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ORIGINAL RESEARCH

Scorpion venom component: AGAP exhibits local anaesthetic effects and attenuates nociceptive pain

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Background: The incidences of systemic toxicity and other complications associated with existing local anaesthetics can occur at clinical concentration level and vary with the anaesthetic techniques, types of surgery and patient factors. This evidence suggests the need for therapeutic interventions in peripheral and regional anaesthesia. *Buthus martensii* Karsch (BmK) scorpion venom is a compound that contains mixtures of peptides that have analgesic properties. This study aimed to investigate the local anaesthetic activity of scorpion venom peptide, AGAP (analgesic-antitumor peptide) in mechanical hyperalgesia or acute inflammatory pain.

Method: Formalin was injected into the left hind paw after 20 minutes of infiltration of drugs. The time of licking or flinching of the injected hind paw was recorded as indicative of nociceptive or acute inflammatory pain. Paw flinching or quick withdrawal was considered a positive response to pain in the partial sciatic nerve ligation. The paw-withdrawal threshold (PWT) was determined by consecutively increasing and decreasing the magnitude of the stimulus.

Results: The results indicated that AGAP exhibited a 67.9% inhibition in licking or flinching time and an 88.1% inhibition in paw withdrawal in mechanical hyperalgesia. The addition of AGAP to lidocaine showed an 89.5% inhibition in paw withdrawal.

Conclusion: The data presented in this study suggest that local infiltration of AGAP significantly reduced mechanical hyperalgesia and acute inflammatory pain.

Keywords: AGAP, lidocaine, mechanical hyperalgesia, acute inflammatory pain, local anaesthetic

Introduction

Peripheral and regional anaesthesia are widely used techniques in anaesthesia to selectively numb a particular nerve distribution or section of the body to facilitate surgery or manage an existing pain.[1-3] These techniques provide many benefits to patients, including superior perioperative pain control, reduction of systemic effects of surgical stress after certain types of surgery, and reduction of general anaesthesia-related side effects.[4,5] Peripheral or neuraxial anaesthesia is accomplished by administering local anaesthetics to a nerve. Local anaesthetics are drugs that act by producing reversible blocks to the transmission of peripheral nerve impulses. Drugs known and used as local anaesthetics have their origin in cocaine. Local anaesthetics are weak bases and are usually formulated as hydrochloride salts to render them water soluble. These local anaesthetics rapidly penetrate the various tissues around the targeted nerve ending and bind to the sodium channel which then inhibits sodium permeability that underlies the action potentials in nerves. The quantity of drug reaching the central axonal core is reduced in large-diameter nerves due to incomplete penetration of the surrounding epineurium, perineurium, lymphatics, endoneurium, fat and blood vessels.[6]

Local anaesthetics also contain a lipophilic aromatic ring and a hydrophilic amine at either end of the molecule, separated by a hydrocarbon chain, and either an ester or an amide bond.[7-9] Despite the many benefits of these existing local anaesthetics, study reports have shown that they produce dose and time-dependent toxicity to a variety of tissues such as the heart and the nerves.[10] The incidences of toxicity and other complications associated with local anaesthetics can occur at the clinical concentration level and vary with anaesthetic techniques, types of surgery and patient factors.[11,12] The evidence of these systemic toxicities and other complications associated with the existing local anaesthetics suggest the need for new therapeutic interventions in peripheral and neuraxial anaesthesia.

Buthus martensii Karsch (BmK) scorpion venom is a natural compound that contains mixtures of peptides that have analgesic properties. BmK venom and its extracts have been used to treat pain in Asia and other parts of the world for many decades. The first BmK analgesic peptide purified from the venom was published in 1994 by Wang et al.[13] A survey of the literature indicates that little is known about the local anaesthetic effects of the scorpion venom analgesic-antitumor peptide (AGAP). This study aimed to investigate the local anaesthetic activity and the synergistic effects of AGAP in mechanical hyperalgesia or acute inflammatory pain.

Materials and methods

Animals and ethical statement

Seventy-two (72) adult male standard deviation (SD) rats weighing between 180 g and 200 g were provided by the Laboratory Animal Centre, Dalian Medical University. The rats were housed in standard transparent plastic cages under a 12-hour/12-hour light-dark cycle regime. They were provided free access to food and water. All the experimental procedures were approved by the animal research committee of the Dalian Medical University as well as by the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health.[14]

Randomisation

Rats were randomly assigned to one of six groups for both the formalin and von Frey filament tests, as follows: Sham (n = 12); 0.9% saline as the negative control (n = 12); 1 mg/kg of AGAP (n = 12); preservative-free lidocaine (0.5%) (n = 12); AGAP (1 mg/kg) and lidocaine (0.5%) combined (n = 12); and AGAP (2 mg/kg) and lidocaine (0.5%) combined (n = 12).

Preparation of AGAP

The process of obtaining the AGAP was the same as previously described.[15] AGAP was dissolved in saline, and its activity was confirmed to be the same as that in our previous study.

Lidocaine

Preservative-free 2% lidocaine (31802232) was purchased from Tianjin Shuicheng Pharmaceutical Limited by Share Ltd, China.

Drugs application

An equal volume of AGAP (1 mg/kg), preservative-free lidocaine (0.5%), and AGAP (1 or 2 mg/kg) and lidocaine (0.5%) combined were prepared using saline (0.9%). An anaesthesiologist who specialises in regional anaesthesia was assigned to carry out the administration of the drugs. An independent anaesthesiologist specialising in pain medicine, who were blinded to the application of the drugs, were assigned to monitor the nociceptive pain or mechanical hyperalgesia in the rats. The doses of the drugs were determined based on the results of preliminary experiments.[16-18] Before administrating the drugs, the sciatic nerve in the left hind paw was located at the upper-thigh level using a nerve stimulator (Stimpod NMS450, Emergo Europe, and the Netherlands) with a 22-gauge short bevel electrically insulated electrode (AB-22025-SS). The motor response elicited by 0.2–0.5 mA (flicking) confirmed the location of the nerve and the proximity of the electrically insulated electrode to the nerve. An equal volume (600 µl) of either one of the drug solutions or the 0.9% saline as the control was injected into the upper-thigh level through the gastrocnemius and biceps femoris muscles to the sciatic nerve.

Formalin test

The formalin-induced pain model is *a valid and reliable model of nociception* and is sensitive for various classes of analgesic drugs. It has an acute nociceptive first phase and a second inflammatory response which is consistent with initial sensory neuron activation and peripheral or central sensitisation. The formalin-induced pain model was the same mode used in the previous study.[19] Before testing, an equal volume (600 µl) of either one of the drug solutions or the 0.9% saline as the control was injected into the left hind paw at the upper-thigh level to the sciatic nerve. Individual rats were placed in a clear plastic observation chamber for 20 minutes for adaptation. The formalin solution was prepared at 5% in saline from a formalin stock and injected intraplantarly into the left hind paw in a volume of 20 µl. After the formalin injection, the rats were immediately placed in a clear transparent box (20 cm × 25 cm × 15 cm) with a mirror set underneath at a 45° angle to view the rats' paws entirely. The rats were observed for 60 minutes after the formalin injection. The time of licking or flinching the injected hind paw from 0 to 5 minutes (1st phase) and 15 to 40 minutes (2nd phase) were recorded as indicative of nociception or acute inflammatory response.

Partial sciatic nerve ligation

The partial nerve ligation model is a nerve injury that induces chronic neuropathic pain in rats or mice, characterised by mechanical and thermal hypersensitivity, ongoing pain and changes in limb temperature, making this model the most appropriate for the preclinical study of neuropathic pain. The procedure used for the partial sciatic nerve ligation (PSL) model was mainly the same as that reported by Seltzer et al.[20] and Bennett et al.[21] Individual rats were anaesthetised with sodium pentobarbital (40 mg/kg, intraperitoneal injection) and a small incision was made at the mid-thigh level to expose the left sciatic nerve. A unilateral tight ligation of one-third of the sciatic nerve was induced with a single ligature (5-0 silk thread). The rats representing the sham group were operated on and the sciatic nerve was exposed but not ligated. The incision made was closed in layers and the surgical wound was treated with antibiotics.

Measurement of mechanical hyperalgesia

Mechanical hyperalgesia was assessed using von Frey filaments (North Coast Medical, Inc., San Jose, CA) starting from 2 g and ending with 0.16 g or 15 g filaments as the cut-off values using the "up-and-down" method. Before testing, an equal volume (600 µl) of either one of the drug solutions or the 0.9% saline as the control was injected into the left or right hind at the upper-thigh level to the sciatic nerve. Individual rats were then placed in a clear plastic box (20 cm × 25 cm × 15 cm) on a metal mesh floor and allowed 20 minutes for adaptation. The filaments were then presented, in ascending order of strength (0.16 g, 0.4 g, 0.6 g, 1 g, 1.4 g, 2 g, 4 g, 6 g, 8 g and 15 g) and held for 6–8 seconds perpendicular to the plantar surface with sufficient force to cause slight bending against the paw. Paw flinching or quick withdrawal were considered positive responses to pain. The paw-withdrawal threshold (PWT) was determined by consecutively increasing and decreasing the magnitude of the stimulus (through the "up-and-down" method). In the PSL model, withdrawal thresholds were measured in the rats using only the ipsilateral (ligated)

paw. The effects of the administration of the drugs and the paw withdrawal threshold were assessed every 30 minutes.

Statistical analysis

We performed each experiment three times. All statistical analyses were carried out using the GraphPad Prism v 7.01 (GraphPad Software, La Jolla, CA, USA). The nonparametric method of Dixon as previously described[22] was used to analyse the data. All values are depicted as a mean ± SD and considered significant if $p < 0.05$. The student's t-test was used to make statistical comparisons between two groups, and two-way analysis of variance (ANOVA) was used for comparisons between three or more groups.

Results

AGAP attenuates nociceptive pain

To investigate the local anaesthetic activity of AGAP, we injected AGAP, lidocaine or AGAP and lidocaine combined into the upper-thigh level of the left hind paw. Concentrations of formalin greater than 0.5% induces a biphasic response in rats. After 20 minutes of administering the drug solutions, 5% formalin was injected into the left hind paw. The time of licking or flinching the injected hind paw was recorded as an indication of nociceptive pain or acute inflammatory response. The data indicated a significant reduction in licking or flinching time among rats from the lidocaine group during the first (0–5 minutes) and second (15–40 minutes) phases (Figure 1). The data also showed a significant reduction in licking or flinching time among rats from the AGAP group during both phases (Figure 1). There was a significant reduction in licking or flinching time during the early and late phases among rats that received AGAP and lidocaine combined (Figure 1). The combined treatment showed a significant difference in licking or flinching time when compared to lidocaine or AGAP (Figure 1). These results showed that AGAP, when infiltrated locally, may exhibit local anaesthetic effects and reduce nociceptive or acute inflammatory pain.

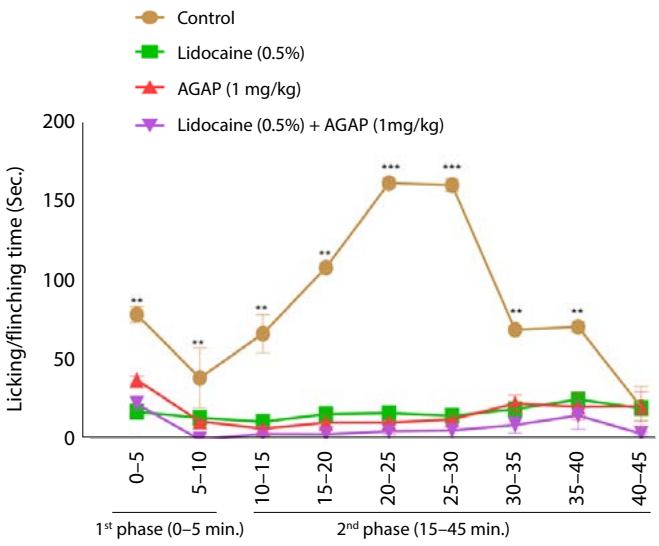

Figure 1: Antinociceptive effect of AGAP

Formalin (5%) solution was administered intraplantarly after AGAP was injected into the left hind paw to determine its local anaesthetic effects. The effect of the AGAP was assessed through the time of licking or flinching by individual rats after the formalin injection which was indicative of response to nociceptive pain or acute inflammatory response. The results showed that AGAP when injected locally, may have some reversible numbness effect within the injected areas which may attenuate nociceptive or acute inflammatory pain. Compared with control, the result was statistically significant at $^{**}p < 0.001$, or $^{***}p < 0.0001$ (Figure 1).

AGAP ameliorates mechanical hyperalgesia

To further investigate the local anaesthetic effect of AGAP, we performed PSL in the left hind paw of the individual rats to produce a rapid onset and long-lasting mechanical hyperalgesia. Mechanical hyperalgesia was first confirmed in all rats from the control group (received 0.9% saline), lidocaine group, AGAP group and the combined treatment group two days after the PSL procedure. Rats from the sham group (operated) recorded no mechanical hyperalgesia on the post PSL day 2. We then infiltrated an equal volume of AGAP, lidocaine, saline or AGAP (1 or 2 mg/kg) and lidocaine combined into the upper-thigh level of the ligated left hind paw. Mechanical hyperalgesia was assessed after 20 minutes of drug infiltration using the von Frey filaments.

The data showed that the PWT significantly increased among rats that received lidocaine or AGAP treatments alone. Also, the data showed no significant difference in the PWT among rats that received lidocaine or AGAP alone. We observed that the PWT slightly increased in rats that received the combined treatments (Figure 2). The data showed 88.1%, 87.6%, 89.5% and 89.53 % inhibition of paw withdrawal in rats that received lidocaine, AGAP or the combined treatments (1 or 2 mg/kg), respectively. These emerging results suggested that, similarly to the lidocaine, AGAP might have exhibited local anaesthetic activity against mechanical hyperalgesia. However, the effect of adding the AGAP to lidocaine did not show any significant increase in PWT when compared to lidocaine or AGAP alone (Figure 2).

Next, we infiltrated an equal volume of AGAP, lidocaine (0.5%), saline (0.9%) or the combined into the upper-thigh level of the right hind paw and then measured the PWT in the ligated left hind to rule out possible systemic effects of the AGAP. The data

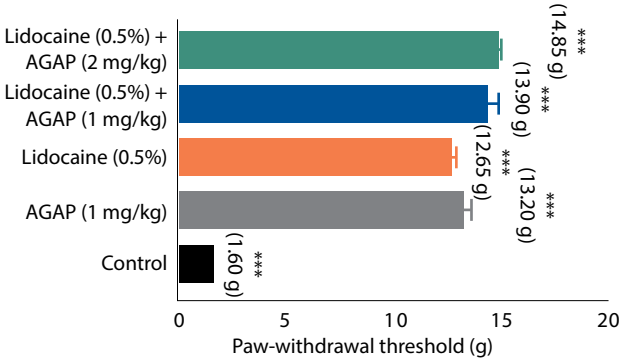

Figure 2: AGAP ameliorated mechanical hyperalgesia

showed that the ligated left hind PWT still remained low (Figure 3). These outcomes indicated that AGAP probably exhibited local anaesthetic effects against mechanical hyperalgesia in this study.

Individual rats were anaesthetised and a small incision made to expose the left sciatic nerve. A unilateral tight ligation of one-third of the sciatic nerve was induced with a single ligature. AGAP was injected and mechanical hyperalgesia was assessed using the von Frey filaments. The infiltration of AGAP improved mechanical hyperalgesia. Compared with control, the result was statistically significant at ***$p < 0.0001$ (Figure 2).

AGAP was injected into the upper-thigh level of the right hind and the PWT measured in the ligated left hind to rule out systemic effects. The data showed a remained decreased PWT in the ligated left hind. Compared with ligated left hind limb PWT, the result was statistically significant at ***$p < 0.0001$ (Figure 3).

AGAP potentiates analgesic activities of lidocaine and prolongs the duration of analgesia

To investigate the duration of blockade, we infiltrated lidocaine or AGAP alone or AGAP (1 or 2 mg/kg) and lidocaine combined into the upper-thigh level of the ligated left hind. The von Frey filaments were then immediately used to assess mechanical hyperalgesia in the rat at 30-minute intervals until the rat started showing signs of mechanical hyperalgesia after treatment (threshold of 5–10 g). The total time (minutes) taken for the paw-withdrawal inhibition by individual treatment was recorded as the duration of analgesia. The data showed a significant increase in the total time taken for the paw-withdrawal inhibition among

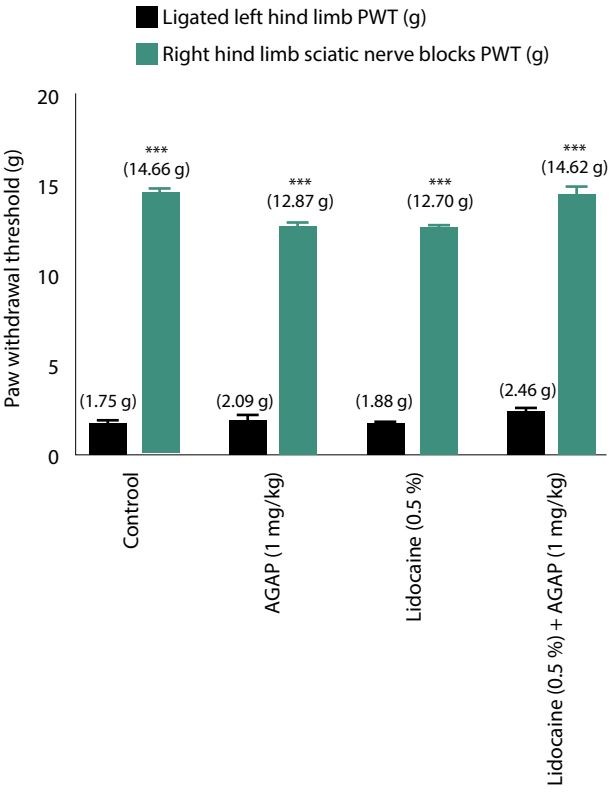

Figure 3: Ipsilateral and contralateral injections of drugs

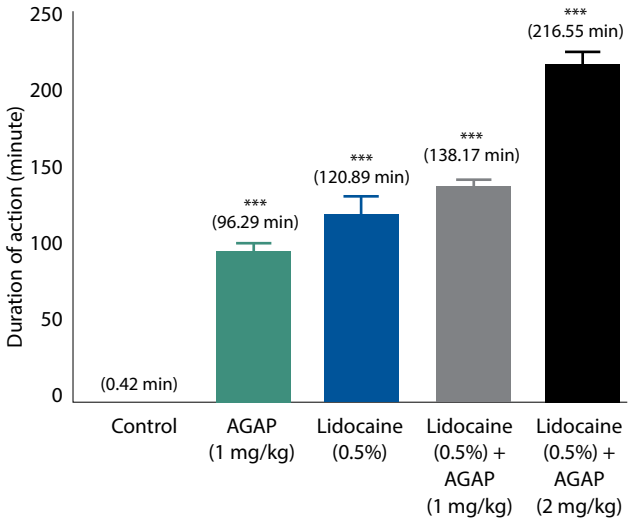

Figure 4: AGAP potentiates the analgesic effects of lidocaine and prolongs the duration of analgesia

rats that received AGAP (1 or 2 mg/kg) and lidocaine (0.5%) combined (Figure 4). These results suggest that scorpion venom peptide, AGAP had synergistic effects with lidocaine.

AGAP and lidocaine was injected into the upper-thigh level of the ligated left hind paw. Mechanical hyperalgesia was assessed every 30 minutes. The time taken for the paw-withdrawal inhibition, was recorded as the duration of the analgesia. Compared with lidocaine, results were statistically significant at ***$p < 0.0001$ (Figure 4).

Discussion

This study aimed to investigate the local anaesthetic activity of a scorpion venom peptide, AGAP. The data showed that AGAP infiltrated close to a nerve ameliorated nociceptive or acute inflammatory pain or mechanical hyperalgesia, suggesting that AGAP might have blocked the nerve to effect its analgesic activity. The data also showed that AGAP administered along with lidocaine increased the duration of pain relief. The findings of this study suggested that AGAP might have exhibited local anaesthetic activity against pain, and also had synergistic effects with lidocaine. Liu et al.[23] demonstrated the analgesic activity of AGAP in 2002 by intraperitoneal injection in mice and realised that AGAP exhibits potent analgesic activity. In another similar study, Li et al.[24] demonstrated the antinociceptive effects of AGAP through the tail vein injection and realised a similar potent analgesic activity. Long-term pain management techniques are frequently needed for acute or chronic pain conditions such as nociceptive pain, neuropathic pain or musculoskeletal pain. The ability of AGAP to attenuate nociceptive pain may offer promise for the development of targeted treatments for illnesses causing chronic pain, perhaps lowering the need for systemic drugs and enhancing the quality of life for people who experience acute or chronic pain.

Despite the existence of many local anaesthetics, there is a therapeutic and scientific concern regarding the prolonged duration of local anaesthetics that can be established by a

single injection. Local anaesthetics are multipurpose drugs applied for infiltration, nerve block, for peripheral or neuraxial anaesthesia and intravenously for perioperative analgesia. The nerve-blocking potency of local anaesthetics increases with increasing molecular weight and increasing lipid solubility. The effectiveness of a given local anaesthetic is influenced by the dose, site of administration, additive and temperature.[7] Local anaesthetics enter nerve cells along three pathways: classic hydrophilic, hydrophobic, or alternative hydrophilic. The clinical effects of local anaesthetics result from the classic hydrophilic pathway.[6] Tetrodotoxin and saxitoxins have high affinity and great specificity for voltage-gated sodium channels. These unique features endow sit-1 neurotoxins with high potency for nerve block.[25,26] Also, neurotoxins such as tetrodotoxin, saxitoxins, and scorpion neurotoxin, and AGAP are naturally occurring sodium channel blockers. Tetrodotoxin has been shown to be synergistic with local anaesthetics and has particularly potent local anaesthetic properties.[25,26] The prospective applications of AGAP in various medical procedures that call for local anaesthesia are suggested by the substance's local anaesthetic qualities. Regional nerve blocks, small operations, dermatological procedures and dental procedures could all fall into this category. The effectiveness and duration of local anaesthesia may be increased with the administration of AGAP, resulting in increased patient comfort and satisfaction.

Infiltrative administration of local anaesthetics for neural blockade in peripheral and regional anaesthesia can be achieved by using a diluted concentration of local anaesthetics. Lidocaine is the most commonly used local anaesthetic for nerve blockade due to its rapid onset, potency and tissue penetration. Larger and more lipophilic local anaesthetics permeate nerve membranes more readily and bind to sodium channels with higher affinity. In clinical practice, local anaesthetics are usually defined by their potency, duration of action, speed of onset and affinity for differential sensory nerve block.

The findings of this study demonstrated that local infiltration of AGAP resulted in nerve block and increased PWT in rats suffering from nociceptive or acute inflammatory pain and mechanical hyperalgesia. The identification of the local anaesthetic properties of AGAP and its capacity to reduce nociceptive pain opens up new avenues for the creation of novel pain management techniques. Potentially, AGAP could be used as a replacement for or a supplement to current local anaesthetics, offering better pain relief, perhaps with fewer side effects.

Many analgesics have been tested and demonstrated to be clinically beneficial when added to local anaesthetics for peripheral nerve blocks or when used for local infiltration. Analgesics such as morphine, fentanyl, buprenorphine and tramadol when added to local anaesthetics reduce the total required dose of local anaesthetic, prolong the sensory block, minimise the central nervous system effects and optimise perioperative analgesia.[27] In this study, the addition of AGAP to lidocaine increased the duration of analgesia. Emerging evidence shows that local anaesthetics do not only target voltage-gated sodium channels but can also interact with calcium channels, potassium channels, G-protein coupled receptors and N-methyl-D-aspartate (NMDA) receptors.[6] AGAP belongs to the group of long-chain scorpion peptide and has a molecular mass of 7 142 Da and the residue of 66 amino acids. AGAP binds to voltage-gated sodium channel independently at site-3 of sodium channels and inactivates the activated sodium channels to mediate potent analgesic activity.[15] Ruan et al.[28] reported in 2018 that intrathecal injection of AGAP inhibits neuropathic and inflammation-associated pain through a mitogen-activated protein kinases (MAPK) mediated mechanism. The study of AGAP and its local anaesthetic characteristics helps to expand the field of venom-based treatments in the pharmaceutical and biological fields. The potential of naturally occurring substances obtained from venomous sources as a source of innovative pharmaceutical medicines is highlighted. Further study on AGAP and other scorpion venom peptides may result in the creation of novel painkillers and other medicinal agents.

Conclusion

The findings of this study showed that local infiltration of AGAP results in nerve block and suggest, for the first time, the local anaesthetic property of AGAP. It may also have synergistic effect with lidocaine. These results demonstrate a potential therapeutic approach to pain treatment. However, further investigation is required to investigate the mechanisms through which AGAP exhibits its local anaesthetic activities.

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Conflict of interest

The authors declare no conflict of interest.

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Ethical approval

All the experimental procedures were approved by the Animal Research Committee of the Dalian Medical University. This study was carried out by a student as a research project under the supervision of the Head of Department who perused the study protocol. Permission was also given by the Head of Department for the student to carry out the research.

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