalin-induced paw licking in both phases compared to the NTG-induce migraine group (Fig. 4A and B, $p < 0.001$ for all cases).

In the presence of CB_1 receptor antagonist AM251 (1 mg/kg), the effects of different doses of WIN 55,212-2 were significantly attenuated on the levels of tail flick (Fig. 3A, 1 and 3 mg/kg, $p < 0.001$ for both cases) and paw withdrawal (Fig. 3B, 1 and 3 mg/kg, $p < 0.001$ for both cases) latencies as well as the level of paw withdrawal threshold (Fig. 3C, 0.33 mg/kg, $p < 0.05$, 1 mg/kg, $p < 0.001$, and 3 mg/kg, $p < 0.001$), in comparison to the equal dose of WIN 55,212-2 without AM251 against NTG-induced migraine group. We also found that the use of AM251 (1 mg/kg) 15 min before WIN 55,212-2, led to significant increases in the duration of formalin-induced paw licking in both first (Fig. 4A, 1 and 3 mg/kg, $p < 0.001$ for both cases) and second (Fig. 4B, 1 mg/kg, $p < 0.001$, and 3 mg/kg, $p < 0.001$) phases, compared to the absence of AM251 in the present of NTG-induced migraine model. However, in the present of AM251 (1 mg/kg), WIN 55,212-2 significantly increased the levels of tail flick (Fig. 3A, 3 mg/kg, $p < 0.001$) and paw withdrawal (Fig. 3B, 3 mg/kg, $p < 0.001$) latencies, and paw withdrawal threshold (Fig. 3C, 1 and 3 mg/kg, $p < 0.001$ for both cases), while notably decreased the duration of paw licking (Fig. 4B, 3 mg/kg, $p < 0.01$) at the second phase of formalin tests, compared to the NTG-induced migraine group.

In the presence of CB_2 receptor antagonist AM630 (1 mg/kg), the effects of high dose of WIN 55,212-2 (3 mg/kg) was markedly subsided on the levels of tail flick (Fig. 3A, $p < 0.001$) and paw withdrawal (Fig. 3B, $p < 0.001$) latencies, and paw withdrawal threshold (Fig. 3C, $p < 0.001$), as well as the duration of paw licking in both first (Fig. 4A, $p < 0.05$) and second (Fig. 4B, $p < 0.001$) phases of formalin test, compared to the equal dose of WIN 55,212-2 in the presence of NTG-induced migraine group. Nevertheless, in the present of AM630 (1 mg/kg), our records elucidated that WIN 55,212-2 significantly increased the levels of tail flick (Fig. 3A, 1 and 3 mg/kg, $p < 0.001$ for both cases) and paw withdrawal (Fig. 3B, 1 and 3 mg/kg, $p < 0.001$ for both cases) latencies as well as the level of paw withdrawal threshold (Fig. 3C, 0.33, 1 and 3 mg/kg, $p < 0.001$ for all cases) in comparison to the NTG-induce migraine group. Additionally, WIN 55,212-2 (1 and 3 mg/kg) markedly abrogated the duration of formalin-induced paw licking in both first (Fig. 4A, $p < 0.01$ for both cases) and second (Fig. 4B, $p < 0.001$ for both cases) phases compared to the NTG-induce migraine group.

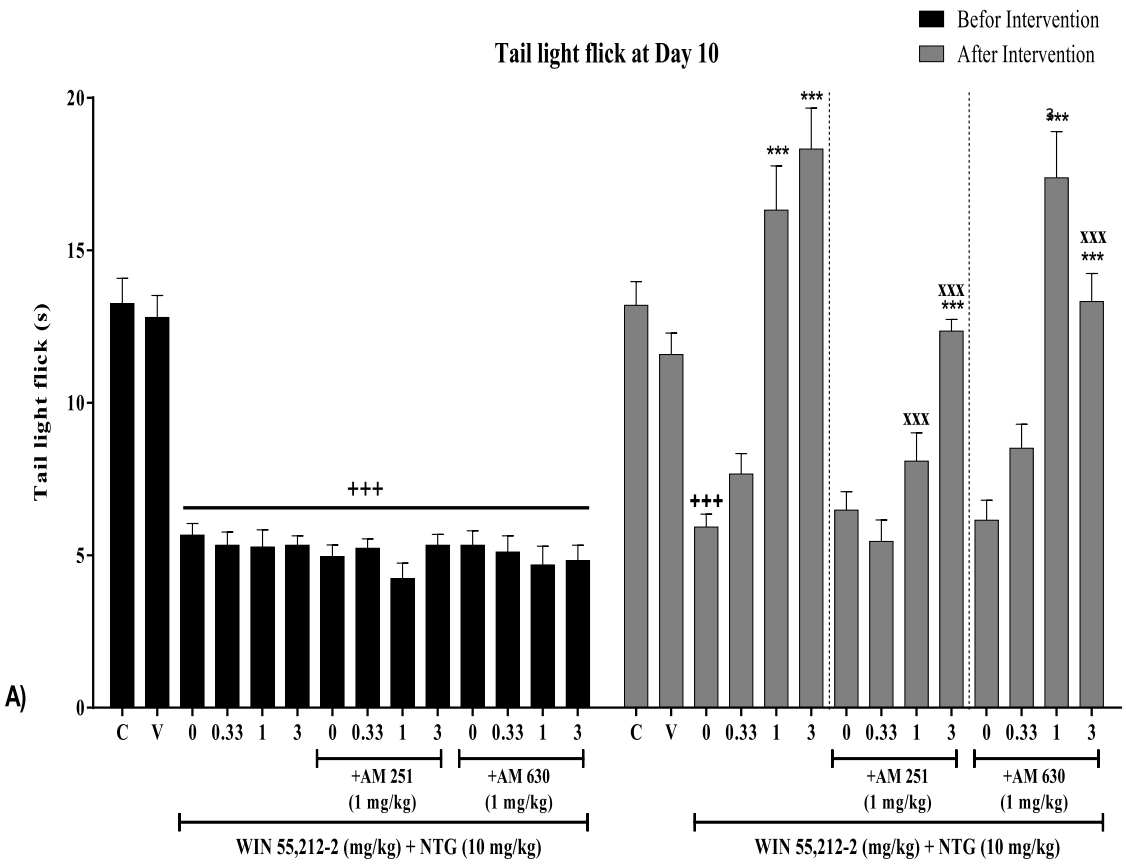
4. Discussion

To the best of our knowledge, this is the first mechanistic study regarding the protective effects of WIN 55,212-2 as non-selective CB_1/CB_2 receptor agonist against NTG-induced acute and chronic animal models of migraine. As results, we found that the administration of NTG (10 mg/kg, i.p.) in both acute and chronic models increased sensitivity to pain in different models of pain. In acute model, we found that the use of WIN 55,212-2 (almost high doses) decreases different models of pain mainly through CB_1 receptors due to CB_1 antagonist abrogates its protective effects; however, in formalin test CB_2 receptors also had a crucial role in both phases at 3 mg/kg of WIN 55,212-2. In the chronic model of migraine, WIN 55,212-2 also significantly attenuated NTG-induced hyperalgesia through both CB_1 and CB_2 receptors which the CB_2 receptor showed a prominent role as well as CB_1 receptor at the high dose of WIN 55,212-2.

For investigation of new medications for treatment of migraine, there are several experimental models which one of the most commonly used model is NTG-induced migraine pain, since it induces a condition of hyperalgesia in the rat and is associated with the activation of both spinal cord and brain structures involving in nociception [17,35,36]. In fact, nitric oxide (NO) donor compounds such as NTG potentiate responses of dorsal horn to afferent stimulation, intensify the subpopulation of trigeminal afferents innervating the dura matter, and also have an important role in inflammatory hyperalgesia [16,20,35,36].

Moreover, important neuronal activity and regulatory roles of NO cannot be ignored in the peripheral, spinal, and supra-spinal levels, as well as in the processing of pain information [20,35,36]. In this regard, in the present study, we used NTG (10 mg/kg, i.p.) for the induction of acute and chronic migraine models in both male and female rats. In agreement to previous reports, we found that there is no difference between male and female nociceptive test responses by NTG [16,17,35]. Based on the pieces of evidence, NTG leads to headaches in normal people and also stimulates a migraine without aura. Moreover, NTG-induced hyperalgesia is associated with the development of sensory hypersensitivity with a migraine. Contextually, the acute administration of NTG led to mechanical and thermal hyperalgesia, which were improved using an anti-migraine medication sumatriptan [35]. Furthermore, it has been indicated that the administration of NTG causes hypersensitivity to light and increases meningeal blood flow in mice [35]. Our results of induction of migraine models may be supported by these studies.

In the present study, WIN 55,212-2 reversed the effects of NTG-induced hyperalgesia in a dose-dependent fashion in both models of acute and chronic migraine. Using the von Frey filaments, we demonstrated that the use of WIN 55,212-2 (0.33, 1 and 3 mg/kg) enhances the paw withdrawal threshold against both acute and chronic models of NTG-induced migraine in a dose-dependent manner. It has been already stated that the tail flick assay evaluates the pain threshold at the spinal level, however upper areas such as the brainstem and cortical regions are able to modify its reflexing responses [37]. Besides, we also performed the radiant heat plantar test (Hargreaves test) to have a better insight onto the distinction between the effects of WIN 55,212-2 on the spinal and supraspinal levels, because of this test requires supraspinal and integration of higher structures to indicate reflexes [29,37]. In this regard, we used both the tail flick assay and radiant heat plantar test to examine the analgesic effect of WIN 55,212-2 at spinal and supraspinal levels. In acute model, we found that middle dose of WIN 55,212-2 (1 mg/kg) reduces the thermal hyperalgesia at the spinal level, while according to the finding from radiant heat plantar test, high dose of WIN 55,212-2 (3 mg/kg) may act through supraspinal structures to reduce the pain. In fact, in the present study, we administrated WIN 55,212-2 systematically (i.p.) and carried out neither specific supraspinal tests nor the cephalic administration study, therefore, we cannot exactly distinguish between spinal and supraspinal sites involving in its analgesic effects. As regards, there are many studies implication that cannabinoid ligands exert their analgesic effects through acting at both spinal and supraspinal sites as well as in the periphery [11,38]. In one study, Ebrahimzadeh and coworker indicated that the administration of WIN 55,212-2 in the nucleus cuneiformis (NCF), the supraspinal neuroanatomical regions involving the modulation of pain, makes dose-dependent analgesic effects against acute and inflammatory pain models (tail-flick and formalin tests) [39]. Indeed, this study also indicates supraspinal analgesic effects of WIN 55,212-2. Moreover, spinally administration of WIN 55,212-2 diminishes thermal hyperalgesia in diabetic rats but this effect is not more effective rather than the systematic administration [40]. Additionally, it has been showed that a systemic administration of cannabinoid ligand WIN55,212-2 is effective



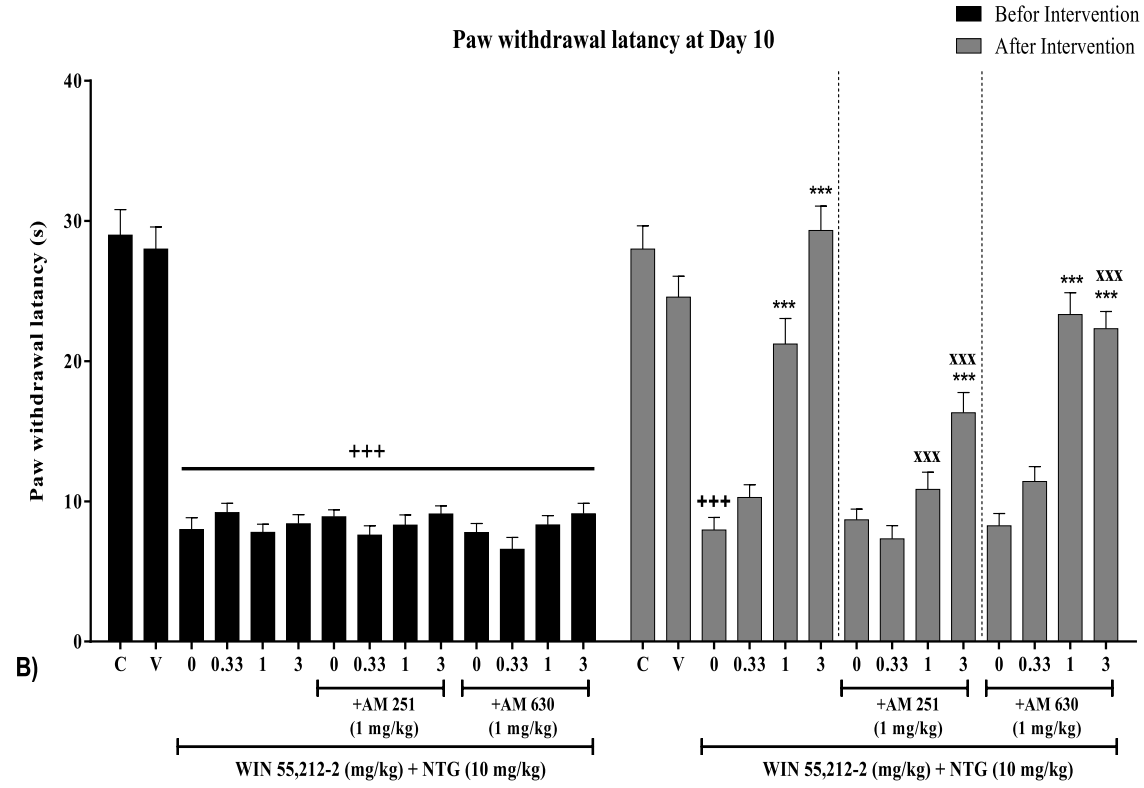


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Fig. 3. The effects of different doses of WIN 55,212-2 on the chronic model of nitroglycerin (NTG)-induced (10 mg/kg, i.p.) thermal allodynia [the tail flick (A) and paw withdrawal latencies (B)] and mechanical allodynia [the paw withdrawal threshold, (C)] tests in the presence or absence of either CB_1 or CB_2 receptor antagonist (AM251 or AM 630, respectively), (n = 12). Following the passing of the normal test Kolmogorov–Smirnov's, statistical analysis was performed by using two-way ANOVA test, and followed by Dunnett's post-test; using GraphPad Prism 6.0 software (San Diego, CA, USA). $^{+}$ shows a comparison between the control or vehicle group with the NTG group, $^{++}$: $p < 0.01$, $^{+++}$: $p < 0.001$; * shows a comparison between WIN 55,212-2 treated groups in the presence or absence of the CB_1 or CB2 receptor antagonist to the respected NTG group, **: $p < 0.01$ and ***: $p < 0.001$; ^{x} indicates a comparison between the equal doses of WIN 55,212-2 groups in the presence or absence of either CB_1 or CB2 receptor antagonist, ^{xxx}: $p < 0.001$.

in decreasing the mechanical allodynia in different animal models of pain [41,42].

Interestingly, in the chronic model, we observed that both middle and high doses of WIN 55,212-2 (1 and 3 mg/kg, respectively) also produce obvious analgesic effects against chronic administration of NTG at both spinal and supraspinal levels. There are several studies notion that either the repetitive or the prolonged pain stimulation, for example, following traumatic injury or an inflammatory process, the expression levels of cannabinoid receptors and of endocannabinoids will be increased both centrally and peripherally, thereby helping to reduction of the pain intensity [11,43–46]. Hence, the effects of the middle dose of WIN 55,212-2 may be explainable via the over-expression of CB receptors, although we performed no investigation in this case as one of this study limitation.

There is some evidence that the stimulation of sensorial areas is not considered supraspinal distribution, which may limit the use of the findings for treatment of migraine [14,47], thereby the formalin test was performed in the present study. Previous studies indicated that plantar injection of formalin in rats leads to significant changes in calcitonin gene-related peptide (CGRP) levels in the superficial laminae I and II, ipsilateral to the injection side and also in the nucleus trigeminalis caudalis (NTC) and that systemic NTG administration similarly causes a reduction in the level of CGRP-immunoreactivity in the NTC, but not in the lumbar dorsal horns [47,48]. In consistent with these studies, in our experiment, we also found that either the acute or the chronic administration of NTG significantly potentiates both phases of the formalin test. Indeed, NTG may induce a direct hyperalgesic effect via formation of NO and liberation of CGRP in the NTC [49], or indirectly via activation of NOS at the meningeal level as a consequence of sensitization of the trigeminovascular system. Collectively, NTG-potentiated formalin test can be considered a relevant model for investigating migraine circuitry, which it has been shown that formalin injection in the paw elevates the expression level of Fos in NTC, and other brainstem areas such as locus coeruleus involving in the modulation of migraine pain [49,50].

Hopefully, it was observed that WIN 55,212-2 attenuates the first phase of formalin at all doses, while the high dose had a significant effect in the second inflammatory phase of formalin test in the acute model. In contrary, both middle and high doses of WIN 55,212-2 had a notable analgesic effect in both phases of formalin test in the chronic model. Likely, different exposures to painful stimuli increase the sensitivity to the pain also the overexpression of endocannabinoid receptors and ligands, therefore WIN 55,212-2 (1 and 3 mg/kg) exerts a putative analgesic effect at the lower dose. However, it needs further investigations in order for this to be confirmed, our findings were in agreement with other studies that indicate the contribution of activation of cannabinoid receptors in reduction of inflammatory responses such as formalin test in NTG-induced model of migraine [12,14,51].

There are several reports regarding the pivotal role of cannabinoids in regulation of many functions in the body, including modulation of

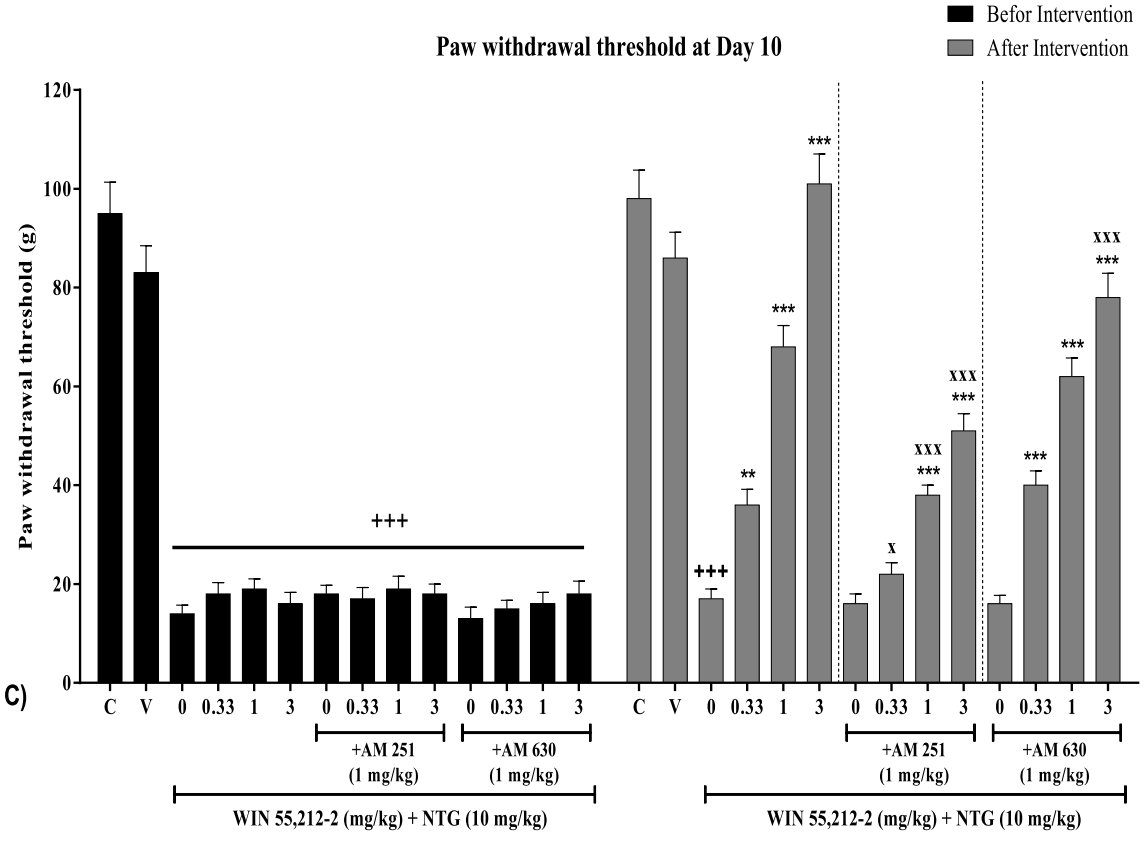

Fig. 3. (*continued*)

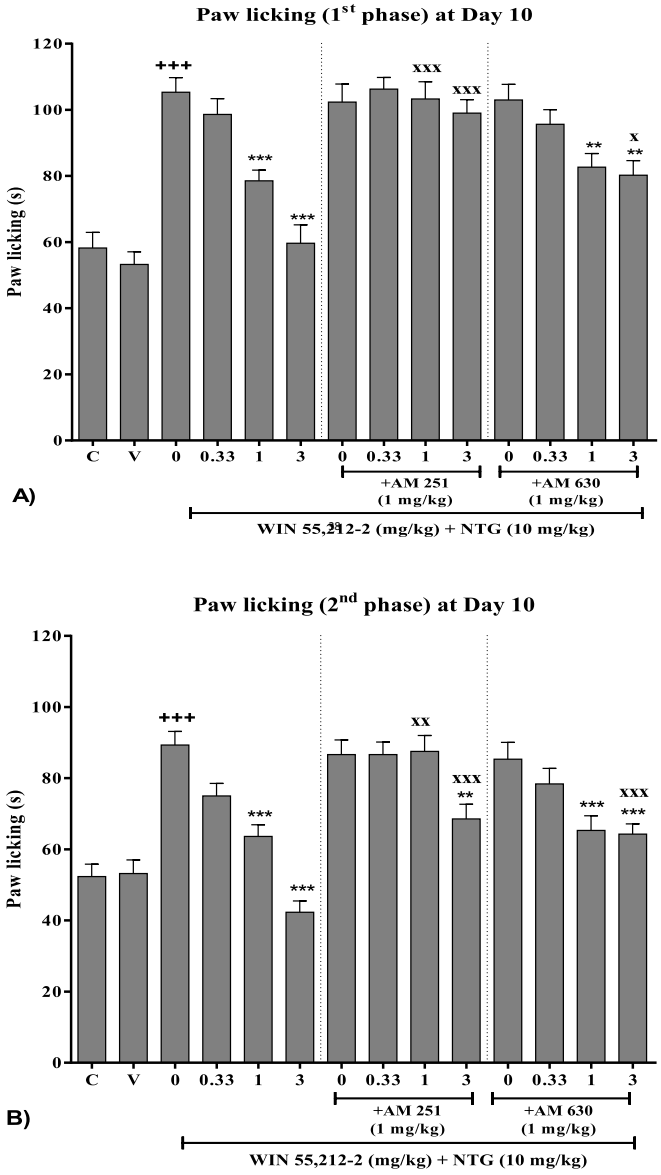

Fig. 4. The effects of different doses of WIN 55,212-2 on the chronic model of nitroglycerin (NTG)-induced (10 mg/kg, i.p.) migraine on first (A) and second (B) phases of formalin test in the presence or absence of the CB_1 or CB2 receptor antagonist (AM251 and AM 630, respectively), (n = 12). Following the passing of the normal test Kolmogorov–Smirnov's, statistical analysis was performed by using one-way ANOVA test, and followed by Dunnett's post-test; using GraphPad Prism 6.0 software (San Diego, CA, USA). $^{+}$ shows a comparison between the control or vehicle group with the NTG group, $^{++}$: $p < 0.01$, $^{+++}$: $p < 0.001$; * shows a comparison between WIN 55,212-2 treated groups in the presence or absence of the CB_1 or CB2 receptor antagonist to the respected NTG group, **: $p < 0.01$ and ***: $p < 0.001$; ^{x} indicates a comparison between the equal doses of WIN 55,212-2 groups in the presence or absence of the CB_1 or CB2 receptor antagonist, ^{xxx}: $p < 0.001$.

pain in peripheral, spinal, and supraspinal nociception. The CB_1 receptor is mainly distributed throughout the CNS and PNS, which mediates psychoactivity, pain regulation, memory processing and motor control. The presynaptic CB_1 heteroreceptor modulates the release of neurotransmitter and neuropeptide and prevents synaptic transmission by the activation of inwardly rectifying potassium channels, and in the inhibition of voltage-sensitive calcium channels [9,14,46,52]. The CB_2 receptor is found predominantly at the periphery and in the immune system cells. Activation of CB_2 receptors lead to the inhibition of cytokine/chemokine release and resolution of chronic inflammatory processes and modulate chronic pain. Additionally, it has been shown that the CB_2 activators lead to the release of β-endorphin acting at μ opioid receptors on peripheral sensory neurons to inhibit nociception [9,14,46,52]. Although the density of CB_2 receptors is at low levels in the CNS and PNS, they may be overexpressed in microglia following the inflammation and after peripheral nerve damage [9,14,46]. Moreover, it is worth mentioning that CB_1/CB_2 receptors may reduce pain through interaction with other receptors and providing hetero receptors including, the putative non-CB_1/CB_2 cannabinoid G protein-coupled receptor (GPCR) 55 (GPR55) or GPR18, opioid receptors, serotonin (5-HT) receptors, nuclear receptors (peroxisome proliferator-activated receptors, PPARs), cys loop ligand-gated ion channels and transient receptor potential (TRP) channels [9,14,46,52].

Therefore, to have a better understanding onto the involved mechanisms during the protective effects of WIN 55,212-2 against NTG-induced model of acute and chronic pain, we also evaluated its effect in the presence of either CB_1 or CB_2 receptors antagonists. Interestingly, we discovered that the CB_1 receptor has a highlighted role in the pain modulation of WIN 55,212-2 in the acute model of migraine due to the use of CB_1 antagonist AM251 completely reversed its protection in contrary the use of CB_2 antagonist AM630. In a similar way, in the chronic model, the use of CB_1 receptor antagonist significantly lessened the protective effects of WIN 55,212-2 but not completely since its protective effects were observed. As another key finding of the present study, we indicated that WIN 55,212-2 at the dose of 3 mg/kg exerted its significant analgesic effects through CB_2 receptors due to the use of an antagonist reduced the protection, although it was more obvious in the chronic model. Indeed, it suggests that analgesic effects induced by WIN 55,212-2 are mediated via both CB_1 and CB_2 receptors, which in lack of each receptor leads to a wide response from deletion to the reduction of analgesic effects. In accordance with our findings, Akerman et al. examined responses of rats following the dural electric stimulation and facial activation in the ophthalmic branch of the trigeminal nerve and the effect of the cannabinoid receptors agonist and antagonist. They showed that WIN 55,212-2 inhibits neuronal responses in type A and C fibers by 25% and 44%, respectively, which these effects are blocked by the CB_1 antagonist but not in the present of the CB_2 antagonist [1]. Furthermore, Kazemi et al. explained the effects of the cannabinoid system on the CSD in rats. The CSD plays a major role in some neurological diseases such as a migraine with aura which is thought to be equivalent to a migraine in humans. The release of CSD-like waves in human neocortical tissues causes aura symptoms in migraine patients. It is argued that CSD may be a stimulant to initiate pain in migraine attacks. Their results indicated that CB_1 agonist WIN 55,212-2 significantly inhibited CSD, while the JWH-133 CB_2 agonist had no effects on CSD [53]. These studies may support our finding regarding the CB_1 receptor-mediated effects of WIN 55,212-2. On the other hand, there are some studies which disagree to the unique role of CB_1 receptors in protective effects of WIN 55,212-2. In one study, protective effects of WIN 55,212-2 were examined on a murine model of bone cancer pain to investigate underlying peripheral neural mechanisms [54]. Their results demonstrated that activation of either CB_1 or CB_2 receptors significantly reduced WIN 55,212-2 effects on the spontaneous activity of C-fiber nociceptors associated with tumor growth, and responses to mechanical stimulation. In fact, this study indicates the involvement of peripheral CB_1 and CB_2 receptors in the reduction of pain. Furthermore, the antinociceptive effect of intrathecal administration of WIN 55,212-2 was investigated in a rat bone tumor pain model [55]. They clarified that both CB_1 and CB_2 receptors involve through the antinociceptive effect of WIN 55,212-2 due to the use of each antagonist significantly provided fewer responses. Indeed, this study indicates the centrally acting role of either CB_1 or CB_2 receptors in addition to the periphery, which they may confirm our findings on modes of action of WIN 55,212-2 in NTG-induced acute and chronic models.

There are several limitations regarding the translational data from

animal studies of headache to the clinical study. The use of NTG in rodents may be effectively modeled migraine-like symptoms, even though doses, type of administrations and the determination of the time of observation need to be carefully monitored when animal models based on NTG are aimed for the study of the trigeminovascular system [56,57]. In fact, the NTG doses in the experimental study are meaningfully more than those seen in the clinical study [58]. It is worth mentioning that several factors should be taken into consideration to estimate the in vivo experimental doses especially the pharmacokinetic of inducing agent including time to maximum effect, rate of metabolism, and body surface area [59]. Therefore, the required effective animal dose may be increased by these underling factors. In addition, although the administration of NO donors such as glycerol trinitrate have been demonstrated to provide an animal model of migraine pain similar to migraineurs, the NTG-induce animal model of migraine does not reduce the cortical-spreading depression (CSD) and liberate CGPR in the blood circulation [56,57]. Tactile allodynia is found a common characteristic of migraine and several methods have been used to explore and examine this. In animal models, NTG leads to a thermal and mechanical allodynia that can be reversed using an anti-migraine medicine sumatriptan [20,56,57,59]. In this model, thermal and mechanical nociceptive thresholds are routinely indicated by hind paw withdrawal, a spread of tactile allodynia to other areas of the body such as limbs is seen during a migraine attack, and not directly examined the nociception in the facial region and craniofacial structures, however [56,57]. Therefore, further preclinical methods of headache should be taken into account to evaluate the protective effects of WIN 55,212-2 on the resolution of headache and migraine.

In conclusion, our data supported the argument that activation of CB_1 and CB_2 receptors by WIN 55,212-2 may be considered a new medication for migraine. However, we found that anti-nociceptive effects of non-selective cannabinoid agonist WIN 55,212-2 are mediated via both CB_1 and CB_2 receptors, which in lack of each receptor leads to different responses from deletion to the reduction of analgesic effects. Finally, we need more experimental and clinical investigations to achieve the argument of the efficacy and safety of the non-selective cannabinoid agonist due to the potential of overuse and psychiatric effects through CB_1 receptor activation, which these may limit their development for pain treatments.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Declaration of transparency and scientific rigor

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigor of preclinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

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