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*Original Article*

# Neurogenic dural inflammation induced by inflammatory soup combined with CGRP: a modified animal model of migraine

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**Abstract:** Neurogenic inflammation has been proposed to play a crucial role in activation and sensitization of the trigeminovascular system in migraine pathogenesis. A dural neurogenic inflammation model was successfully induced by topical infusion of inflammatory soup (IS) over the dura mater. Evidence has demonstrated that calcitonin gene-related peptides (CGRP) are involved in the pathophysiology of migraine. The aim of this study was to establish a modified animal model of migraine involving mixed mechanisms through dural infusion of IS combined with CGRP. This study investigated the combined effects of IS and CGRP on periorbital mechanical thresholds and dural neurogenic inflammation including meningeal artery dilation, vascular permeability, and mast cell degranulation. Dural infusion of IS combined with CGRP induced significantly decreased periorbital mechanical thresholds. Meningeal blood flow showed a significant increase after dural treatment of combined IS and CGRP compared with that in rats receiving IS or CGRP, separately. Dural infusion of combined IS and CGRP resulted in significant plasma protein extravasation within the dura mater. In addition, topical administration of IS combined with CGRP over the dura produced an increased percentage of degranulated mast cells in the dura. These results suggest that stimulation of the dura by a combination of IS and CGRP produces a greater effect upon neurogenic inflammation than IS or CGRP alone. This modified and integrated animal model can be a useful tool for better understanding the pathophysiology of migraine and finding new therapeutic targets.

**Keywords:** Migraine, animal model, neurogenic inflammation, inflammatory soup, calcitonin gene-related peptides

## Introduction

migraine are a debilitating neurological disorder affecting approximately 12% of the population, worldwide. Migraine are listed as the sixth most prevalent cause of disability by the World Health Organization [1]. Various experimental models for migraine, based on vascular and neuronal mechanisms, have been developed [2]. Nevertheless, establishment of an animal model to explain all of the features of this complicated disorder has remained a challenge. Continuing evolution of available experimental models, with minimized limitations, is pivotal for understanding migraine pathogenesis and the discovery of antimigraine drugs.

Although the pathophysiology of migraine is not completely understood, it has been accepted that this disorder is mainly attributed to activation and sensitization of the trigeminovascular system [3]. Activated trigeminal nociceptive afferents release vasoactive peptides, such as calcitonin gene-related peptide (CGRP) and substance P, which subsequently cause sterile neurogenic inflammation in the dura mater. Neurogenic inflammation is characterized by vasodilatation of dural vessels, increased vascular permeability, plasma protein extravasation, and mast cell degranulation [4, 5]. Neurogenic inflammation has been proposed to activate meningeal nociceptors and induce peripheral and central sensitization [6]. To stim-

ulate neurogenic inflammation, inflammatory agents can be topically infused over the dura mater. A dural neurogenic inflammation model, induced by inflammatory soup (IS) containing histamine, serotonin, bradykinin, and prostaglandin E2, was successfully used to prompt the understanding of migraine pathophysiology and to predict the efficacy of antimigraine drugs [7].

Previous studies have shown that intracisternal administration of IS activates the trigeminal nerve system by releasing CGRP [8]. CGRP is the most abundant neuropeptide in the trigeminal nerve, expressed in nearly 50% of neurons [9]. There has been a convergence of evidence demonstrating that CGRP plays a key role in the pathogenesis of migraine [10]. It has been reported that CGRP levels were elevated in serum and saliva during migraine attacks [11, 12]. Intravenous injections of CGRP provoke a delayed migraine-like headache in patients with migraine [13, 14]. Drugs targeting CGRP, including CGRP receptor antagonists and CGRP antibodies, have been shown to be effective in the treatment of migraine [15]. CGRP has been hypothesized to be involved in the pathogenesis of migraine by both peripheral and central mechanisms. In the periphery, CGRP contributes to both neurogenic inflammation and peripheral sensitization. CGRP is the most potent vasodilatory peptide, having been found to induce dilation of intracranial arteries in migraineurs [16]. CGRP also plays an indirect role in plasma extravasation, primarily caused by substance P [17]. Importantly, CGRP can also trigger mast cell degranulation, which can release proinflammatory and inflammatory compounds [18, 19]. Nevertheless, previous studies have shown that CGRP infusion to normal individuals does not evoke migraine-like attacks [13, 14]. Consistently, animal studies have suggested that topical and intravenous infusion of CGRP in anesthetized rats did not activate or sensitize trigeminal nociceptors [20]. Similarly, the physiological effects of nitric oxide donor glycerol trinitrate (GTN) are clearly different in humans, with and without recurrent migraine [21]. Intravenous GTN infusions induce a long-lasting headache in subjects with recurrent migraines. Conversely, GTN causes a mild headache for a short time in subjects without migraine. Animal studies have demonstrated that GTN facilitates sensory responses in rats with repetitive IS infusions [22]. Thus, it was hypothesized that rats are hyperresponsive to CGRP following IS infusions, possibly serving as a model for migraine hypersensitivity to CGRP.

The aim of this study was to establish a modified animal model of migraine through dural infusion of IS combined with CGRP. This study investigated the nociceptive behaviors of rats following stimulation of the dura with IS and CGRP and the combined effects on dural neurogenic inflammation including meningeal artery dilation, vascular permeability, and mast cell degranulation.

## Materials and methods

### *Animals*

A total of forty-eight adult Sprague-Dawley rats, weighing 180-200 g, were used for experiments. Experimental procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University and were consistent with ethical guidelines recommended by the International Association for the Study of Pain. The animals were randomly divided into four groups: IS group, CGRP group, IS+CGRP group, and control group. Each experimental group consisted of 12 animals. Rats were housed individually under a 12-hour light-dark cycle in a temperature controlled (21-22°C) environment, with food and water ad libitum.

### *Surgical procedure*

Surgical procedures were performed according to Oshinsky et al. [22, 23]. Briefly, after anesthesia with 10% chloral hydrate (4 mL/kg intraperitoneally), the rats were placed in a stereotactic frame (KOPF instruments, Tujunga, CA, USA). After an incision to expose the skull completely, a 3-mm-diameter craniotomy was drilled above the junction of the superior sagittal and transverse sinuses in the left frontal bone to expose the dura mater, which extended 3 mm posteriorly to the bregma and 1.5 mm laterally to the midline. The above procedures were conducted under sterile conditions.

### *Infusion of inflammatory soup, CGRP, or saline*

Rats were placed in a glass chamber in which they could move freely during the infusion. Rats in the IS group, CGRP group, and IS+CGRP

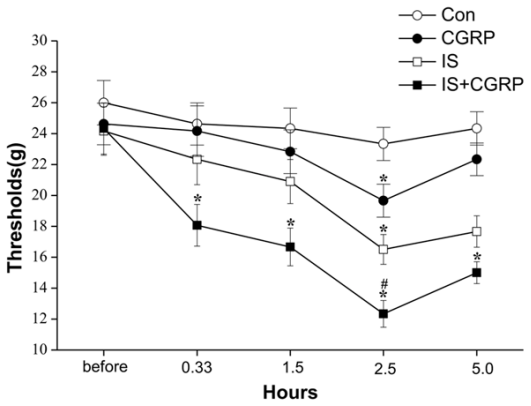


**Figure 1.** Periorbital mechanical thresholds significantly decreased after dural infusion of IS combined with CGRP. Periorbital mechanical thresholds were assessed before dural stimulation and at 0.33, 1.5, and 2.5, and 5 hours after dural administration of saline, IS, CGRP, and IS+CGRP. Data are presented as mean ± SEM. *p<0.01 compared with the control group. #p<0.01 compared with the IS group and CGRP group. n = 4 per group.

group were separately infused with 10 μL of IS, 10 μL of CGRP (0.1 mM), or a combination of IS and CGRP over the dura. IS contained 2 mM histamine, serotonin, bradykinin, and 0.2 mM prostaglandin E2 in phosphate-buffered saline (PBS) at pH 7.4 (adapted from Strassman et al.) [24]. Rats infused with the same volume of saline served as the control group.

*Assessment of periorbital mechanical thresholds*

Periorbital mechanical thresholds were determined by applying von Frey monofilaments (Stoelting Co., Wood Dale, IL, USA) to the periorbital region on the left side of the face, as described by Oshinsky and Gomonchareonsiri [22]. The von Frey stimuli were applied to the skin 5 times for 5 seconds each in a sequential descending order to determine thresholds of response. Mechanical thresholds were defined as a positive response to 3 of 5 trials of the von Frey monofilament. Rats that did not respond to the 26 g stimulus were assigned 26 g as their threshold for analysis. Mechanical thresholds were determined prior to dural infusion and at 0.33, 1.5, and 2.5, and 5 hours after dural stimulation with saline, IS, CGRP, or IS+CGRP.

*Dural blood flow recording*

Laser doppler flowmetry was used to monitor dural blood flow. A cranial window of about 5 mm in diameter was drilled to expose the dura mater as a monitoring location, as previously reported [25]. Exposed dura mater was covered with pieces of gauze soaked with isotonic saline before testing. The needle-type probe of laser doppler flowmetry (UK Moor Company) was positioned over a branch of the middle meningeal artery on the dural surface. MoorVMS-PC V1.0 software was used to collect data and calculate average dural blood flow, before and after dural stimulation. Changes in meningeal blood flow after dural stimulation were determined by comparing mean flow values within the period of 30 minutes after stimulation with the baseline measured as mean flow during the 5-minute period prior to dural stimulation. Changes are expressed as percentages.

*Measurement of plasma protein extravasation*

Evans blue (EB), a kind of fluorescent dye with the characteristic of binding to plasma protein, has been widely used to assess plasma protein extravasation. Evans Blue (50 mg/kg) was injected intravenously to the rats 5 minutes prior to dural stimulation. One hour after stimulation, rats were perfused transcardially with phosphate-buffered saline (PBS) for 5 minutes. Next, the dura mater was dissected carefully, subsequently rinsed with water, and mounted flat onto the glass slide. A fluorescence microscope was used to quantify the amount of Evans blue dye extravasation of harvested dura mater.

*Observation of mast cell degranulation*

After dural infusion, rats were transcardially perfused with PBS, followed by 4% paraformaldehyde. Ipsilateral dura mater was quickly dissected out and immersed in 4% paraformaldehyde for fixation overnight at 4°C. The dura mater was then mounted flat onto the glass slide and stained with 0.5% toluidine blue. Mast cell counts were performed at a magnification of ×100 in 5 randomly selected fields per slice, then averaged. Mast cells with empty cavities or granules outside the cell shape were considered as degranulation. Data are presented as the percentage of degranulated mast cells.

*Statistical analysis*

All statistical analysis was performed with SPSS software, version 21.0 (SPASS Inc, Chicago, IL, USA). Data are presented as mean ±

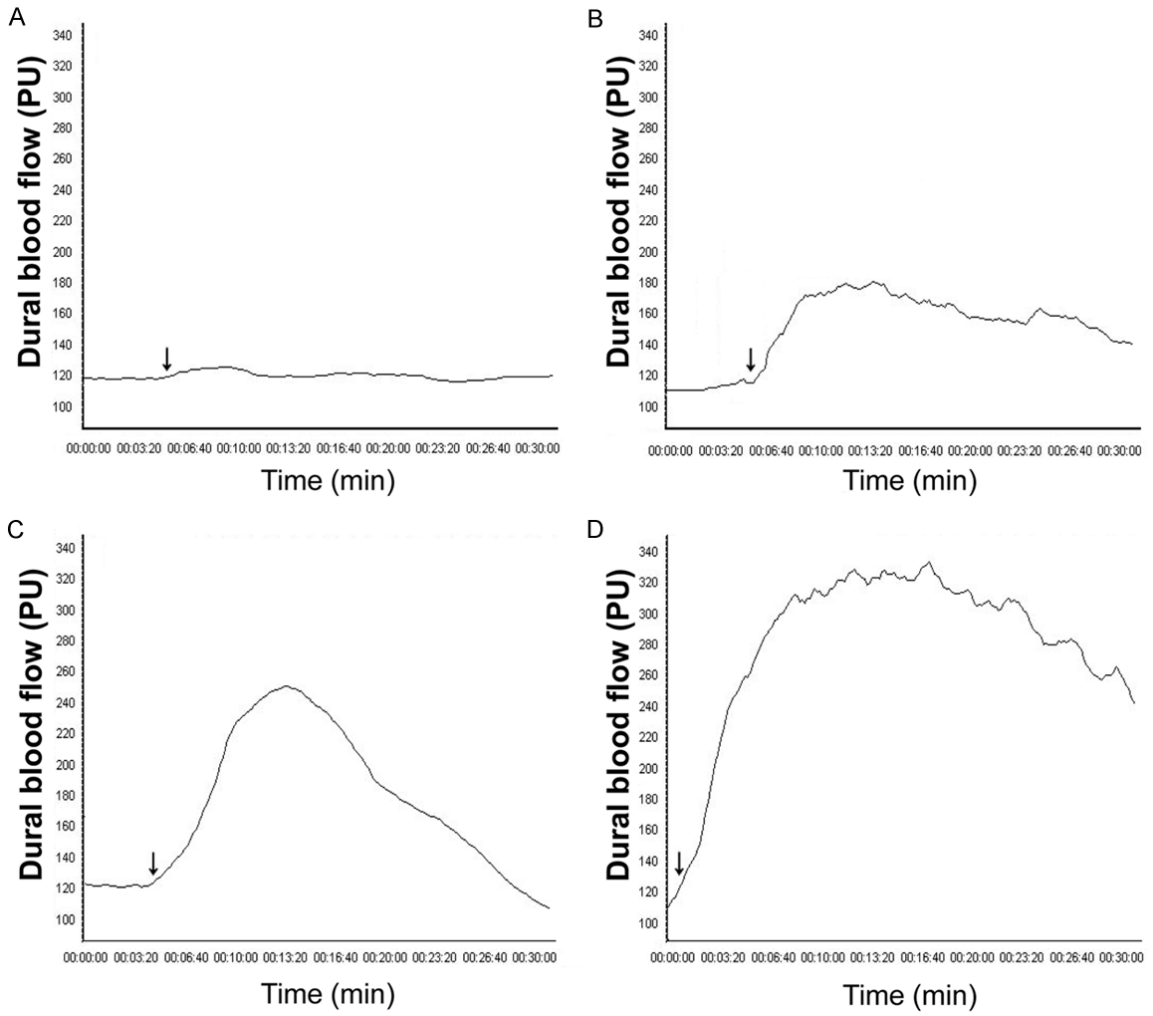


**Figure 2.** Representative recordings demonstrating the effects of IS and CGRP on dural blood flow. Dural blood flow was measured by a laser doppler flowmetry within the period of 30 minutes after topical administration of saline (A), IS (B), CGRP (C), or IS+CGRP (D). PU: perfusion unit.

standard error of the mean (SEM). Significance of differences in mechanical thresholds was analyzed with two-way analysis of variance (ANOVA) and significance of other variables was analyzed with one-way ANOVA, followed by Tukey's multiple comparison tests using SPSS software, version 21. Differences are considered statistically significant at $p<0.05$.

## Results

*Dural infusion of IS combined with CGRP induced significantly decreased periorbital mechanical thresholds*

To investigate whether dural infusion of IS combined with CGRP induces decreased periorbital mechanical thresholds, this study measured periorbital pressure thresholds with von Frey monofilament before dural stimulation and at 0.33, 1.5, and 2.5, and 5 hours after the infusion of saline, IS, CGRP, or IS+CGRP over the dura. As indicated in **Figure 1**, there were no differences in periorbital mechanical thresholds between rats in the four groups prior to dural infusion. Periorbital mechanical thresholds were significantly decreased at 2.5 hours after infusion of IS, CGRP, or IS+CGRP. Rats receiving dural administration of IS+CGRP induced significantly decreased periorbital pre ssure thresholds compared with the IS group and CGRP group (**Figure 1**).

*Dural infusion of IS combined with CGRP caused a significant increase in dural blood flow*

In the control group, topical administration of saline over the dura did not significantly change

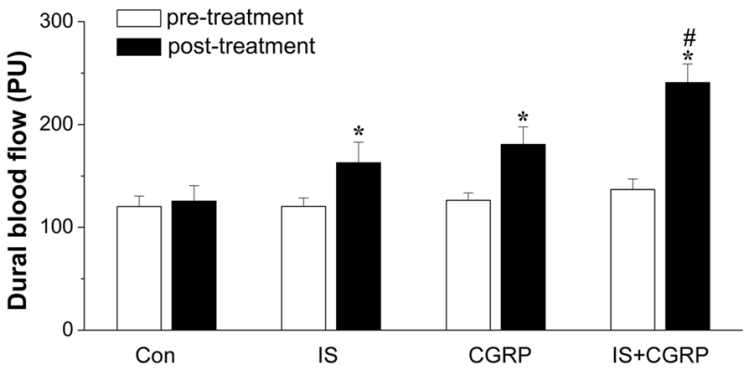


**Figure 3.** Dural infusion of IS combined with CGRP causes a significant increase in dural blood flow. Bar graphs depicting the summary data of dural blood flow before and after dural treatment with saline, IS, CGRP, or IS+CGRP. Data are presented as mean ± SEM. *p<0.01 compared with the pre-treatment in the same group. #p<0.01 compared with the control group, IS group and CGRP group. n= 4 per group.

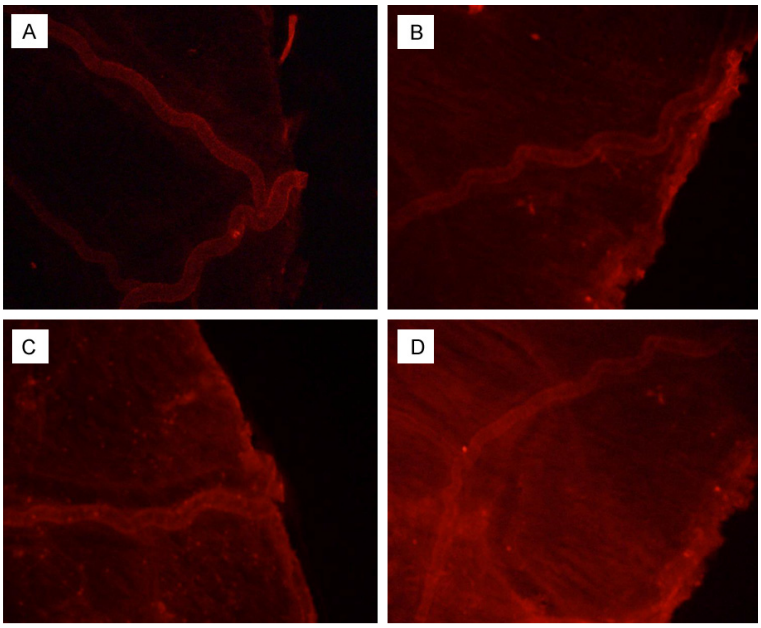


**Figure 4.** Dural infusion of IS combined CGRP induced significant plasma protein extravasation. Dural plasma protein extravasation was assessed by the amount of Evans blue dye extravasation with a fluorescence microscope. Original magnification ×100. n = 4 per group.

meningeal blood flow (**Figure 2A**). Meningeal blood flow showed a significant increase after stimulation of the dura mater in the IS group (**Figure 2B**), CGRP group (**Figure 2C**), and IS+CGRP group (**Figure 2D**). An average increase of 26.6% in meningeal blood flow was observed during the 30 minutes after IS treatment (**Figure 3**, p<0.01). In the CGRP group, meningeal blood flow increased by 38.1% of the control response in the period of 30 minutes after CGRP stimulation (**Figure 3**, p<0.01). Rats receiving dural administration of IS+CGRP showed maximal effects on dural blood flow compared with rats receiving IS or CGRP separately (**Figure 3**, p<0.01). Topical application of IS+CGRP over the dura mater induced an immediate and prolonged increase in meningeal blood flow, within a few minutes, outlasting the study period of 30 minutes (**Figure 2D**).

*Dural infusion of IS combined CGRP induced significant plasma protein extravasation*

Dural plasma protein extravasation was assessed by leakage of the fluorescent dye Evans Blue in dura mater. The control group showed little or no leakage within the dura (**Figure 4A**). Significantly increased leakage of Evans Blue dye was observed in the dura mater in rats stimulated with IS (**Figure 4B**), CGRP (**Figure 4C**), or IS+CGRP (**Figure 4D**). Rats receiving dural infusion of IS+CGRP resulted in significant extravasation of Evans Blue within the dura mater compared with the IS group and CGRP group. There were no significant differences in leakage of Evans Blue dye within the dura between the IS group and CGRP group.

*Dural infusion of IS combined CGRP produced extensive dural mast cell degranulation*

In the dura of rats in the control group, mast cells were cycloidal or ovoid around the vessels and granules were stained purple in the cytoplasm (**Figure 5A**). In the dura of rats in other groups, mast cells were degranulated characterized by granules outside the cell shape or loss of cellular staining (**Figure 5B-D**). Dural administration of IS, CGRP, or IS+CGRP significantly increased the degranulation ratio of mast cells in the dura (**Figure 6**, p<0.01). The

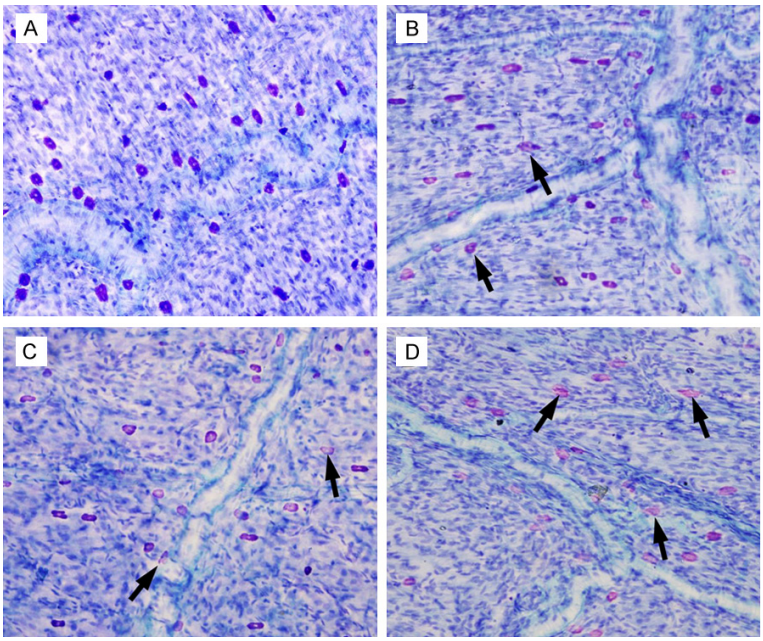


**Figure 5.** Histological analysis of the effects of IS and CGRP on dural mast cell degranulation. Dural mast cell degranulation was assessed by toluidine blue staining after dural infusion of saline (A), IS (B), CGRP (C), or IS+CGRP (D). Arrows indicate the degranulated mast cells. Original magnification ×20. n = 4 per group.

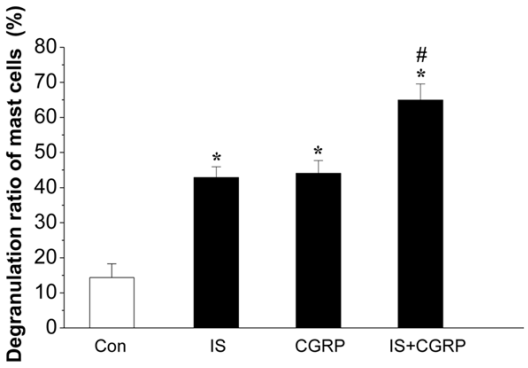


**Figure 6.** Dural infusion of IS combined with CGRP produced an increased degranulation ratio of dural mast cells. Quantification of the percentage of degranulated mast cells after dural stimulation with saline, IS, CGRP, or IS+CGRP. Data are presented as mean ± SEM. *p<0.01 compared with the control group. #p<0.01 compared with the IS group and CGRP group. n = 4 per group.

percentage of degranulated mast cells in the dura of rats in the IS+CGRP group was significantly higher than the IS group and CGRP group (**Figure 6**, p<0.01), but no significant differences were found in the degranulation ratio of mast cells between the IS group and CGRP group.

## Discussion

The present results indicate that dural infusion of IS combined with CGRP produces significantly decreased periorbital mechanical thresholds and greater neurogenic inflammation compared with infusion of IS or CGRP, separately. This combination is characterized by more potent effects on dural vasodilatation, plasma protein extravasation, and mast cell degranulation. The present study successfully established a modified and more effective animal model of migraines by inducing neurogenic inflammation with a combination of IS and CGRP.

CGRP has a pivotal effect on dilation of vessels, especially intracranial arteries. Previous studies have found that CGRP can induce dilation of middle meningeal arteries and middle cerebral arteries during migraine attacks [16]. It has been proposed that CGRP causes vascular dilation mainly by a nitric oxide-independent and endothelium-independent pathway through a direct action on the smooth muscle cells via increases in cyclic adenosine monophosphates [26]. The present results demonstrate that CGRP has combined effects with IS on the induction of significantly increased meningeal blood flow. Topical infusion of IS can lead to increased CGRP levels, adding to the vascular effects of CGRP alone. Although the vascular theory of migraine has been largely questioned and spontaneous migraine headaches are not accompanied by direct extracranial vasodilatation [27], intracranial arterial dilatation could potentially cause pain by activating perivascular nociceptors. Findings from previous studies have shown that mechanical dilatation in the cerebral part of internal carotid arteries and middle cerebral arteries can cause ipsilateral moderate to severe frontotemporal and periorbital headaches [28]. Therefore, vasodilatory effects of IS combined with CGRP could potentially be linked to a neurogenic mechanism by activating trigeminal vascular nociceptors, promoting sensitization during migraine attacks.

CGRP also plays an indirect role in plasma extravasation, which is primarily induced by substance P and neurokinin A. These peptides are often co-released with CGRP. CGRP can further increase substance P release, leading to plasma extravasation. Similarly, IS can also enhance expression levels of substance P and induce plasma extravasation [29, 30]. Consistently, the present results suggest that combined CGRP and IS has much stronger effects on plasma protein extravasation than CGRP or IS, separately. The concept of plasma protein extravasation in migraine pathogenesis is based on results demonstrating that trigeminal ganglion stimulation produces plasma protein extravasation into the dura mater on the ipsilateral side in rats [31]. Clinically effective antimigraine medications, such as sumatriptan [32], can inhibit this neurogenic inflammation, indicating that reduction of plasma protein extravasation could be predictive of antimigraine therapeutic strategies.

CGRP can also trigger mast cell degranulation, a process in which mast cells release bradykinin, histamine, prostaglandins, and several inflammatory and proinflammatory mediators [18]. A direct role for CGRP in degranulation is supported by the identification of CGRP receptors on dural mast cells [33]. Results demonstrated that IS combined with CGRP produced more increased degranulation of mast cells than CGRP or IS, separately. It is believed that mast cell degranulation plays a key role in the pathogenesis of migraine. Dural mast cell degranulation results in the activation of meningeal nociceptors in electrophysiological recordings and increases the activity of neurons in the trigeminal nucleus caudalis [34].

In this study, combination of IS with CGRP showed much higher potency upon neurogenic inflammation than IS or CGRP alone. Underlying mechanism may be mainly associated with integrated neurovascular effects of IS and CGRP on the pathogenesis of migraine.

Previous studies have shown that administration of IS onto the dura leads to the activation of peripheral trigeminal ganglion neurons and brainstem trigeminal neurons [24, 35]. In these investigations, topical application of IS induced hypersensitivity to mechanical stimulation, leading to expanded dural and cutaneous receptive fields. Thus, IS has been used for the study of peripheral and central sensitization in several experimental models of migraine [23, 36]. Moreover, it has been shown that topical administration of IS caused significantly increased levels of CGRP, a crucial marker of trigeminal nerve activation [8, 37]. It has been suggested that CGRP does not directly excite or sensitize trigeminal nociceptors [20]. Previous studies have also shown that intravenous administration of CGRP to normal individuals does not induce migraine-like attacks [13, 14]. The present investigation showed that IS can potentiate the sensory response along with greater neurogenic inflammation in rats receiving CGRP administration over the dura. This demonstrates that inflammatory stimulation of the dura with IS is required to model the hyperresponsiveness of humans with migraine to CGRP.

CGRP is one of the most potent vasodilators that has been identified to date. CGRP plays an important role in the pathophysiology of migraine in both periphery and central sites [10]. The periphery site of action of CGRP in migraine involves neurogenic inflammation and peripheral sensitization. In the central nervous system, CGRP could act as a neuromodulator of cortical spreading depression and central sensitization [20]. Previous studies have reported that intravenous administration of CGRP causes headaches and migraine attacks in migraineurs [13, 14], suggesting that CGRP may play a causative role in migraine. Consistently, the present results demonstrate that CGRP facilitates sensory responses in rats receiving IS infusion. Mechanisms may be associated with greater neurogenic inflammation after dural administration of CGRP combined with IS. Thus, this study combined these two different chemical agents to enhance characterized pathophysiological changes of the neurogenic inflammation model. Further studies are required to investigate the effects of dural stimulation with IS and CGRP on central sensitization, a crucial central mechanism underlying migraine pathophysiology.

In conclusion, the present results suggest that dural stimulation with a combination of IS and CGRP produced greater effects upon neurogenic inflammation than IS or CGRP alone, representing a modified and effective animal model of migraine. This modified and integrated ani-

mal model is recommended for use to better understand the pathophysiology of migraine and find new targets for treatment of migraine.

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## Disclosure of conflict of interest

None.

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