Journal of Zhejiang University-SCIENCE B 2026 27(6):615-627
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

Check for updates

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

Ayca Nur GONUL[1], Ibrahim Ethem TORUN[1], Yasemin Baranoglu KILINC[2], Erkan KILINC[3]✉

[1]*Department of Physiology, Faculty of Medicine, Bolu Abant Izzet Baysal University, Bolu 14030, Türkiye*
[2]*Department of Pediatrics, Faculty of Medicine, Bolu Abant Izzet Baysal University, Bolu 14030, Türkiye*
[3]*Department of Physiology, Cerrahpasa Faculty of Medicine, İstanbul University-Cerrahpaşa, İstanbul 34098, Türkiye*

Abstract: Growing evidence suggests that esculetin, a 5-lipoxygenase inhibitor, has pharmacotherapeutic potential due to its various pharmacological properties, such as potent anti-inflammatory, anti-nociceptive, and γ-aminobutyric acid type A ($GABA_A$) receptor partial agonist activities. However, the effects of this promising agent on migraine remain unexplored. This study therefore examined the impact of esculetin on 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 (NTG)-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, trigeminal nucleus, 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 cellular Fos proto-oncogene (c-Fos) levels. It also decreased 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 degranulating, and vasodilatory properties (Ottosson and Edvinsson, 1997; Kilinc et al., 2017b). 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.

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., 2010). Esculetin

✉ Erkan KILINC, erkan.kilinc@iuc.edu.tr
Erkan KILINC, https://orcid.org/0000-0001-9261-2634

Received Mar. 26, 2025; Revision accepted Sept. 1, 2025;
Crosschecked Mar. 20, 2026; Published online May 1, 2026

also exhibits a broad array of pharmacological effects, with potent anti-inflammatory and antioxidant properties (Liang et al., 2017; Zhang et al., 2022; Danis et al., 2023). It has been found to exhibit antinociceptive 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., 2017b; 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; and 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 (NTG)-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. They were housed in separate Plexiglas chambers and maintained on a 12-h light/dark cycle at (22±2) ℃. Water and chow were provided ad libitum.

2.2 Chemicals and their preparation

NTG in solution 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), enzyme-linked immunosorbent assay (ELISA) kits for rat CGRP detection from Elabscience (Texas, USA, Cat. #E-EL-R0135), ELISA kits for rat cellular Fos proto-oncogene (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% (volume fraction) DMSO, was employed as the vehicle for esculetin (Veh1). The solvent of stock NTG 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% (volume fraction) propylene glycol, 6% (volume fraction) alcohol, and 0.9% (9 g/L) saline) was employed as the vehicle for NTG (Veh2). Sumatriptan was dissolved in saline solution.

2.3 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. The 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 at 10 mL/kg body weight. NTG was administered at a single dose of 10 mg/kg to establish an 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 with seven animals in each group: Veh1+Veh2

(control), Veh1+NTG (model), low-dose esculetin (ES5)+NTG, medium-dose esculetin (ES10)+NTG, high-dose esculetin (ES20)+NTG, sumatriptan (SUM)+NTG, and ES20+Veh2. Veh1 was injected into both the control and model groups once daily for five days. A dose of 5 mg/kg 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 SUM+NTG group. In the withdrawal hyperalgesia test, an effective dose of 20 mg/kg esculetin was administered to the ES20+Veh2 group. Thirty minutes after the injections on Day 5, Veh2 was administered to the control and ES20+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.

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*g* 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

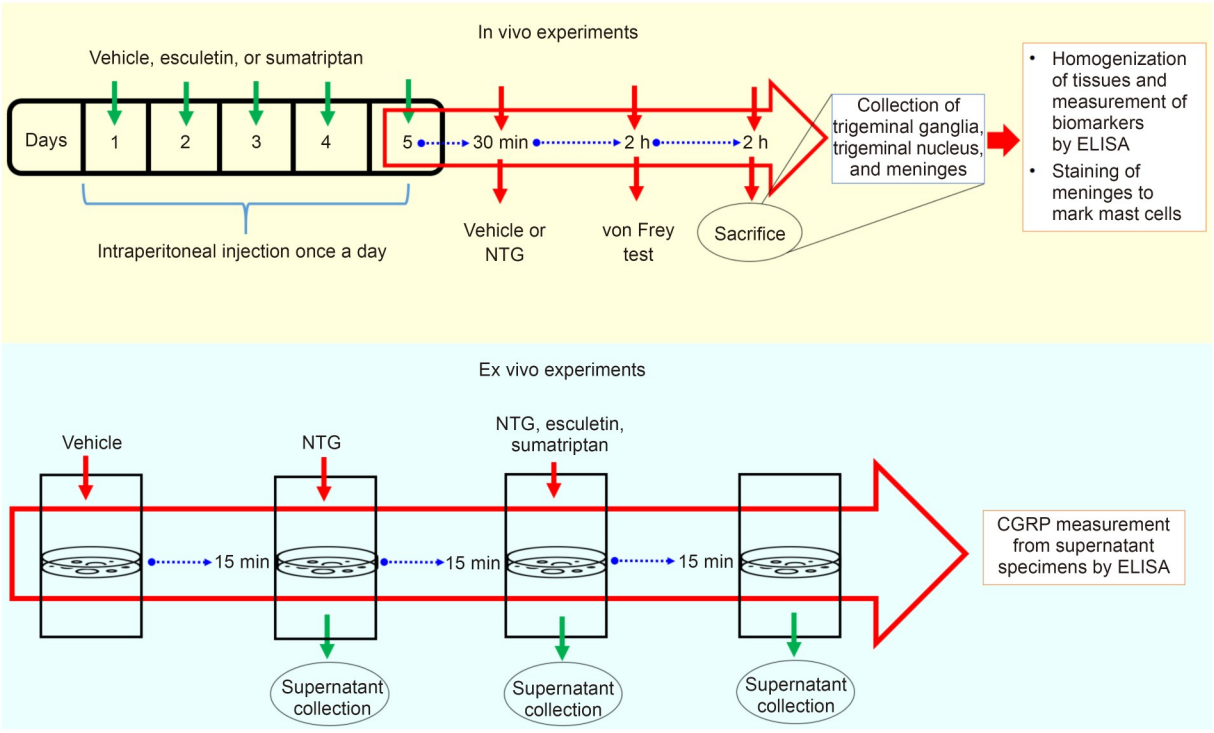

Fig. 1 Schematic illustration of the in vivo and ex vivo experimental processes. NTG: nitroglycerin; ELISA: enzyme-linked immunosorbent assay; CGRP: calcitonin gene-related peptide.

was withdrawn from the right chamber of the heart by means of an injector. The blood samples were transferred to ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged at 4000 r/min for 15 min at 4 ℃. The plasma samples were preserved at −20 ℃. 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 resection 1 mm from the diverging part of the mandibular branch of the trigeminal nerve. The collected samples were stored at −20 ℃. The dura mater connected to the hemiskull was placed in the fixative solution (4% (0.04 g/mL) paraformaldehyde (PFA)) overnight for immune mast cell fixation.

2.7 Trigeminal ganglia and trigeminal nucleus homogenization

Trigeminal ganglion and trigeminal nucleus specimens were homogenized at a constant quantity of 100 mg/mL using a mild-work Ultra-Turrax homogenization device in supercooled PBS (pH 7.4) containing 20 IU/mL aprotinin. The homogenized samples were subsequently centrifuged for 30 min at 4000 r/min at 4 ℃. The supernatant fluid obtained from the homogenates was preserved at −20 ℃.

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 PFA, 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% (1 g/L), 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 considered 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

The 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., 2017b; 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 used 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 was isolated by a transverse cut at 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 min in 400 mL of synthetic brain extracellular fluid (SBEF; pH 7.4) (Kilinc et al., 2017b). The explants were subsequently treated

with vehicle (SBEF containing 0.2% (volume fraction) 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 or in various combinations. The preparations were incubated with 350 μL of SBEF solution containing drugs for 15 min in an incubator at 37 ℃. The explants were then washed with SBEF. At 15-min intervals, 240 μL of supernatant was collected and immediately stirred with 25 μL of aprotinin to prevent CGRP destruction. The samples were kept at −20 ℃ until the time of assay. Fig. 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 ℃ for the specified time and the addition of other reagents, the absorbance of the ELISA plates was quantified at 450 nm using an optical reader device (Epoch BioTek Instruments Inc., Winooski, VT, USA). Peptide concentrations were then calculated.

2.11 Statistical analysis

The data were expressed as mean±standard deviation (SD). The Shapiro-Wilk test was used to evaluate normal distribution. For the in vivo data, one-way analysis of variance (ANOVA) was performed, followed by Tukey's post-hoc test for pairwise comparisons between the groups. For the ex vivo data, one-way repeated measures ANOVA 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 in NTG-induced hyperalgesia and c-Fos expression by esculetin

In the in vivo research sets, compared to vehicle treatment, NTG treatment induced mechanical hyperalgesia by reducing the nociceptive threshold (P< 0.001; Fig. 2a), and increased the expression of the neural activation marker c-Fos in the trigeminal nucleus (P<0.001; 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 (P=0.05 and P=0.001, respectively; Fig. 2a) and elevated levels of c-Fos transcription factor (P=0.005 and P=0.0001, respectively; Fig. 2b), no statistically significant effect was observed with low-dose (5 mg/kg) esculetin (P>0.05; Fig. 2). 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; Fig. 2). For the pain behavior experiments, a

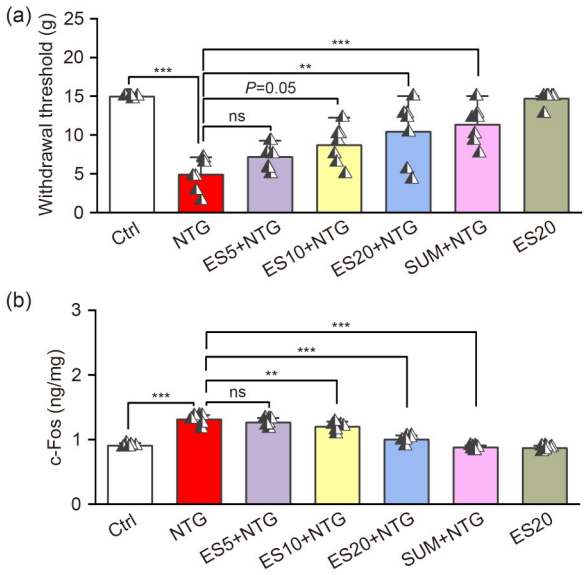

Fig. 2 Effects of different doses of esculetin on the withdrawal threshold (a) and c-Fos expression in the brainstem trigeminal nucleus (b) in nitroglycerin (NTG)-evoked migraine model rats (n=7 per group). The data are expressed as mean±standard deviation (SD) and were analyzed using a one-way analysis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

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=0.05; 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 (20 mg/kg) 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; Fig. 2).

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

In the in vivo research sets, NTG injection increased CGRP concentrations in the plasma, trigeminal nucleus, and trigeminal ganglion compared with the vehicle treatment (P<0.001; Fig. 3). Both medium and high doses of esculetin lowered NTG-stimulated CGRP increases in these structures (P<0.001; Fig. 3), while no statistically significant effect was observed with low-dose esculetin (P>0.05; Fig. 3). Additionally, sumatriptan lowered the concentrations of CGRP that were increased by NTG in these structures (P=0.0001; Fig. 3). High-dose esculetin with no combination did not significantly change baseline CGRP concentrations in these three structures (control vs. ES20, P=0.996 for plasma, P=1.0 for trigeminal ganglion and trigeminal nucleus; Fig. 3).

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; Fig. 4). The high dose of esculetin significantly reduced the NTG-induced increase in mast cell activation (P=0.025); the moderate dose also reduced it, but the reduction was not statistically significant (P=0.054) (Fig. 4). Both medium and high doses of esculetin significantly mitigated the NTG-enhanced mast cell count (P=0.001 and P=0.002, respectively; Fig. 4). Sumatriptan also suppressed NTG-induced increases in both the degranulation (P=0.005) and count (P=0.001) of mast cells (Fig. 4). High-dose esculetin alone had no statistically significant effect on basal activation or mast cell count compared with the control group (P=0.976 and P=0.991, respectively; Fig. 4). Selected photomicrographs of mast cells in the different groups are shown in Fig. 5.

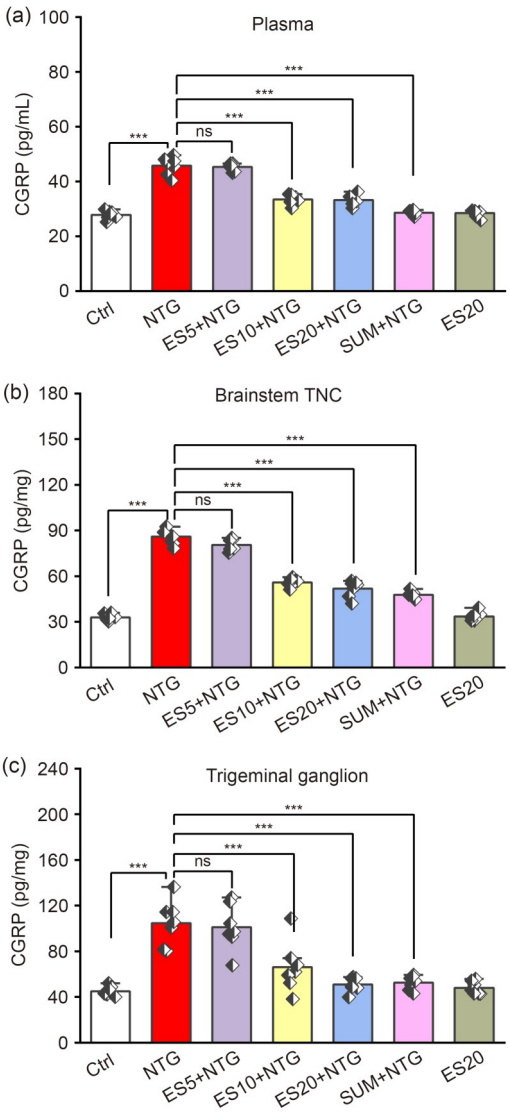

Fig. 3 Impacts of different doses of esculetin on calcitonin gene-related peptide (CGRP) levels in the plasma (a), brainstem trigeminal nucleus caudalis (TNC) (b), and trigeminal ganglion (c) in vivo in nitroglycerin (NTG)-evoked migraine model rats (n=7 per group). The data are expressed as mean±standard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

3.4 Reduction in NTG-induced CGRP secretion from explants of the trigeminal nucleus 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–6c), trigeminal nucleus (P<0.001; Figs. 6d–6f), and meninges (hemiskull, P<

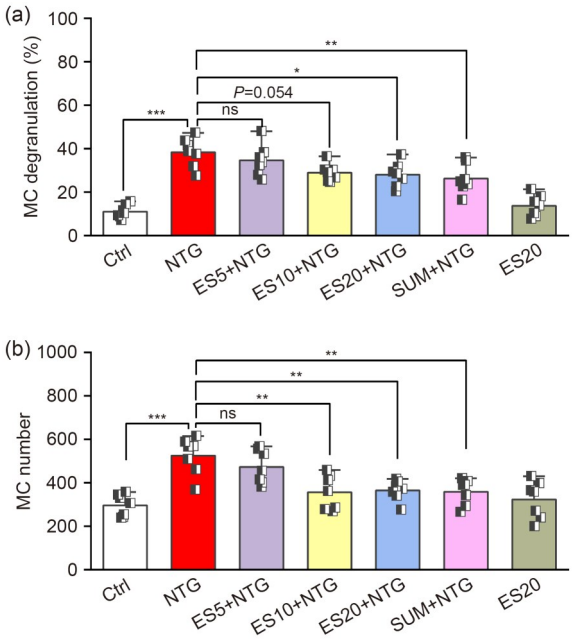

Fig. 4 Effects of different doses of esculetin on the degranulation (a) and number (b) of meningeal mast cells (MCs) in vivo in nitroglycerin (NTG)-evoked migraine model rats (n=7 per group). The data are expressed as mean± standard deviation (SD) and were analyzed using one-way analysis of variance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

0.001; Figs. 6g–6i), compared with their control treatments. Esculetin reduced NTG-elicited CGRP secretion from explants of the trigeminal ganglia (P< 0.001; Fig. 6a) and trigeminal nucleus (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. 6g).

The antimigraine medication sumatriptan suppressed NTG-elicited CGRP secretion from explants of the trigeminal ganglia, trigeminal nucleus, and meninges (P<0.001 for all; Figs. 6b, 6e, and 6h). However, esculetin alone did not significantly alter baseline CGRP secretion from explants of the trigeminal nucleus or meninges compared to their vehicle treatments (P=1.0 and P=0.825, respectively; Figs. 6f and 6i). In contrast, esculetin alone slightly reduced baseline CGRP secretion from explants of the trigeminal ganglia compared with the control group (P=0.023; Fig. 6c). Additionally, sumatriptan was more effective than esculetin in reducing NTG-stimulated CGRP secretion in all explants (P=0.0001; Fig. 7).

4 Discussion

This research showed that esculetin attenuated multiple NTG-induced effects in in vivo experimental sets, including mechanical hyperalgesia, enhanced c-Fos concentrations in the nucleus trigeminalis,

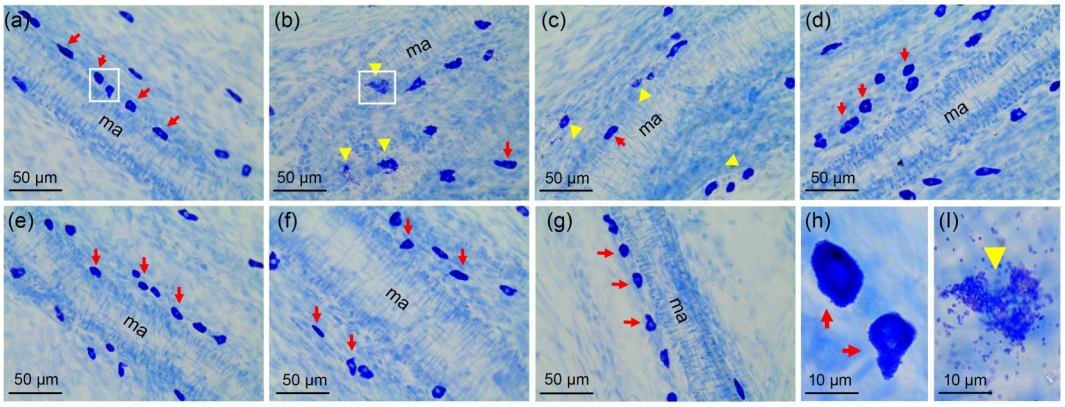

Fig. 5 Representative photomicrographs of meningeal mast cells in the in vivo groups. (a) Intact mast cells in the control group; (b) Degranulated mast cells in the model group; (c) 5 mg/kg dose of esculetin did not suppress nitroglycerin (NTG)-induced degranulation of mast cells; (d, e) 10 mg/kg (d) or 20 mg/kg (e) dose of esculetin reduced mast cell degranulation elicited by NTG, as a positive control; (f) Sumatriptan inhibited NTG-induced degranulation; (g) Esculetin (20 mg/kg) alone did not affect mast cell degranulation; (h, i) Intact (h) and degranulated (i) mast cells from the control (a) and model (b) groups (white frame), respectively. The open red arrows denote the intact cells, while the thick, straight yellow arrowheads represent the degranulated cells. Notably, the grain-like mast cell granules spread in close proximity to the activated cells. ma: mast cells.

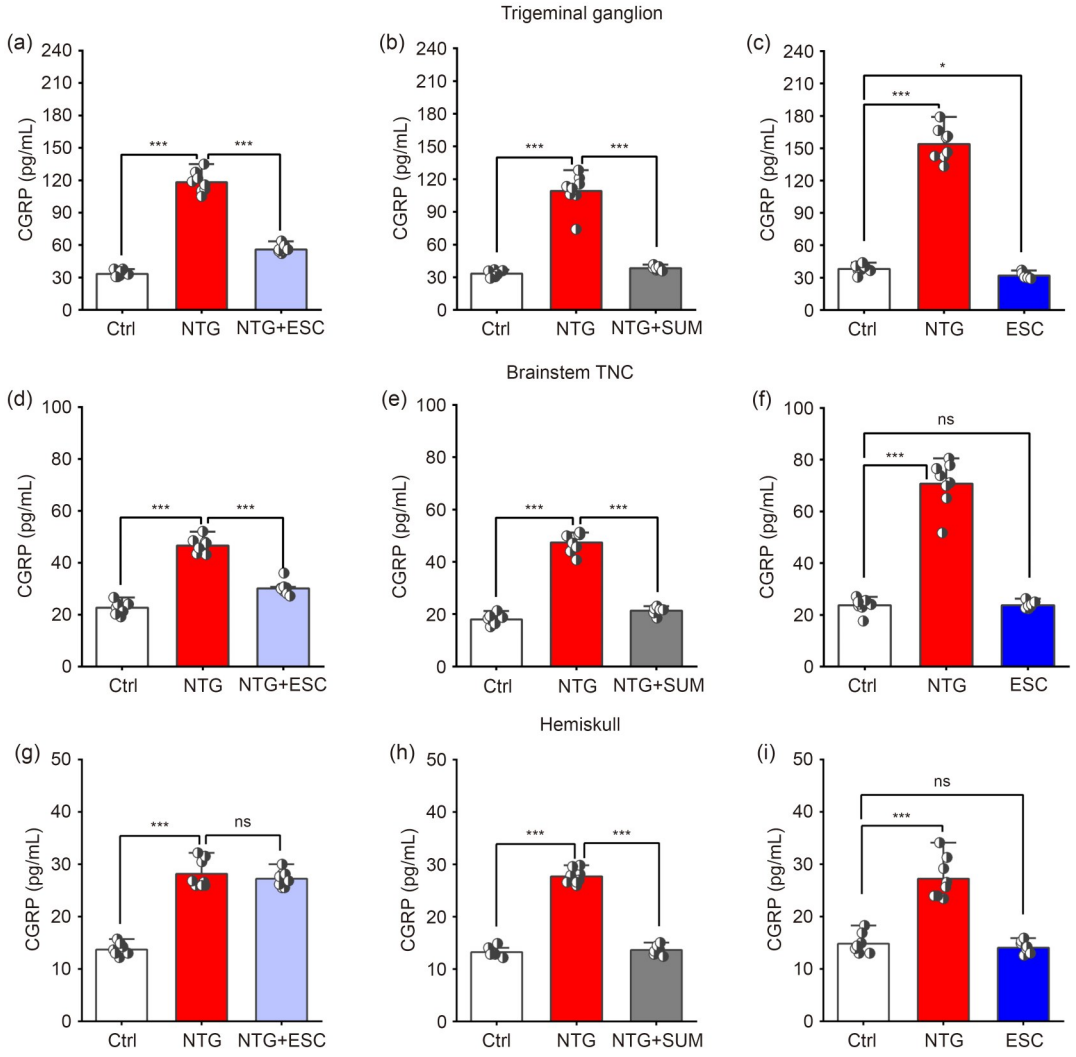

Fig. 6 Effects of esculetin (ESC) on the nitroglycerin (NTG)-stimulated calcitonin gene-related peptide (CGRP) release from isolated trigeminal ganglion (a–c), brainstem trigeminal nucleus caudalis (TNC) (d–f), and hemiskull (meningeal) (g–i). *n*=8 per group. The data are expressed as mean±standard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

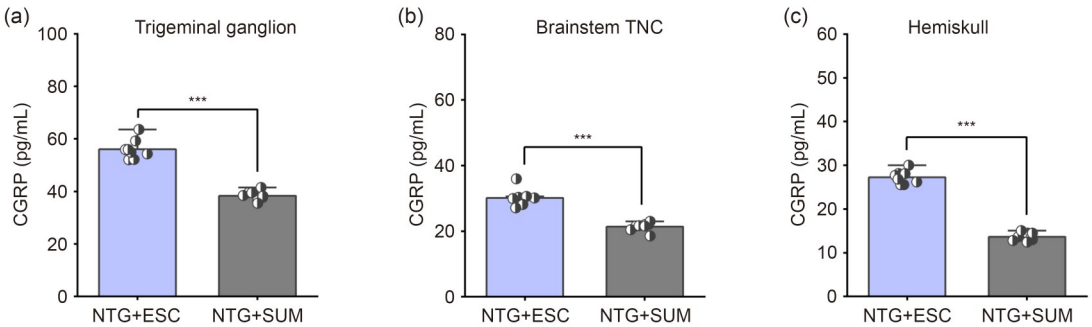

Fig. 7 Comparisons of the effectiveness of esculetin (ESC) and sumatriptan (SUM) in reducing nitroglycerin (NTG)-stimulated release of CGRP from isolated trigeminal ganglion (a), brainstem trigeminal nucleus caudalis (TNC) (b), and hemiskull (c). *n*=8 per group. The data are expressed as mean±standard deviation (SD). Comparisons between ESC and SUM were carried out using an independent samples *t*-test. *** *P*<0.001.

elevated CGRP concentrations in the plasma, trigeminal ganglia, and trigeminal nucleus, and increased activation and number of immune mast cells. It also reduced the NTG-induced secretion of CGRP from explants of the trigeminal ganglia and trigeminal nucleus in ex vivo experimental sets. The NTG-induced changes indicate that the in vivo and ex vivo migraine models were 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, 2024; Baranoglu 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 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 bidirectional interplay between CGRP and dural mast cells, novel multi-targeted approaches capable of blocking both may represent attractive alternative approaches 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, 2016; Zhu et al., 2016; Singh et al., 2020; Cheng et al., 2021). In the present 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 trigeminal nucleus. 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 present study, esculetin lowered the raised concentrations of CGRP in the plasma, trigeminal ganglia, and trigeminal nucleus 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., 2017a; 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 Fc epsilon receptor I (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 in the future. New-generation anti-migraine drugs target only 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 trigeminal nucleus. 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 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-Woźniak et al., 2016).

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 mechanism. 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.

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 paws 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.

5 Conclusions

The findings of this study suggest that esculetin ameliorates migraine-like pain by reducing CGRP secretion from the 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.

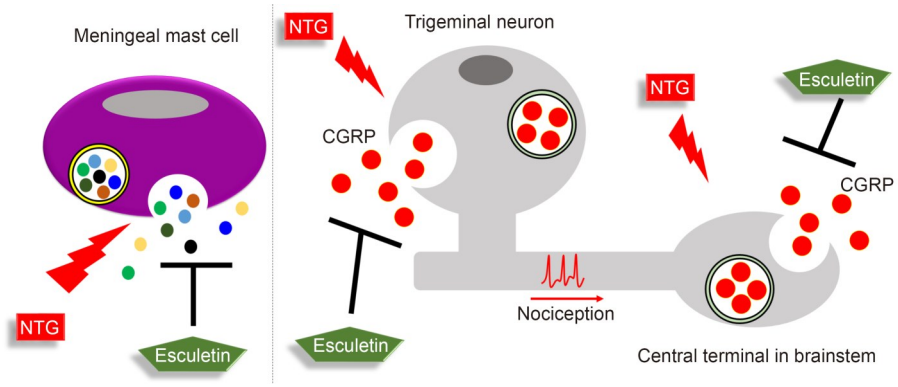

Fig. 8 Proposed mechanisms of action of esculetin in reducing hyperalgesia in nitroglycerin (NTG)-induced migraine-like experimental conditions. Esculetin alleviates NTG-induced hyperalgesia by reducing calcitonin gene-related peptide (CGRP) release from peripheral (trigeminal neurons) and central (brainstem 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.

Data availability statement

The data will be made available upon 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) (No. 122S014).
Funding note: Open access funding provided by the Scientific and Technological Research Council of Türkiye (TÜBİTAK).

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 the experiments, collected the data, and prepared the original draft. Yasemin Baranoglu KILINC analyzed the data, interpreted the results, produced the graphs, and prepared the original draft. All the authors reviewed the results and approved the final version of the manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Ayca Nur GONUL, Ibrahim Ethem TORUN, Yasemin Baranoglu KILINC, and Erkan KILINC declare that they have no conflicts of interest.

The experimental implementations were permitted by the regional ethics council for animal experiments of the Bolu Abant Izzet Baysal University, Türkiye (protocol Nos. 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.

Declaration on the use of generative AI tools

No generative AI tools were used in the preparation of this manuscript.

Open Access

References

Baranoglu Kilinc Y, Torun IE, Kilinc E, 2024. D2 dopamine receptor-mediated mechanisms of dopaminergic system modulation in in vivo and in vitro experimental models of migraine. *Eur J Neurosci*, 59(6):1177-1193. https://doi.org/10.1111/ejn.16106

Bolay H, Reuter U, Dunn AK, et al., 2002. Intrinsic brain activity triggers trigeminal meningeal afferents in a migraine model. *Nat Med*, 8(2):136-142. https://doi.org/10.1038/nm0202-136

Cetinkaya A, Kilinc E, Camsari C, et al., 2020. Effects of estrogen and progesterone on the neurogenic inflammatory neuropeptides: implications for gender differences in migraine. *Exp Brain Res*, 238(11):2625-2639. https://doi.org/10.1007/s00221-020-05923-7

Cheng YJ, Tian XL, Zeng YZ, et al., 2021. Esculetin protects against early sepsis *via* attenuating inflammation by inhibiting NF-κB and STAT1/STAT3 signaling. *Chin J Nat Med*, 19(6):432-441. https://doi.org/10.1016/S1875-5364(21)60042-0

Citak A, Kilinc E, Torun IE, et al., 2022. The effects of certain TRP channels and voltage-gated KCNQ/Kv7 channel opener retigabine on calcitonin gene-related peptide release in the trigeminovascular system. *Cephalalgia*, 42(13):1375-1386. https://doi.org/10.1177/03331024221114773

Danis A, Baranoglu Kilinc Y, Torun IE, et al., 2023. Esculetin alleviates pentylenetetrazole-induced seizures, cognitive impairment and pro-inflammatory cytokines and suppresses penicillin-induced epileptiform activity in rats. *Life Sci*, 313:121300. https://doi.org/10.1016/j.lfs.2022.121300

Frederiksen SD, Bekker-Nielsen Dunbar M, Snoer AH, et al., 2020. Serotonin and neuropeptides in blood from episodic and chronic migraine and cluster headache patients in case-control and case-crossover settings: a systematic review and meta-analysis. *Headache*, 60(6):1132-1164. https://doi.org/10.1111/head.13802

Giménez-Bastida JA, González-Sarrías A, Laparra-Llopis JM, et al., 2021. Targeting mammalian 5-lipoxygenase by dietary phenolics as an anti-inflammatory mechanism: a systematic review. *Int J Mol Sci*, 22(15):7937. https://doi.org/10.3390/ijms22157937

Jeong NH, Yang EJ, Jin ML, et al., 2018. Esculetin from *Fraxinus rhynchophylla* attenuates atopic skin inflammation by inhibiting the expression of inflammatory cytokines. *Int Immunopharmacol*, 59:209-216. https://doi.org/10.1016/j.intimp.2018.04.005

Kilinc E, Dagistan Y, Kotan B, et al., 2017a. Effects of *Nigella sativa* seeds and certain species of fungi extracts on number and activation of dural mast cells in rats. *Physiol Int*, 104(1):15-24. https://doi.org/10.1556/2060.104.2017.1.8

Kilinc E, Guerrero-Toro C, Zakharov A, et al., 2017b. Serotonergic mechanisms of trigeminal meningeal nociception: implications for migraine pain. *Neuropharmacology*, 116:160-173. https://doi.org/10.1016/j.neuropharm.2016.12.024

Kilinc E, Dagistan Y, Kukner A, et al., 2018. Salmon calcitonin ameliorates migraine pain through modulation of CGRP release and dural mast cell degranulation in rats. *Clin Exp Pharmacol Physiol*, 45(6):536-546. https://doi.org/10.1111/1440-1681.12915

Kilinc E, Tore F, Dagistan Y, et al., 2020. Thymoquinone inhibits neurogenic inflammation underlying migraine through modulation of calcitonin gene-related peptide release and stabilization of meningeal mast cells in glyceryltrinitrate-induced migraine model in rats. *Inflammation*, 43:264-273. https://doi.org/10.1007/s10753-019-01115-w

Kilinc E, Ankarali S, Torun IE, et al., 2022. Receptor mechanisms mediating the anti-neuroinflammatory effects of endocannabinoid system modulation in a rat model of migraine. *Eur J Neurosci*, 55(4):1015-1031. https://doi.org/10.1111/ejn.14897

Kilinc E, Torun IE, Baranoglu Kilinc Y, 2024. Meningeal mast cell-mediated mechanisms of cholinergic system modulation in neurogenic inflammation underlying the pathophysiology of migraine. *Eur J Neurosci*, 59(9):2181-2192. https://doi.org/10.1111/ejn.15888

Kilinc YB, Kilinc E, Danis A, et al., 2023. Mitochondrial metabolism related markers GDF-15, FGF-21, and HIF-1α are elevated in pediatric migraine attacks. *Headache*, 63(8):1076-1086. https://doi.org/10.1111/head.14618

Kim D, Jeong S, Lee S, et al., 2025. Esculetin inhibits mast cell-mediated allergic inflammation and anaphylaxis by suppressing the FcεRI signaling pathway (Abstract ID: 162178). *J Pharmacol Exp Ther*, 392(S3):100683. https://doi.org/10.1016/j.jpet.2024.100683

Koyuncu Irmak D, Kilinc E, Tore F, 2019. Shared fate of meningeal mast cells and sensory neurons in migraine. *Front Cell Neurosci*, 13:136. https://doi.org/10.3389/fncel.2019.00136

Labastida-Ramírez A, Rubio-Beltrán E, Villalón CM, et al., 2019. Gender aspects of CGRP in migraine. *Cephalalgia*, 39(3):435-444. https://doi.org/10.1177/0333102417739584

Labastida-Ramírez A, Caronna E, Gollion C, et al., 2023. Mode and site of action of therapies targeting CGRP signaling. *J Headache Pain*, 24:125. https://doi.org/10.1186/s10194-023-01644-8

Lee CR, Shin EJ, Kim HC, et al., 2011. Esculetin inhibits *N*-methyl-D-aspartate neurotoxicity via glutathione preservation in primary cortical cultures. *Lab Anim Res*, 27(3):259-263. https://doi.org/10.5625/lar.2011.27.3.259

Levy D, Moskowitz MA, 2023. Meningeal mechanisms and the migraine connection. *Annu Rev Neurosci*, 46:39-58. https://doi.org/10.1146/annurev-neuro-080422-105509

Liang CY, Ju WH, Pei SM, et al., 2017. Pharmacological activities and synthesis of esculetin and its derivatives: a mini-review. *Molecules*, 22(3):387. https://doi.org/10.3390/molecules22030387

Neichi T, Koshihara Y, Murota SI, 1983. Inhibitory effect of esculetin on 5-lipoxygenase and leukotriene biosynthesis. *Biochim Biophys Acta*, 753(1):130-132. https://doi.org/10.1016/0005-2760(83)90106-6

Okubo M, Yamanaka H, Kobayashi K, et al., 2010. Leukotriene synthases and the receptors induced by peripheral nerve injury in the spinal cord contribute to the generation of neuropathic pain. *Glia*, 58(5):599-610. https://doi.org/10.1002/glia.20948

Ottosson A, Edvinsson L, 1997. Release of histamine from dural mast cells by substance P and calcitonin gene-related peptide. *Cephalalgia*, 17(3):166-174. https://doi.org/10.1046/j.1468-2982.1997.1703166.x

Ramachandran R, 2018. Neurogenic inflammation and its role in migraine. *Semin Immunopathol*, 40(3):301-314. https://doi.org/10.1007/s00281-018-0676-y

Ramachandran R, Bhatt DK, Ploug KB, et al., 2014. Nitric oxide synthase, calcitonin gene-related peptide and NK-1 receptor mechanisms are involved in GTN-induced neuronal activation. *Cephalalgia*, 34(2):136-147. https://doi.org/10.1177/0333102413502735

Rasmussen RH, Jansen-Olesen I, Kristensen DM, et al., 2022. *Ex vivo* release of calcitonin gene-related peptide from the trigeminovascular system in rodents. *J Vis Exp*, 183:e63723. https://doi.org/10.3791/63723

Rzodkiewicz P, Gasinska E, Maslinski S, et al., 2015. Antinociceptive properties of esculetin in non-inflammatory and inflammatory models of pain in rats. *Clin Exp Pharmacol Physiol*, 42(2):213-219. https://doi.org/10.1111/1440-1681.12346

Rzodkiewicz P, Gąsińska E, Gajewski M, et al., 2016. Esculetin reduces leukotriene B4 level in plasma of rats with adjuvant-induced arthritis. *Reumatologia*, 54(4):161-164. https://doi.org/10.5114/reum.2016.62469

Silvestro M, Orologio I, Siciliano M, et al., 2023. Emerging drugs for the preventive treatment of migraine: a review of CGRP monoclonal antibodies and gepants trials. *Expert Opin Emerg Drugs*, 28(2):79-96. https://doi.org/10.1080/14728214.2023.2207819

Singh L, Kaur A, Garg S, et al., 2020. Protective effect of esculetin, natural coumarin in mice model of fibromyalgia: targeting pro-inflammatory cytokines and MAO-A. *Neurochem Res*, 45(10):2364-2374. https://doi.org/10.1007/s11064-020-03095-y

Singhuber J, Baburin I, Ecker GF, et al., 2011. Insights into structure-activity relationship of $GABA_A$ receptor modulating coumarins and furanocoumarins. *Eur J Pharmacol*, 668(1-2):57-64. https://doi.org/10.1016/j.ejphar.2011.06.034

Skalicka-Woźniak K, Orhan IE, Cordell GA, et al., 2016. Implication of coumarins towards central nervous system disorders. *Pharmacol Res*, 103:188-203. https://doi.org/10.1016/j.phrs.2015.11.023

Sureda-Gibert P, Romero-Reyes M, Akerman S, 2022. Nitroglycerin as a model of migraine: clinical and preclinical review. *Neurobiol Pain*, 12:100105. https://doi.org/10.1016/j.ynpai.2022.100105

Torun IE, Kilinc YB, Kilinc E, et al., 2024. TRESK channel activation ameliorates migraine-like pain via modulation of CGRP release from the trigeminovascular system and meningeal mast cells in experimental migraine models. *Life Sci*, 357:123091. https://doi.org/10.1016/j.lfs.2024.123091

Woo TS, Yoon SY, Pena ICD, et al., 2011. Anticonvulsant effect of *Artemisia capillaris* Herba in mice. *Biomol Ther*, 19(3):342-347. https://doi.org/10.4062/biomolther.2011.19.3.342

Wu KC, Chen YH, Cheng KS, et al., 2013. Suppression of voltage-gated Na^+ channels and neuronal excitability by imperatorin. *Eur J Pharmacol*, 721(1-3):49-55. https://doi.org/10.1016/j.ejphar.2013.09.056

Yan J, Dussor G, 2014. Ion channels and migraine. *Headache*, 54(4):619-639. https://doi.org/10.1111/head.12323

Zhang LL, Xie QX, Li XF, 2022. Esculetin: a review of its pharmacology and pharmacokinetics. *Phytother Res*, 36(1): 279-298. https://doi.org/10.1002/ptr.7311

Zhang WY, Mulatia S, Zhang MM, et al., 2025. Esculetin inhibited fever, pain, and inflammatory responses via binding to HSC70. *J Ethnopharmacol*, 350:120022. https://doi.org/10.1016/j.jep.2025.120022

Zhu LP, Nang C, Luo F, et al., 2016. Esculetin attenuates lipopolysaccharide (LPS)-induced neuroinflammatory processes and depressive-like behavior in mice. *Physiol Behav*, 163:184-192. https://doi.org/10.1016/j.physbeh.2016.04.051