Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) in press
www.jzus.zju.edu.cn; www.springer.com/journal/11585
E-mail: jzus_b@zju.edu.cn

Research Article

https://doi.org/10.1631/jzus.B2500147

Esculetin attenuates migraine-like pain via CGRP suppression and meningeal mast cell modulation in rat models

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Abstract: Growing evidence suggests that esculetin, a 5-lipoxygenase inhibitor, has pharmacotherapeutic potential due to various pharmacological properties, such as potent anti-inflammatory, anti-nociceptive, and GABA(A) receptor partial agonist activities. However, the effects of this promising agent on migraine remain unexplored. This study therefore examined the impact of esculetin on the relevant mechanisms in migraine-like conditions in rats. The systemic effects of esculetin at three distinct doses (5, 10, and 20 mg/kg) were tested in a nitroglycerin-induced migraine model using in vivo experimental sets. The direct action of esculetin on the release of calcitonin gene-related peptide (CGRP) from critical structures of the trigeminovascular system (trigeminal ganglion, nucleus trigeminalis, and meningeal afferents) was also tested in ex vivo experimental sets. Sumatriptan was used as a positive control in both sets of experiments. The in vivo results showed that esculetin reduced NTG-induced mechanical hyperalgesia and decreased trigeminal CGRP and c-Fos levels. It also lowered degranulation and meningeal mast cell numbers. The ex vivo results revealed that esculetin reduced NTG-stimulated CGRP release from trigeminovascular explants, with the exception of meningeal explants. Sumatriptan reversed the NTG-induced changes in both experimental sets. Our findings suggest that esculetin exhibits anti-nociceptive activities in experimental migraine conditions, alleviating trigeminovascular CGRP concentrations and the degranulation of meningeal mast cells. Esculetin may thus represent a therapeutic option for relieving migraine headaches, although further research is needed to confirm this.

Key words: Esculetin; Neuroinflammation; Nitroglycerin model; Natural compound; Mast cells; Calcitonin gene-related peptide (CGRP)

1 Introduction

Migraine is a major debilitating headache condition, affecting over one billion individuals worldwide. The interplay among immune mast cells, trigeminal afferents, and blood vessels in the cranial meninges elicits sterile inflammation, which in turn leads to activation of the trigemino-vascular nociceptive tract, resulting in migraine headache (Koyuncu Irmak et al., 2019; Levy and Moskowitz, 2023). Calcitonin gene-related peptide (CGRP), released from trigeminal nerve fibers, plays a central role in the sensitization and subsequent activation of the trigeminovascular system due to its pronociceptive, mast cell degranulator, and vasodilator properties (Ottosson and Edvinsson, 1997; Kilinc et al., 2017a). Current migraine medications are either symptomatic or prophylactic, do not provide complete recovery, and are not effective in all patients. It is therefore imperative to investigate novel, multi-targeted therapeutic strategies that are capable of regulating critical contributors to meningeal inflammation, such as CGRP and meningeal mast cells.

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Received Mar. 26, 2025; Revision accepted Sept. 1, 2025;
Crosschecked xxx. xx, 20xx; Published online xxx. xx, 20xx

Esculetin is a plant-derived chemical compound that inhibits 5-lipoxygenase and leukotriene biosynthesis (Neichi et al., 1983). The 5-lipoxygenase pathway is the central source of potent proinflammatory lipid leukotrienes that play crucial roles in acute and chronic inflammation (Giménez-Bastida et al., 2021). Previous research has shown that inhibition of 5-lipoxygenase suppresses neuropathic pain-induced hyperalgesia in rats (Okubo et al., 2017). Esculetin also exhibits a broad array of pharmacological effects, with potent anti-inflammatory and antioxidant properties (Liang et al., 2017; Zhang et al., 2022). It has been found to exhibit anti-nociceptive effects by reducing mechanical hyperalgesia in inflammatory and non-inflammatory pain models (Rzodkiewicz et al., 2015; Singh et al., 2020). Additionally, it suppresses mast cell activation (Kim et al., 2025), which is also associated with neurogenic inflammation in the pathophysiology of migraine (Kilinc et al., 2017a; Ramachandran, 2018; Levy and Moskowitz, 2023). Furthermore, it restrains neuronal activity by enhancing the Cl^- influx into neuroblastoma cells (Woo et al., 2011).

These multi-target pharmacological properties of esculetin provide a scientific rationale for investigating its therapeutic potential in migraine, which is characterized by inflammation and increased trigeminal neuronal activation. Esculetin is an ideal candidate for testing in a migraine model compared to other 5-lipoxygenase inhibitors or coumarins because it is a plant-derived phytochemical; its analgesic effects have been demonstrated in various non-migraine pain models; it exhibits a combination of multiple pharmacological effects, such as the inhibition of inflammation, oxidative stress, and mast cell activation, which also play a role in migraine pathophysiology. Additionally, the effects of this promising multifaceted phytochemical on migraine have not yet been investigated.

Although current anti-migraine medicines targeting CGRP or its receptors (e.g., monoclonal antibodies and gepants) have achieved significant success, a substantial proportion of migraineurs remain resistant to them (Labastida-Ramírez et al., 2023). Novel therapeutic agents that may be effective in all migraine patients—or at least in those who do not benefit from existing treatments—must therefore be investigated.

This study is the first to examine the impacts of multiple doses of esculetin on nociceptive and neuroinflammatory parameters related to the pathobiology of migraine in nitroglycerin-induced rodent models of migraine.

2. Materials and methods

2.1 Experimental animals

This study was performed using male Wistar rats weighing 180–220 g (aged 8–11 weeks) obtained from the animal generation and welfare center at Bolu Abant Izzet Baysal University, Türkiye. Approval for the experimental procedures was granted by the university regional ethics council for animal experiments (protocol No. 2021/38, and additional decision No. 2022-13). The rats were treated in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. They were housed in separate Plexiglas chambers, maintained on a 12-h light/dark cycle at (22±2) °C. Water and chow were provided ad libitum.

2.2 Chemicals and their preparation

Nitroglycerin in solution form was purchased from Adeka Pharmaceutical Industry (Samsun, Türkiye, Cat.#C01DA02), esculetin from Santa Cruz Biotechnology (Dallas, USA, Cat.#sc-200486), sumatriptan from TCI Chemical Industry (Tokyo, Japan, Cat.#S0851), dimethyl sulfoxide (DMSO) from Sigma-Aldrich (Schnelldorf, Germany, Cat.#D8418), ELISA kits for rat CGRP detection from Elabscience (Texas, USA, Cat.#E-EL-R0135), ELISA kits for rat c-Fos detection from Sunredbio (Shanghai, China, Cat.#201-11-0047), and toluidine-blue from Carlo Erba Reagents (Val de Reuil, France, Cat.#CE.429282). Esculetin was initially dissolved in DMSO and subsequently diluted with normal saline to yield the treatment dose. An equal amount of saline, including 1% (v/v) DMSO, was employed as the vehicle for esculetin (Veh1). The solvent of stock

nitroglycerin consisted of a mixture of propylene glycol and alcohol, which was further diluted with normal saline to obtain the treatment dose. An equal quantity of this mixture (6% (v/v) propylene glycol, 6% (v/v) alcohol, and 0.9% (w/v) saline) was employed as the vehicle for nitroglycerin (Veh2). Sumatriptan was dissolved in saline solution.

2.3 The in vivo experimental design and creation of the migraine model

Male rats were used to avoid the confounding (bias) effect of female sex hormone fluctuations on the measured behavioral and molecular biomarkers. Levels of estrogen and progesterone depend on the estrous cycle, and both hormones are capable of affecting migraine-related biomarkers such as CGRP (Labastida-Ramírez et al., 2019; Cetinkaya et al., 2020). All in vivo drug administrations were performed via the intraperitoneal route and at 10 ml/kg body weight. Nitroglycerin (NTG) was administered at a single dose of 10 mg/kg to establish the in vivo migraine model (Baranoglu Kilinc et al., 2024; Kilinc et al., 2024; Torun et al., 2024). Forty-nine rats were randomly allocated to seven groups of seven animals each: Veh1+Veh2 (control), Veh1+NTG (model), low-dose esculetin (ES5+NTG), medium-dose esculetin (ES10+NTG), high-dose esculetin (ES20+NTG), Sumatriptan+NTG, and ES20+Veh2. Veh1 injection was administered to both the control and model groups once daily for five days. A dose of 5 mg/kg of esculetin was administered to the ES5+NTG group, 10 mg/kg esculetin to the ES10+NTG group, 20 mg/kg esculetin to the ES20+NTG group, and 600 μg/kg of the anti-migraine drug sumatriptan to the Sumatriptan+NTG group. In the withdrawal hyperalgesia test, the effective dose of 20 mg/kg of esculetin was administered to the ES20+Veh2 group. Thirty minutes after the injections on day 5, Veh2 was administered to the control and Es-20+Veh2 groups, and 10 mg/kg NTG to the other groups. Due to the nature of the model, mechanical hyperalgesia was tested 2 h after the final injection, and the rats were euthanized four hours after the final drug administration (Kilinc et al., 2018, 2020, 2022). Fig. 1 illustrates the timeline of the in vivo experiments.

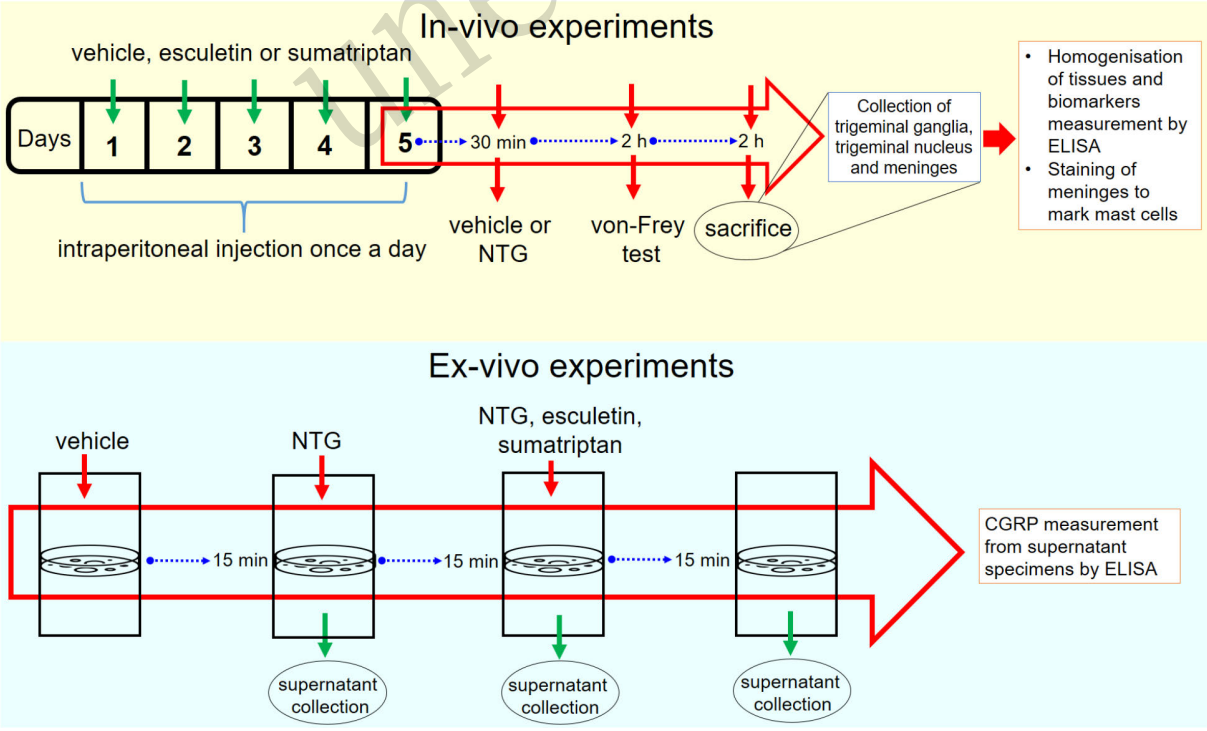

Fig. 1 Schematic illustration of in vivo and ex vivo experimental processes. NTG: nitroglycerin.

2.4 Testing mechanical hyperalgesia

In experimental migraine models, a nociceptive reaction in the hind paw following NTG injection represents extracephalic hyperalgesia in human migraineurs (Sureda-Gibert et al., 2022; Baranoglu Kilinc et al., 2024; Torun et al., 2024). Hind paw mechanical hyperalgesia was assessed using von Frey filaments (North Coast Medical, Morgan Hill, CA, USA) with the up/down procedure, as described in previous studies (Baranoglu Kilinc et al., 2024; Kilinc et al., 2024; Torun et al., 2024). Briefly, a range of von Frey hairs (warping power ranging between 0.008 and 300 g) was applied to the plantar area of the hind paws. The test stages were performed in a blinded manner. The test commenced with a 2 g filament. If the rats' paws lifted or trembled immediately after stimulation with the relevant filament, this was interpreted as a positive reaction. Each filament application to the hind paw was performed three times without interruption until the animal withdrew its hind paw or the filament curved into a “letter C” shape. The nociceptive reaction threshold was considered the minimum gauge filament that elicited at least two withdrawal reactions during three consecutive tests with the same filament.

2.5 Blood collection

The animals were first anesthetized with 90 mg/kg ketamine, after which approximately 4 mL of blood was withdrawn from the right chamber of the heart by means of an injector. The blood samples were transferred to EDTA tubes and centrifuged at 4000 r/min for 15 min at 4 ˚C. Plasma samples were preserved at -20 ˚C. Following blood retraction, the head region was perfused transcardially with approximately 200 mL of phosphate-buffered saline (PBS) (pH: 7.4) via a tube inserted in the left chamber of the heart.

2.6 Harvesting of trigeminal structures

Following blood collection, the cranial dura mater, trigeminal ganglia, and brainstem nucleus (trigeminal nucleus caudalis) were removed as described in previous studies (Citak et al., 2022; Rasmussen et al., 2022; Kilinc et al., 2024). First, the entire brain was extracted, and the brain stem was separated. Under a stereomicroscope, the trigeminal nucleus, lying caudally 13–16 mm from bregma, was excised bilaterally from the brainstem region in accordance with stereotaxic coordinates. The skull to which the dura mater was attached was halved across the longitudinal plane. The trigeminal ganglia were subsequently collected by resectioning 1 mm from the diverging part of the mandibular branch of the trigeminal nerve. The collected samples were stored at -20 ˚C. The dura mater connected to the hemiskull was placed in fixative (4% (w/v) PAF) overnight for immune mast cell fixation.

2.7 Trigeminal ganglia and nucleus trigeminalis homogenization

Trigeminal ganglion and trigeminal nucleus specimens were homogenized at a constant quantity of 100 mg per milliliter using a mild-work Ultra-Turrax homogenization device in supercooled PBS (pH: 7.4) containing 20 IU per milliliter aprotinin. The homogenized samples were subsequently centrifuged for 30 min at 4000 rpm at 4 ˚C. Supernatant fluid obtained from the homogenates was preserved at -20 ˚C.

2.8 Visualization of immune mast cells in the meninges

Mast cell staining was performed as described in previous studies (Baranoglu Kilinc et al., 2024; Kilinc et al., 2024; Torun et al., 2024). Briefly, a meningeal specimen was removed from the paraformaldehyde, washed with PBS, and placed on a microscope slide. It was then air-dried in the laboratory for 20 min. The meningeal specimen was then stained with toluidine blue solution (0.1% (w/v), pH: 2.5) for 15 min by dripping a sufficient quantity to cover the samples. The mast cells were explored in a blinded manner under a light microscope in 10 distinct bifurcation fields of the arteria meningea in each dura mater. The cells were classified as either whole or activated (degranulation). A cell was regarded as degranulated when more than 15 granules were scattered in its

immediate vicinity or when the content coloration disappeared. The numbers of whole and activated cells were calculated. Finally, the proportion of activated cells was computed as a percentage value. Mast cells were photomicrographed using the microscope camera attachment.

2.9 CGRP secretion from explants of trigeminal structures

Rats were randomly allocated to the various groups. The author administered the test drugs in a blinded manner. For CGRP release experiments, isolated trigeminal ganglion, trigeminal nucleus, and meningeal explants were established as previously described (Kilinc et al., 2017a; Citak et al., 2022; Torun et al., 2024). Three general groups were constituted for each isolated preparation. Owing to their two-part anatomical locations, two trigeminal ganglia or meningeal (hemiskull) explants were produced from each animal (n=8/each group, four rats). Twenty-four rats were employed for trigeminal ganglion and meningeal preparations.

However, due to the smaller extent of the trigeminal nucleus in the brainstem, a single preparation containing a pair of trigeminal nuclei was prepared from one rat (n=8/each group, eight rats). Ultimately, a total of 48 animals were recruited for the CGRP release experiments from trigeminal explants. Following CO_2 anesthesia, the head was removed and cleaned of muscles and other tissues. The skull was then split into two halves along the sagittal plane. The brainstem area was harvested by cutting from the caudal side of the brain. As described under the heading “harvesting of trigeminal structures”, the trigeminal ganglion and trigeminal nucleus areas were harvested in conformity with the stereotaxic coordinates. The trigeminal explants were first washed for 30 minutes in 400 mL of synthetic brain extracellular fluid (SBEF, pH: 7.4) (Kilinc et al., 2017a). The explants were subsequently treated with vehicle (SBEF containing 0.2% (v/v) propylene glycol and alcohol), 100 μmol/L NTG (Kilinc et al., 2022), 100 μmol/L esculetin (Lee et al., 2011), or 30 μmol/L of the anti-migraine drug sumatriptan (Torun et al., 2024), alone and in various combinations. The preparations were incubated with 350 μL of SBEF solution containing drugs for 15 min in an incubator at 37 °C. The explants were then washed with SBEF. At 15-min intervals, 240 μL of supernatant was collected and immediately stirred with 25 μL aprotinin to prevent CGRP destruction. The samples were kept at -20 °C until the time of assay. Figure 1 displays the timeline of the *ex vivo* experiments.

2.10 Determination of CGRP and c-Fos concentrations

CGRP and c-Fos concentrations in specimens obtained from in vivo experiments and CGRP concentrations in specimens acquired from trigeminal explants were determined using ELISA equipment. The lowest detectable concentrations were 9.38 pg/mL for CGRP and 0.159 ng/mL for c-Fos. The assay procedures were performed in accordance with the manufacturer’s guidelines. Briefly, the CGRP or c-Fos standard was placed in the relevant slots of the ELISA plate, and the samples were placed in the remaining slots. Following incubation at 37 °C for the specified times and the addition of other reagents, the absorbance of the ELISA plates was quantified at 450 nm using an optical reader device. Peptide concentrations were then calculated.

2.11 Statistical analysis

Data were expressed as mean±standard deviation. The Shapiro–Wilk test was used to evaluate normal distribution. For the in vivo data, a one-way analysis of variance was performed, followed by Tukey’s post-hoc test for pairwise comparisons between the groups. For the ex vivo data, a one-way repeated measures analysis of variance was performed, followed by Bonferroni’s post-hoc test for pairwise comparisons. An independent samples t-test was used for comparisons of two independent groups from the ex vivo research. SPSS Statistics for Windows software (ver. 22.0, Armonk, NY, USA) was employed for the statistical comparisons. A P value of ≤ 0.05 was considered statistically significant.

3. Results

3.1 Reduction of NTG-induced hyperalgesia and c-Fos expression by esculetin

In the in vivo research sets, NTG treatment induced mechanical hyperalgesia by reducing the nociceptive threshold ($P < 0.0001$, Fig. 2a). Compared to vehicle treatment, it also increased the expression of the neural activation marker c-Fos in the nucleus trigeminalis ($P < 0.0001$, Fig. 2b). This confirmed the successful establishment of the in vivo model of migraine in line with previous studies (Baranoglu Kilinc et al., 2024; Kilinc et al., 2024; Torun et al., 2024). However, while both medium (10 mg/kg) and high (20 mg/kg) doses of esculetin alleviated NTG-induced hyperalgesia and elevated levels of c-Fos transcription factor ($P = 0.050$ and $P = 0.001$, respectively, Figs. 2a and 2b), no statistically significant effect was observed with low-dose (5 mg/kg) esculetin ($P > 0.05$, Fig. 2a). Used as a positive control, the anti-migraine drug sumatriptan reversed the NTG-induced changes in withdrawal thresholds and c-Fos levels ($P < 0.0001$ for both; Figs. 2a and 2b). For the pain behavior experiments, a high dose of esculetin ($P = 0.001$, Fig. 2a) was selected as the effective dose since it was more efficacious than the medium dose ($P = 0.050$, Fig. 2a) compared to the model group. Therefore, to test whether the effective dose of esculetin altered the basal levels of the relevant parameters, it was administered alone without inducing a migraine. The results showed that esculetin alone did not significantly alter the pain threshold or c-Fos levels compared with the vehicle control ($P = 1.0$ and $P = 0.875$, respectively, Figs. 2a and 2b).

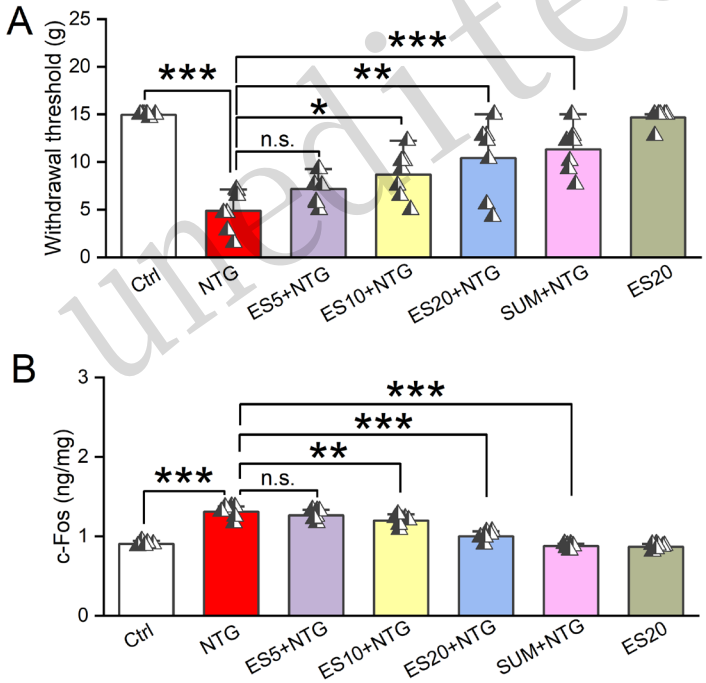

Fig. 2 The effects of esculetin on nitroglycerin-evoked mechanical hyperalgesia and c-fos expression in vivo. (a) The influences of different doses of esculetin on the withdrawal threshold, and (b) c-fos expression in the brainstem trigeminal nucleus caudalis in nitroglycerin-evoked migraine rats. n = 7 per group. The data are expressed as mean±SD. The data were analyzed using a one-way analysis of variance followed by a post-hoc Tukey's test. *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001. Ctrl: control; NTG: nitroglycerin; ES5: 5 mg/kg dose of esculetin; ES10: 10 mg/kg dose of esculetin; ES20: 20 mg/kg dose of esculetin; SD: standard deviation; SUM: sumatriptan; n.s.: non-significance.

3.2 Alleviation of NTG-induced CGRP concentrations in plasma, the nucleus trigeminalis, and the trigeminal ganglion by esculetin

In the in vivo research sets, NTG injection increased CGRP concentrations in plasma, the nucleus trigeminalis, and the trigeminal ganglion compared with the vehicle treatment ($P < 0.001$, Figs. 3a-c). Both medium and high doses of esculetin lowered NTG-stimulated CGRP increases in these structures ($P < 0.001$ for all; Figs. 3a-c), while no statistically significant effect was observed with low-dose esculetin ($P > 0.05$, Figs. 3a-c). Additionally, sumatriptan lowered the concentrations of CGRP that had been increased by NTG in these structures ($P < 0.0001$ for all; Figs. 3a-c). High-dose esculetin with no combination did not significantly change baseline CGRP concentrations in these three structures ($P > 0.05$ for all; Figs. 3a-c).

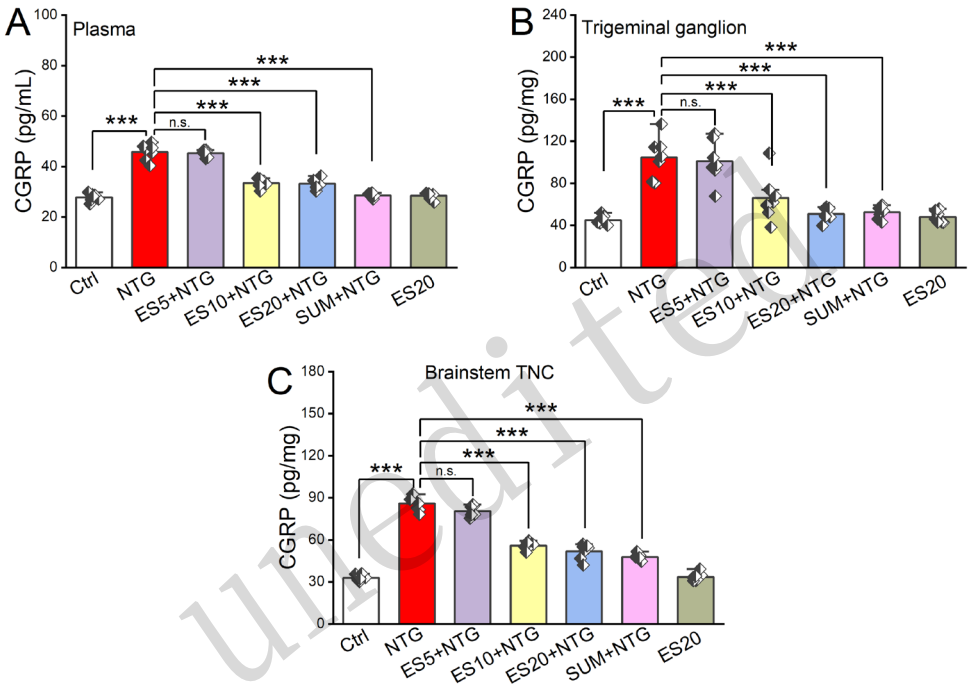

Fig. 3 The effects of esculetin on the nitroglycerin-evoked CGRP levels in plasma, trigeminal ganglion, and trigeminal nucleus caudalis in vivo. (a) The impacts of different doses of esculetin on calcitonin gene-related peptide levels in plasma, (b) trigeminal ganglion, and (c) brainstem trigeminal nucleus caudalis in nitroglycerin-evoked migraine rats. n = 7 per group. The data are expressed as mean±SD. The data were analyzed using a one-way analysis of variance followed by a post-hoc Tukey's test. *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001. Ctrl: control; NTG: nitroglycerin; ES5: 5 mg/kg dose of esculetin; ES10: 10 mg/kg dose of esculetin; ES20: 20 mg/kg dose of esculetin; SD: standard deviation; SUM: sumatriptan; n.s.: non-significance.

3.3 Alleviation of NTG-induced immune mast cell degranulation and counts by esculetin

In the in vivo research sets, NTG led to degranulation/activation and increased immune mast cell numbers compared with the vehicle treatment ($P < 0.001$, Figs. 4a and 4b). Both medium and high doses of esculetin mitigated activation and increased mast cell counts caused by NTG ($P = 0.054$ and $P = 0.025$, respectively, for activation; $P = 0.001$ and $P = 0.002$ for the counts; Figs. 4a and 4b). Sumatriptan reduced the NTG-induced increases in mast cell degranulation and numbers ($P = 0.005$, $p = 0.001$, separately; Figs. 4a and 4b). We observed no statistically significant effect of high-dose esculetin alone on basal activation or mast cell numbers ($P > 0.05$ for both; Figs. 4a and 4b). Selected photomicrographs of mast cells in the different groups are shown in Fig. 5.

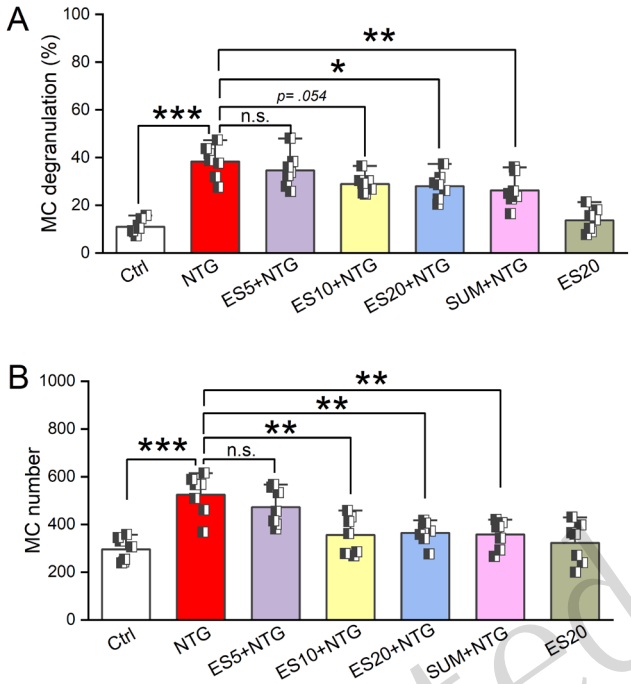

Fig. 4 The effects of esculetin on the nitroglycerin-evoked degranulation and number of meningeal mast cells in vivo. (a) The actions of different doses of esculetin on the degranulation and (b) number of meningeal mast cells in nitroglycerin-evoked migraine rats. n = 7 per group. The data are expressed as mean±SD. The data were analyzed using a one-way analysis of variance followed by a post-hoc Tukey's test. *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001. Since the p-value (0.054) for the comparison between the "NTG" and "ES10+NTG" groups (panel A) is almost equal to 0.050, its exact numerical value is shown on the graph as an exception. Ctrl: control; MC: mast cell; NTG: nitroglycerin; ES5: 5 mg/kg dose of esculetin; ES10: 10 mg/kg dose of esculetin; ES20: 20 mg/kg dose of esculetin; SD: standard deviation; SUM: sumatriptan; n.s.: non-significance.

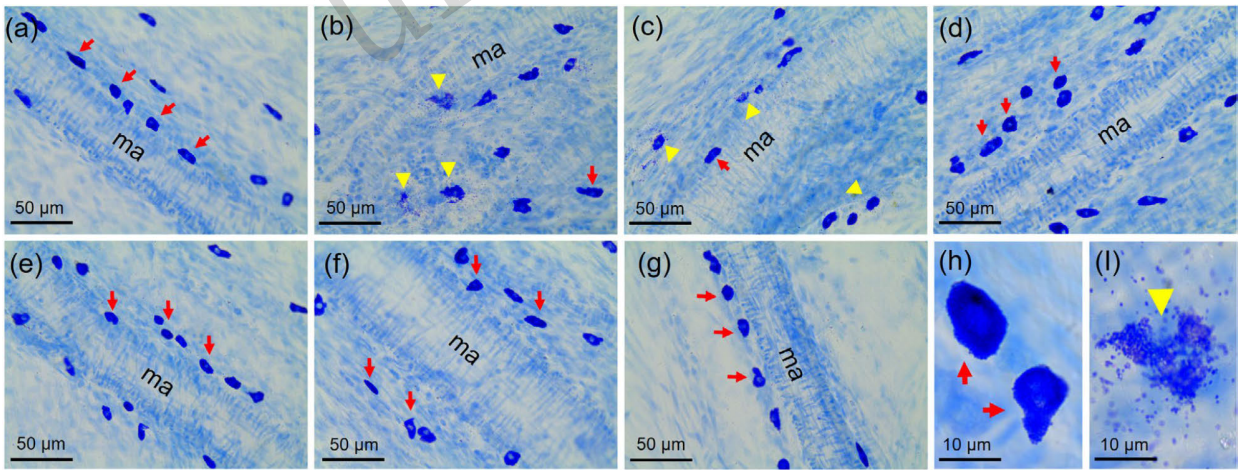

Fig. 5 Representative photomicrographs of meningeal mast cells in the in vivo groups.Photomicrographs of meningeal mast cells in the in vivo groups were taken with a magnification of ×40. (a) Intact mast cells in the control group, (b) degranulated mast cells in the model group, (c) 5 mg/kg dose of esculetin did not suppress nitroglycerin-induced degranulation of mast cells, (d) however 10 mg/kg or (e) 20 mg/kg dose of esculetin reduced mast cell degranulation elicited by nitroglycerin, as a positive control, (f) sumatriptan inhibited nitroglycerin-induced degranulation, (g) esculetin (20 mg/kg) alone did not affect mast cell degranulation. (h) Intact and (i) degranulated mast cells with a magnification of ×100 from the control and model groups, re-

spectively. Open red arrowheads denote the intact cells, while thick, straight yellow arrows represent the degranulated cells. Please note the grain-like mast cell granules spread in close proximity to the activated cells. Scale bars are shown on the images; the scale bar for panels (a)–(g) is 50 μm, while that for panels (h) and (i) is 10 μm.

3.4 Reduction of NTG-induced CGRP secretion from explants of the nucleus trigeminalis and trigeminal ganglion, but not the meninges, ex vivo by esculetin

In the ex vivo research sets, NTG treatment stimulated CGRP secretion from explants of the trigeminal ganglion ($P < 0.001$, Figs. 6a-c), nucleus trigeminalis ($P < 0.001$, Figs. 6d-f), and meninges (hemiskull, $P < 0.001$, Figs. 7a-c) compared with their control treatments. Esculetin reduced NTG-elicited CGRP secretion from explants of the trigeminal ganglia ($P < 0.001$, Fig. 6a) and nucleus trigeminalis ($P < 0.001$, Fig. 6d). However, we observed no statistically significant effect of esculetin on NTG-stimulated CGRP secretion from meningeal explants ($P = 0.481$, Fig. 7a).

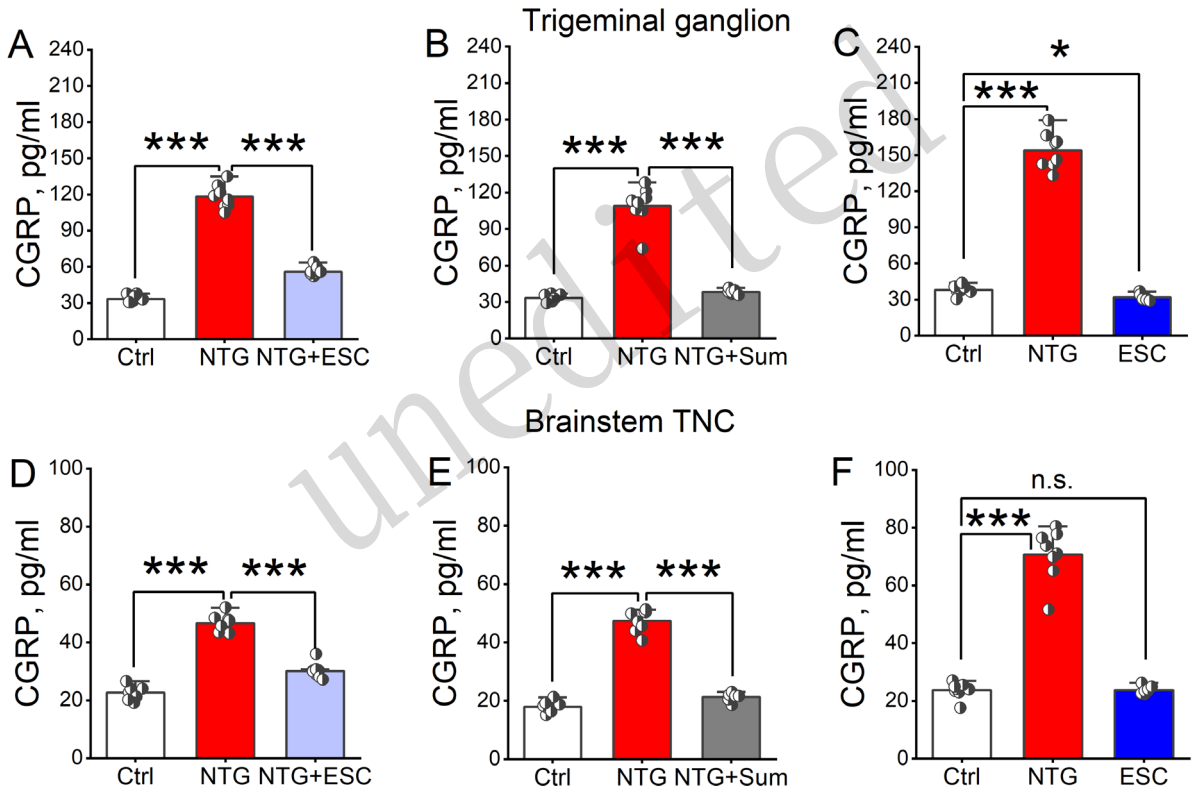

Fig. 6 The effects of esculetin on the nitroglycerin-stimulated CGRP release from isolated trigeminal ganglion and brainstem TNC. (a-c) The influences of esculetin on the nitroglycerin-stimulated release of calcitonin gene-related peptide from isolated trigeminal ganglion and (d-f) brainstem trigeminal nucleus caudalis. n = 8 per group. The data are expressed as mean±SD. The data were analyzed using a one-way repeated measures analysis of variance followed by Bonferroni's post-hoc test. *p ≤ 0.05 and *** p ≤ 0.001. Ctrl: control; NTG: nitroglycerin; ESC: esculetin; Sum: sumatriptan; n.s.: non-significance; TNC: trigeminal nucleus caudalis; SD: standard deviation; CGRP: calcitonin gene-related peptide.

The antimigraine medication sumatriptan suppressed NTG-elicited CGRP secretion from explants of the trigeminal ganglia, nucleus trigeminalis, and meninges ($P < 0.001$ for all; Figs. 6b, 6e and 7b). However, esculetin alone did not significantly alter baseline CGRP secretion from explants of the nucleus trigeminalis and me-

ninges compared to their vehicle treatments ($P = 1.0$ and $P = 0.825$, Figs. 6f and 7c). In contrast, esculetin alone slightly reduced baseline CGRP secretion from explants of trigeminal ganglia ($P = 0.023$, Fig. 6c). Additionally, we observed no statistically significant difference between the effects of esculetin and sumatriptan in reducing NTG-stimulated CGRP secretion in all explants ($P > 0.05$, Figs. 7d-f).

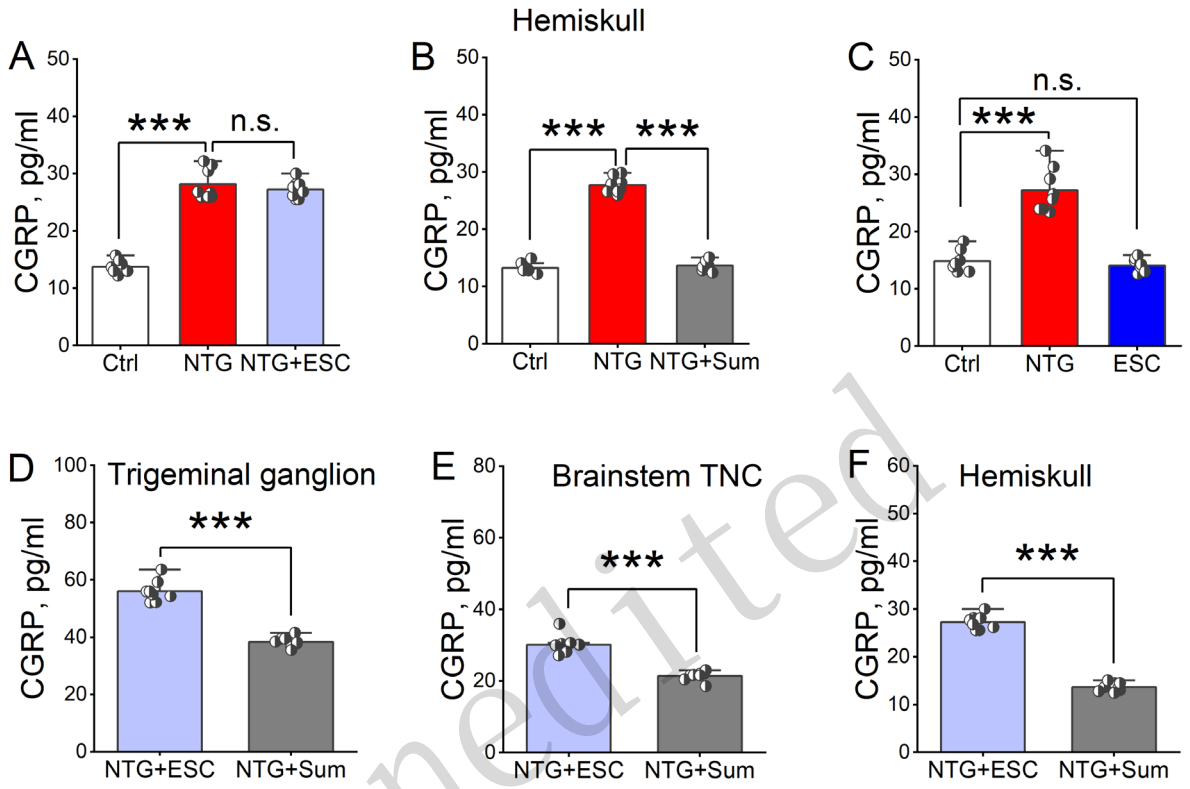

Fig. 7 The effects of esculetin on the nitroglycerin-stimulated CGRP release from isolated hemiskull. (a-c) The influences of esculetin on the nitroglycerin-stimulated release of calcitonin gene-related peptide from isolated hemiskull (meningeal) preparations. (d-f) The comparison of the effectiveness of esculetin and sumatriptan in reducing nitroglycerin-stimulated release of calcitonin gene-related peptide. n = 8 per group. The data are expressed as mean±SD. The data from isolated hemiskull experiments were analyzed using a one-way repeated measures analysis of variance followed by Bonferroni's post-hoc test. Comparisons between esculetin and sumatriptan (in panels D, E, and F) were carried out using an independent samples t-test. ***p ≤ 0.001. Ctrl: control; NTG: nitroglycerin; ESC: esculetin; Sum: sumatriptan; n.s.: non-significance; SD: standard deviation; CGRP: calcitonin gene-related peptide.

4. Discussion

This research showed that esculetin attenuated NTG-induced mechanical hyperalgesia, enhanced c-Fos concentrations in the nucleus trigeminalis, elevated CGRP concentrations in plasma, the trigeminal ganglia, and the nucleus trigeminalis, and increased the activation and numbers of immune mast cells in the in vivo experimental sets. It also reduced the NTG-induced secretion of CGRP from explants of the trigeminal ganglia and nucleus trigeminalis in the ex vivo experimental sets. These NTG-induced changes indicate that the in vivo migraine model was successfully established. NTG-induced alterations were reversed by sumatriptan, used as a positive control, further confirming the migraine model's validity. These findings align with those of similar previous

studies (Ramachandran et al., 2014; Kilinc et al., 2018; Baranoglu Kilinc et al., 2024; Kilinc et al., 2024; Torun et al., 2024).

Trigeminovascular system activation contributes to pathophysiological processes in migraine by leading to vascular dilatation, trigeminal CGRP secretion, and degranulation of dural mast cells (Ramachandran, 2018; Koyuncu Irmak et al., 2019). These three critical events result in the development of a sterile inflammation in the meninges, which is well established in animal studies (Bolay et al., 2002; Levy and Moskowitz, 2023). Additionally, circulating CGRP concentrations rise during and between migraine attacks in humans (Frederiksen et al., 2020; Kilinc et al., 2023). New generation anti-migraine drugs targeting CGRP or its receptors (gepants and monoclonal antibodies) have been shown to be efficacious in migraineurs (Silvestro et al., 2023). However, some patients are still resistant to existing medications, including these novel drugs. Due to the bi-directional interplay between CGRP and dural mast cells, novel multi-targeted approaches capable of blocking both may represent an attractive alternative approach for migraine therapy.

Esculetin is a molecule worth investigating for its effects on migraine-like conditions due to its multiple biological impacts (Singhuber et al., 2011; Rzodkiewicz et al., 2015; Rzodkiewicz et al., 2016; Zhu et al., 2016; Singh et al., 2020; Cheng et al., 2021). In the current study, esculetin ameliorated NTG-induced mechanical hyperalgesia by raising the hind paw withdrawal threshold. This was supported by decreased expression of the activation-related c-Fos protein in the nucleus trigeminalis. Esculetin has previously been shown to exhibit analgesic effects in animal models of non-migraine pain (Rzodkiewicz et al., 2015; Singh et al., 2020; Zhang et al., 2025). The results of these prior studies are consistent with our findings regarding the analgesic effect of esculetin. However, our study is the first in the literature to demonstrate the analgesic effect of esculetin in a migraine model.

In the current study, esculetin lowered the raised concentrations of CGRP in plasma, the trigeminal ganglia, and the nucleus trigeminalis in a migraine-like state. Since the release of CGRP from the trigeminovascular system is a critical target of new-generation migraine treatments, esculetin's ability to reduce CGRP expression is indicative of its anti-migraine potential.

Esculetin suppressed NTG-stimulated degranulation and immune mast cell numbers in the meninges, regarded as the area of migraine pain onset. CGRP induces the degranulation of mast cells, and in response, mediators released from these cells give rise to further activation of meningeal trigeminal nerves, as well as meningeal vasodilation (Kilinc et al., 2017b; Levy and Moskowitz, 2023). This interplay between mast cells and nerves exacerbates neurogenic inflammation through a vicious cycle. The stabilization of mast cells is therefore important for breaking this cycle. This is the first research to demonstrate the potency of esculetin in stabilizing meningeal mast cells. In a previous study, esculetin prevented the activation of mouse tumor mast cells by inhibiting leukotriene synthesis (Neichi et al., 1983). Furthermore, a recent study has shown that esculetin inhibited mast cell-mediated allergic inflammation and anaphylaxis by suppressing the FcεRI signaling pathway (Kim et al., 2025). Our findings are consistent with these previous studies regarding esculetin's mast cell-stabilizing effect. However, further studies are required to elucidate the stabilizing mechanism.

Esculetin also reduced the number of meningeal mast cells that had been increased by NTG. This is consistent with a previous study reporting that esculetin reduced mast cell infiltration of the lesion area in a mouse model of acute atopic skin inflammation (Jeong et al., 2018). Compared with the control group, the effective dose of esculetin alone in the absence of migraine induction did not alter the baseline values of the parameters measured in in vivo experiments. The fact that esculetin produced no change in the physiological biomarker levels adds to its potential for development into a drug form in the future. New-generation anti-migraine drugs only target the CGRP signaling pathway and are not effective in all patients (Labastida-Ramírez et al., 2023). Esculetin may therefore represent a multi-target therapeutic option for patients for whom existing drugs are ineffective, as it acts by suppressing both CGRP release from trigeminal structures and meningeal mast cell activation. However, studies investigating the therapeutic effects of esculetin in different migraine models and clinical trials are now

needed.

We also tested the impact of esculetin in ex vivo experimental sets to reveal whether it exerts direct or indirect effects on CGRP release. Esculetin was administered directly ex vivo but systemically in vivo. It reduced the NTG-stimulated secretion of CGRP from explants of the trigeminal ganglia and nucleus trigeminalis. The results of these experiments with trigeminal explants suggest that esculetin suppresses the excitability of CGRP-containing peptidergic neurons and nerve endings in the trigeminal ganglion and the trigeminal nucleus caudalis, as CGRP is released from these structures via the vesicular exocytosis mechanism, which requires neuronal depolarization. This finding is consistent with previous studies showing that esculetin inhibited neuronal excitability (Singhuber et al., 2011; Wu et al., 2013; Skalicka-Wozniak et al., 2016).

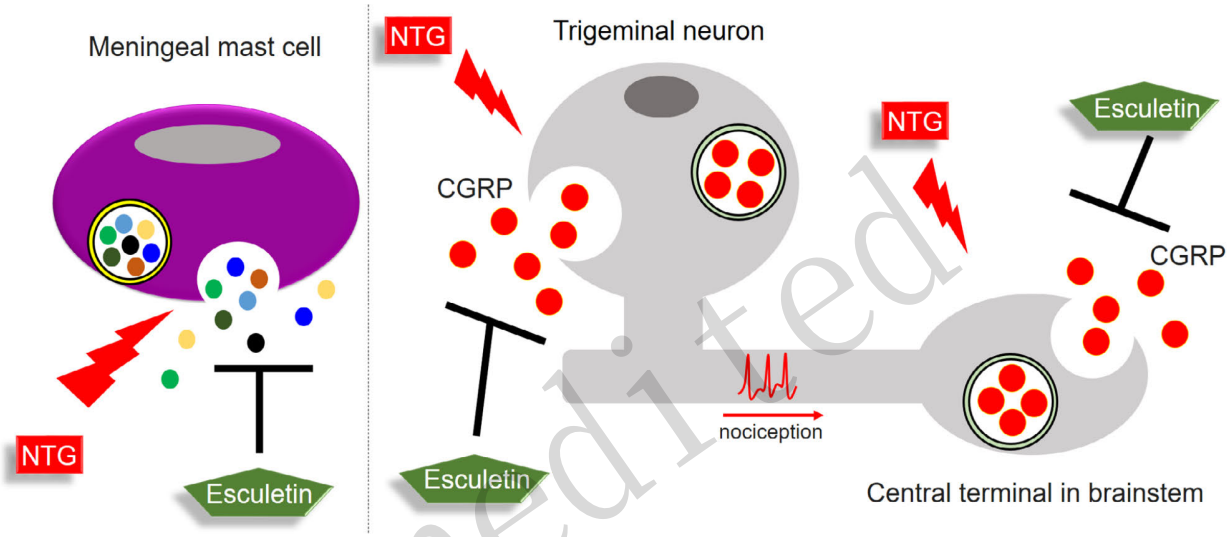

Fig. 8 Proposed mechanisms of action of esculetin in reducing hyperalgesia in nitroglycerin-induced migraine-like experimental conditions. Esculetin alleviates nitroglycerin-induced hyperalgesia by reducing CGRP release from peripheral (trigeminal neurons) and central (brainstem trigeminal nucleus caudalis) components of the trigeminovascular system and suppressing the activation of meningeal mast cells. The red lightning bolt indicates activation (induction) while the black T shape indicates inhibition. CGRP: calcitonin gene-related peptide; NTG: nitroglycerin.

Esculetin had no effect on the NTG-induced secretion of CGRP from hemiskull explants. It is not easy to establish the exact reason for this based on the current findings. Different mechanisms may exist by which esculetin interacts in the meninges, since the meningeal afferents of the trigeminal nerve contain a wide range of receptors and ion channels that can detect almost all types of stimuli (Yan and Dussor, 2014). Further mechanistic studies are therefore needed to elucidate this. The migraine drug sumatriptan was superior to esculetin in reducing CGRP secretion from trigeminal explants. However, this by no means trivializes esculetin's multi-target therapeutic effects on migraine-related parameters in vivo.

5. Conclusion

The findings of this study suggest that esculetin ameliorates migraine-like pain by reducing CGRP secretion from peripheral and central areas of the trigeminal complex and by repressing the activation of meningeal mast cells. Fig. 8 illustrates the proposed mechanisms of action of esculetin in this study. The findings also provide a

preliminary scientific basis for possible future human trials. With its multiple pharmacological features, esculetin may represent a multifaceted therapeutic candidate for future migraine therapy.

Limitations

This study employed an acute migraine model to investigate the effects of esculetin. However, since migraine has both episodic and chronic forms, it would be interesting for future research to test the impact of esculetin in a chronic migraine model. We evaluated mechanical hyperalgesia in the hind paw of rats following NTG injection, which mimics migraine-like extracephalic allodynia in patients. However, if, in addition to the hind paw, we had tested for hyperalgesia in the face, such as the periorbital area, this would have further supported our findings, since the facial area is innervated by the trigeminal nerve, which also transmits migraine pain.

Data availability statement

Data will be made available on request. Some of the data from the work were represented at the 48th Turkish Physiology Conference held in Türkiye on 01-04.11.2023.

Acknowledgments

This work was supported by The Scientific and Technological Research Council of Türkiye (TÜBİTAK) (Grant-number: 122S014). We thank the The Scientific and Technological Research Council of Türkiye for the financial support.

Author contributions

Erkan Kilinc supervised the study, provided resources and prepared the first draft of the manuscript. Ayca Nur Gonul and Ibrahim Ethem Torun carried out experiments, collected the data and contributed to original draft preparation. Yasemin Baranoglu Kilinc analyzed data, interpreted the results, produced graphs, and contributed to original draft preparation. All authors reviewed the results and approved the final version of the manuscript.

Compliance with ethics guidelines

The authors Erkan Kilinc, Ayca Nur Gonul, Ibrahim Ethem Torun and Yasemin Baranoglu Kilinc declare that they have no potential conflicts of interest.

The experimental implementations were permitted by regional ethics council for animal experiments of the University (protocol no: 2021/38 and 2022-13). The animals were treated in harmony with the National Institutes of Health guide for the care and use of laboratory animals.

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