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Antinociceptive effect of intrathecal ginsenosides through α-2 adrenoceptors in the formalin test of rats

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Editor's key points

- The role of α-2 adrenoceptors in the analgesic effect of ginsenosides was studied in rats.
- Intrathecal ginsenosides act *via* α-2A, -B, and -C adrenoceptors.
- Ginsenosides interact with clonidine.
- This study adds to our understanding of the mechanisms of action of ginseng.

Background. We defined the nature of the pharmacological interaction after intrathecal co-administration of ginsenosides with clonidine, and clarified the contribution of the α-2 adrenoceptors on the effect of ginsenosides.

Methods. Pain was evoked by injection of a formalin solution (5%, 50 μl) into the hindpaw of male Sprague–Dawley rats. Isobolographic analysis was performed to characterize the drug interaction between ginsenosides and clonidine. The antagonism of ginsenosides-mediated antinociception was determined with α-2A (BRL 44408), α-2B (ARC 239), and α-2C (JP 1302) adrenoceptor antagonists. The expression of α-2 adrenoceptor subtypes was examined by reverse transcriptase–polymerase chain reaction.

Results. Intrathecal ginsenosides (n=29) and clonidine (n=31) displayed an antinociceptive effect. The ED_{50} values (95% confidence intervals) of ginsenosides and clonidine for phases 1 and 2 were 109.5 (63–190.3) and 110.9 (57.1–215.5), and 11.8 (3.7–37.1) and 4.9 (3.1–6.7) μg, respectively. With an isobolographic study (n=48), the ED_{50} values (95% confidence intervals) of ginsenosides in the combination of ginsenosides and clonidine for phases 1 and 2 were 58.2 (38.9–87.3) and 57.2 (46.5–70.3) μg, respectively. Intrathecal BRL 44408 (n=6), ARC 239 (n=5), and JP 1302 (n=5) reversed the antinociception of ginsenosides in both phases (P<0.01, <0.001). The injection of formalin increased the expression of α-2C adrenoceptor in the spinal cord (P<0.05).

Conclusions. Intrathecal ginsenosides additively interacted with clonidine in the formalin test. Furthermore, α-2A, -B, and -C adrenoceptors contributed to the antinociception of intrathecal ginsenosides.

Keywords: clonidine; drug interaction; ginsenosides; pain; receptors adrenergic, alpha-2

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The root of *Panax ginseng* C. A. Meyer, or ginseng, has been used as a herbal medicine.[1] The major active constituents of ginseng are ginsenosides.[2] Many studies have shown antinociceptive effects of spinal ginsenosides in a variety of nociceptive states.[3–6]

Previous experiments have indicated that Ca^{2+} channels could be the pharmacological basis of the antinociceptive effect of ginsenosides.[7–9] In contrast, the antinociceptive effect of ginsenosides is not mediated through α-2, muscarinic, or opioid receptors.[7 10] However, it has been reported that intrathecal Korean red ginseng produced a potent antinociceptive effect in formalin-induced nociception, and that such antinociception was reversed by an intrathecal α-2 antagonist,[11] providing evidence of possible involvement of spinal α-2 adrenoceptors in the antinociceptive action of ginsenosides. α-2 adrenoceptors are subdivided into three subtypes: α-2A, B, and C.[12–15]

Intrathecal clonidine reduces both acute pain and tissue injury hyperalgesia,[16 17] and the antinociception is mediated through spinal α-2 adrenoceptors.[18]

We evaluated the characteristics of the spinally mediated interaction between ginsenosides and clonidine in formalin-induced nociception. In addition, we examined the involvement of α-2 adrenoceptor subtypes in the effect of ginsenosides at the spinal level.

Methods

Animal preparation

This study was conducted according to the IASP guidelines for the Care of Experimental Animals. The protocol was approved by The Institutional Animal Care and Use Committee, Medical Science of Chonnam National University. Experiments were done on adult male Sprague–Dawley rats

weighing 250–300 g. The rats were housed in individual cages in a temperature-controlled room [22 (0.5°C)] with an alternating 12 h light/dark cycle. Animals were allowed free access to food and water. Intrathecal catheterization was performed to administer experimental drugs as described previously.[19] Briefly, the rats were anaesthetized with sevoflurane (3–4%) under spontaneous respiration, and placed in a stereotaxic head holder. Adequate anaesthesia was ascertained by no withdrawal response to a paw pinch. A polyethylene-10 catheter was inserted through an incisional slit in the atlanto-occipital membrane. The catheter was advanced caudally to 8.5 cm from the slit such that its tip was located at the lumbar enlargement of the spinal cord. The skin margins were closed, leaving only 2 cm of the catheter above the skull exposed for injections and plugged with a piece of steel wire. Local anaesthetic solution (1% lidocaine 0.5 ml) was infiltrated into the incised sites in cases of distress on emergence. If any motor or sensory deficit was present after intrathecal catheter placement, the rats were dropped from the study and killed immediately with an overdose of volatile anaesthetics. A period of not less than 5 days was allowed for post-surgical recovery.

Drugs

The following drugs were used in this study: ginsenosides, clonidine hydrochloride (Sigma Aldrich Co., St Louis, MO, USA), BRL 44408 maleate (Tocris Cookson Ltd, Bristol, Avon, UK), ARC 239 (Tocris), and JP 1302 (Tocris). Ginsenosides were kindly provided by the Korea Ginseng and Tobacco Research Institute (Daejon, Korea). Ginsenosides were dissolved in dimethyl sulphoxide (DMSO). The remaining drugs were dissolved in normal saline. Intrathecal administration of these agents was performed using a hand-driven, gear-operated syringe pump. All drugs were delivered in a 10 μl solution, followed by an additional 10 μl of normal saline to flush the catheter.

Nociceptive test

The formalin test was performed as a nociceptive test.[16] Animals were injected s.c. with 50 μl of 5% formalin solution into the plantar surface of the hindpaw using a 30 G needle. The formalin injection produced characteristic flinching behaviour, rapid and brief withdrawal, or flexing of the injected paw, which was regarded as a pain response. The flinching behaviour appeared biphasically. Such pain behaviour was therefore quantified by periodically counting the number of flinches of the injected paw after injection according to each phase. The number of flinches was counted for 1 min periods from 1 to 2 min, 5 to 6 min, and every 5 min from 10 to 60 min. The interval from 0 to 9 min was defined as phase 1 of the formalin test and the interval from 10 to 60 min was defined as phase 2. After the entire observation period, the rats were killed with an overdose of volatile anaesthetics.

Study design

After acclimatization for 15–20 min in a restraint cylinder, the rats were randomly allocated into one of the drug-treatment groups. The control study was performed using intrathecal DMSO or saline, depending on the solvent for the agents. Each rat was used only once. The total number of rats used in the behavioural study was 145, with 5–8 rats per group. The investigator was unaware of the drug-treatment in each animal.

Effects of ginsenosides and clonidine

To examine the time course and dose-dependency of the antinociceptive effect of ginsenosides [DMSO (n=8), 30 (n=7), 100 (n=7), 300 μg (n=7)] and clonidine [saline (n=5), 1 (n=7), 3 (n=7), 10 (n=6), 30 (n=6) μg], each agent was administered intrathecally. The formalin test was performed 10 min after drug delivery. The ED_{50} value (effective dose producing a 50% reduction in control formalin response) for the drugs was calculated separately in each phase.

Drug interaction

Isobolographic analysis was used to assess the characteristics of pharmacological interactions between ginsenosides and clonidine in the formalin test.[16] This method is based on the comparison of doses determined to be equi-effective. First, each ED_{50} value was determined from the dose–response curves of agents alone. Next, ginsenosides and clonidine were intrathecally co-administered at a dose calculated using the ED_{50} values (n=12) and fractions [1/2 (n=12), 1/4 (n=12), and 1/8 (n=12)] of the ED_{50} for each drug. The ED_{50} values of the mixture were calculated from the dose–response curves of the combined drugs, and the combinations were used to plot the isobologram. The isobologram was constructed by plotting the ED_{50} values of the single agents on the x- and y-axes, respectively. The theoretical additive dose combination was then calculated. From the variance of the total dose, the individual variances for the combined agents were obtained. Moreover, a total fraction value was calculated to describe the magnitude of the interaction.

$$\text{Total fraction value} = \frac{ED_{50}\ \text{of drug 1 combined with drug 2}}{ED_{50}\text{for drug 1 given alone}} + \frac{ED_{50}\ \text{of drug 2 combined with drug 1}}{ED_{50}\ \text{for drug 2 given alone}}$$

The fraction values indicate what portion of the single ED_{50} value was accounted for by the corresponding ED_{50} value for the combination. Values near 1 indicate an additive interaction, values >1 indicate an antagonistic interaction, and values <1 indicate a synergistic interaction. The formalin test was performed 10 min after co-administration of two drugs, and the pharmacological characteristics were evaluated in phases 1 and 2, respectively.

α-2 adrenoceptor subtypes and ginsenosides

The possible interaction between ginsenosides and α-2 adrenoceptors was examined. Accordingly, several α-2 adrenoceptor antagonists were administered intrathecally 10 min before the delivery of ginsenosides (300 μg). The α-2 adrenoceptor antagonists used in this study were as follows: α-2A receptor antagonist, BRL 44408 (100 μg, $n=6$); α-2B receptor antagonist, ARC 239 (1 μg, $n=5$); and α-2C receptor antagonist, JP 1302 (0.01 μg, $n=5$). The types and maximal doses of the three α-2 adrenoceptor antagonists were selected based on their lack of significant effect on the control formalin response from previous studies[20–24] and our preliminary experiments. The formalin test was conducted 10 min after administration of ginsenosides. The reversal effect was examined in phases 1 and 2, respectively.

General behaviour

The behavioural effects of ginsenosides and clonidine were examined in separate rats ($n=10$). They received the highest doses of the two drugs used here, and were examined for 60 min after intrathecal administration. Motor functions were assessed by examining the righting and placing-stepping reflexes.[25] The former was evaluated by placing the rat horizontally with its back on the table, which normally gives rise to an immediate co-ordinated twisting of the body to an upright position. The latter was evoked by drawing the dorsum of either hindpaw across the edge of the table. Normally, rats try to put their paws forward into a position for walking. Pinna and corneal reflexes, reflecting central nervous system function, were evaluated with a paper string.[25] Each reflex was evoked by stimulation of the ear canal or the cornea with a paper string. Normal rats spontaneously shook their heads or blinked, respectively. All reflexes were judged as present or absent.

Detection of α-2 adrenoceptor expression

α-2 adrenoceptor subtype (α-2A, -B, and -C) mRNA expression was measured in the dorsal spinal cord of naïve and formalin-injected rats ($n=6$) using reverse transcriptase–polymerase chain reaction (RT–PCR). At 5 and 35 min after formalin injection, rats were killed by decapitation and the spinal cord was quickly removed and stored at −80°C. The area of the spinal cord from L4 to L6 was dissected and total RNA in the spinal cord was isolated according to the manufacturer's protocol for the RNeasy kit (Cat. no. 74104, Qiagen). Purified total RNA was quantified spectrophotometrically at A_{260}. The isolated mRNA was reverse transcribed using the Omniscript RT kit (Cat. no. 205111, Qiagen), following the manufacturer's instructions. Previously published primer sets were used for the rat α-2 adrenoceptors.[26] The RT–PCR conditions were standardized for each type of receptor using PCR PreMix (Cat. no. K-2012, Bioneer) containing 1× PCR buffer, 1.5 mM $MgCl_2$, 250 μM dNTPs, 20 pmol each of forward and reverse oligonucleotide primers coding for rat α-2A (α-2A-1: 5′-GCG CCC CAG AAC CTC TTC CTG GTG-3′, α-2A-2: 5′-CCA GCG CCC TTC TTC TCT ATG GAG-3′), α-2B (α-2B-1: 5′-AAA CGC AGC CAC TGC AGA GGT CTC-3′, α-2B-2: 5′-ACT GGC AAC TCC CAC ATT CTT GCC-3′), or α-2C (α-2C-1: 5′-CTG GCA GCC GTG GTG GGT TTC CTC-3′, α-2C-2: 5′-GTC GGG CCG GCG GTA GAA AGA GAC-3′), followed by 32 thermal cycles (1 min at 95°C, 1 min at 57°C, and 1 min at 72°C) for α-2A and 40 cycles (1 min at 95°C, 1 min at 64°C, and 1 min at 72°C) for α-2B, and 36 cycles (1 min at 95°C, 1 min at 59°C, and 1 min at 72°C) for α-2C. The final cycle was followed by a 5 min extension step at 72°C before reducing the temperature to 4°C for storage. Amplification of rat β-actin (GenBank accession no. NM_031144) was used as an internal and loading control. The PCR products were separated by gel electrophoresis in a 2% agarose gel containing 0.5 μg ml^{-1} ethidium bromide, observed under ultraviolet light and photographed. Densitometry was performed using Multi gauge V3.0 (Life-science, Fujifilm Global) and analysis software to determine the ratio between α-2A, -B, -C, and β-actin.

Statistical analysis

Data are expressed as means (SD). The time–response data are presented as the number of flinching responses. The dose–response data are presented as a percentage of the control in each phase. The numbers of flinching responses were converted to a percentage of the control to calculate the ED_{50} values of each drug. Percentage of the control=[(Sum of Phase 1(2) count with drug)/(Sum of control Phase 1(2) count)]×100. The dose–response data were analysed using the Kruskal–Wallis test with the Mann–Whitney test *post hoc*. The dose–response lines were fitted using least-squares linear regression and the ED_{50} and its 95% confidence intervals were calculated, and the difference between the theoretical and experimental ED_{50} was analysed using the method reported by Tallarida and Murray.[27] The antagonism of ginsenosides and RT–PCR data were analysed using the Mann–Whitney test. A P-value of <0.05 was considered significant.

Results

The vehicle (control) groups exhibited a typical biphasic flinching response of the injected paw after the formalin injection. The sums of the number of flinches in each phase did not differ [saline:DMSO; 18 (4):17 (3) in phase 1, 142 (18):143 (40) in phase 2].

Figure 1 shows the time course of intrathecal ginsenosides and clonidine administered 10 min before the formalin injection. As illustrated, the duration of action of the two drugs at the highest dose examined was nearly complete over the entire observation period.

Intrathecal ginsenosides and clonidine produced a dose-dependent reduction in the flinching response during phases 1 and 2 in the formalin test (Fig. 2). Phase 1 ED_{50} values (95% confidence intervals) of ginsenosides and clonidine were 109.5 (63–190.3) and 11.8 (3.7–37.1) μg, respectively. Phase 2 ED_{50} values (95% confidence intervals) of

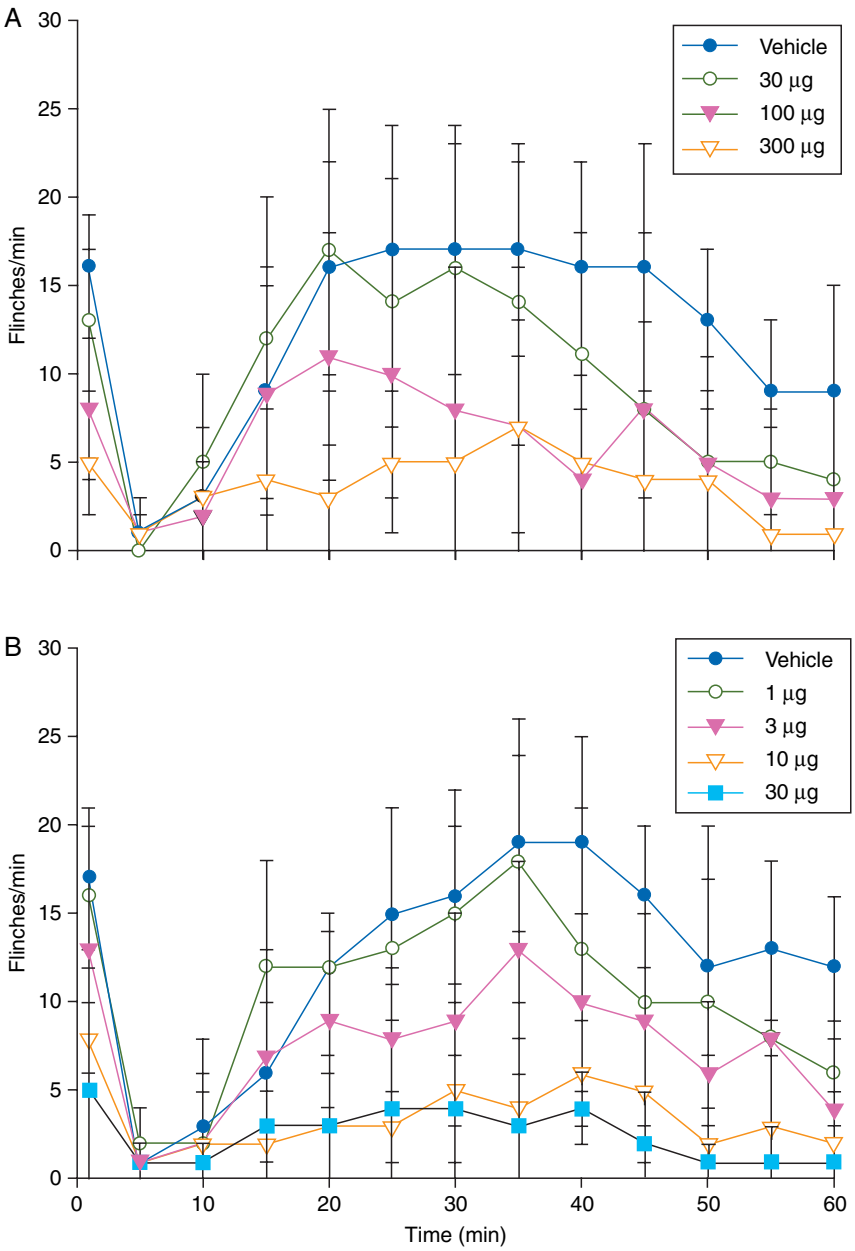

Fig 1 Time–effect curve of intrathecal ginsenosides (A) and clonidine (B) for flinching in the formalin test. Each drug was administered 10 min before the formalin injection. Formalin was injected at time 0. Data are presented as the number of flinches. Each line represents means (SD) of 5–8 rats.

ginsenosides and clonidine were 110.9 (57.1–215.5) and 4.9 (3.1–6.7) μg, respectively.

Isobolographic analysis revealed an additive interaction between ginsenosides and clonidine during phases 1 and 2 in the formalin test (Fig. 3). Accordingly, the ED_{50} values (95% confidence intervals) of ginsenosides in the mixture of ginsenosides and clonidine for phases 1 and 2 were 58.2 (38.9–87.3) and 57.2 (46.5–70.3) μg, respectively. These experimental ED_{50} values were not significantly different from the theoretical ED_{50} values in either phase. Each total fraction value for the mixture of ginsenosides and clonidine in phases 1 and 2 were 0.96 and 0.98, respectively, indicating an additive interaction.

Intrathecal BRL 44408, ARC 239, and JP 1302 reversed the antinociceptive effect of intrathecal ginsenosides during phases 1 and 2 in the formalin test (Fig. 4).

All righting, placing-stepping, pinna, and corneal reflexes were present after intrathecal administration of ginsenosides and clonidine (data not shown).

The naïve rat spinal cord, analysed by RT–PCR for the presence of α-2 adrenoceptors using previously published primer pairs, yielded 312, 456, and 425 bp fragments from cDNAs,

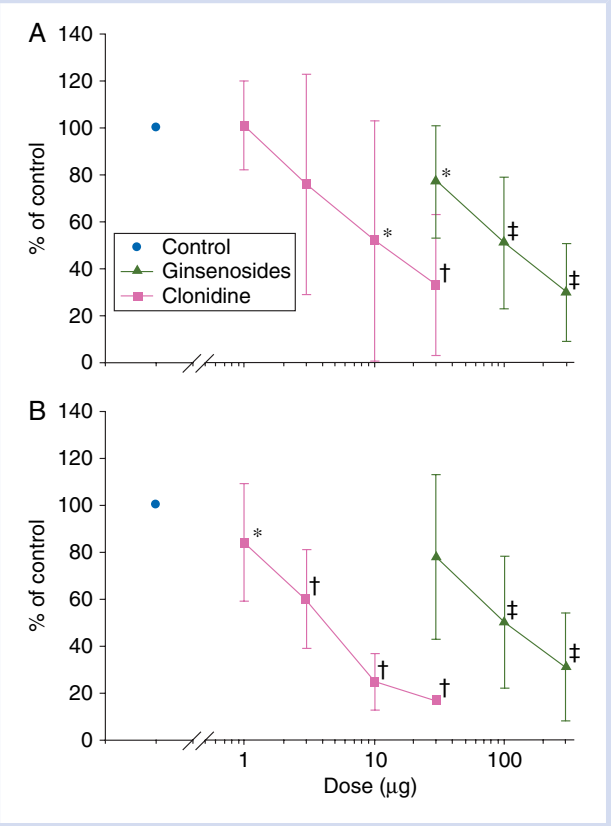

Fig 2 Dose–response curve of intrathecal ginsenosides and clonidine for flinching in the formalin test. Data are presented as a percentage of the control. Ginsenosides and clonidine produced a dose-dependent inhibition of flinches in Phase 1 (A) and Phase 2 (B). Each line represents means (SD) of 5–8 rats. *$P<0.05$, †$P<0.01$, ‡$P<0.001$, *vs* control.

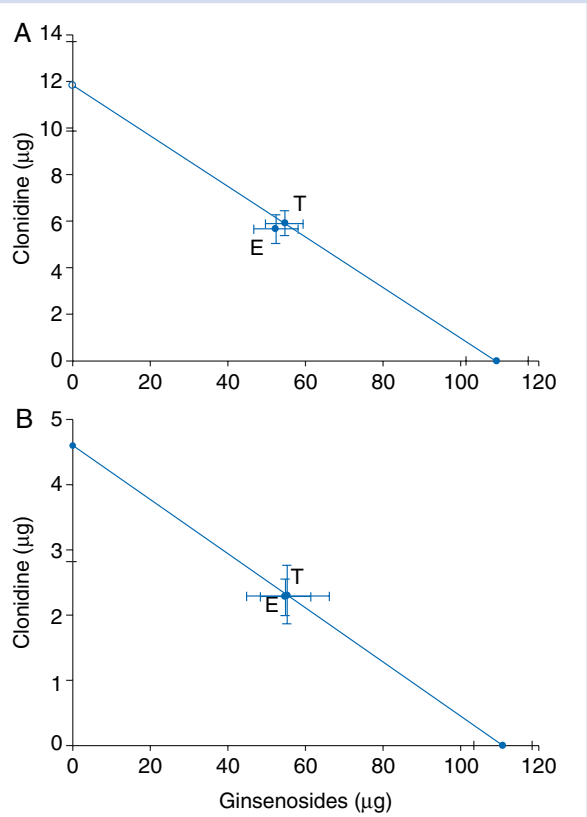

Fig 3 Isobologram for the interaction between intrathecal ginsenosides and clonidine during Phase 1 (A) and Phase 2 (B) in the formalin test. The ED_{50} values for each agent are plotted on the *x*- and *y*-axes, respectively. Horizontal and vertical bars indicate confidence intervals. The straight line connecting each ED_{50} value is the theoretical additive line and the point on this line is the theoretical additive ED_{50}. The experimental ED_{50} point was not significantly different from the theoretical ED_{50} points, indicating an additive interaction.

respectively (Fig. 5). After formalin injection, the level of α-2C adrenoceptor mRNA was increased, whereas the α-2A adrenoceptor mRNA level was unchanged; however, α-2B adrenoceptor mRNA levels were decreased, compared with those of naïve rats (Fig. 5).

Discussion

In the present study, intrathecal ginsenosides and clonidine suppressed the flinching response during phases 1 and 2 in the formalin test in rats. In the formalin test, phase 1 flinching response occurs as a result of the immediate and intense increase in primary afferent activity, reflecting acute pain. On the other hand, phase 2 response results from the activation of a wide dynamic range of dorsal horn neurones with a very low level of ongoing activity of primary afferents, reflecting a facilitated state. Thus, these observations suggest that ginsenosides and clonidine attenuate the facilitated state and acute nociception at the spinal level, consistent with previous studies.[3 11 16–18] On the other hand, the ED_{50} value of phase 1 was higher than that of phase 2 in clonidine; however, no significant difference was seen between the two phases. These findings suggest that both ginsenosides and clonidine seem to be equally effective in acute pain and the facilitated state.

Ginsenosides have been shown to be effective for a variety of ailments;[1] thus, they have been used for a long time to relieve various types of pain, such as toothaches, abdominal pain, and neuralgia in traditional folk medicine. Despite extensive research, the binding sites and the mechanism(s) of action are not fully understood. Several studies have shown that ginsenosides inhibit voltage-dependent Ca^{2+} channels in sensory neurones.[7–9 28] Furthermore, ginsenosides have a four-ring, steroid-like structure and show properties similar to acetylcholine, epinephrine, histamine, and opioids.[29] Thus, it is reasonable that the antinociceptive action of ginsenosides may involve Ca^{2+} channels, neurotransmitters, or both. On the other hand, the inhibitory effect of ginsenosides on the Ca^{2+} current was not altered by α-2, muscarinic, opioid, or GABA receptor antagonists,[7 10] so it seems likely that those receptors are not involved in the effects of ginsenosides. However, one recent study reported that the antinociception

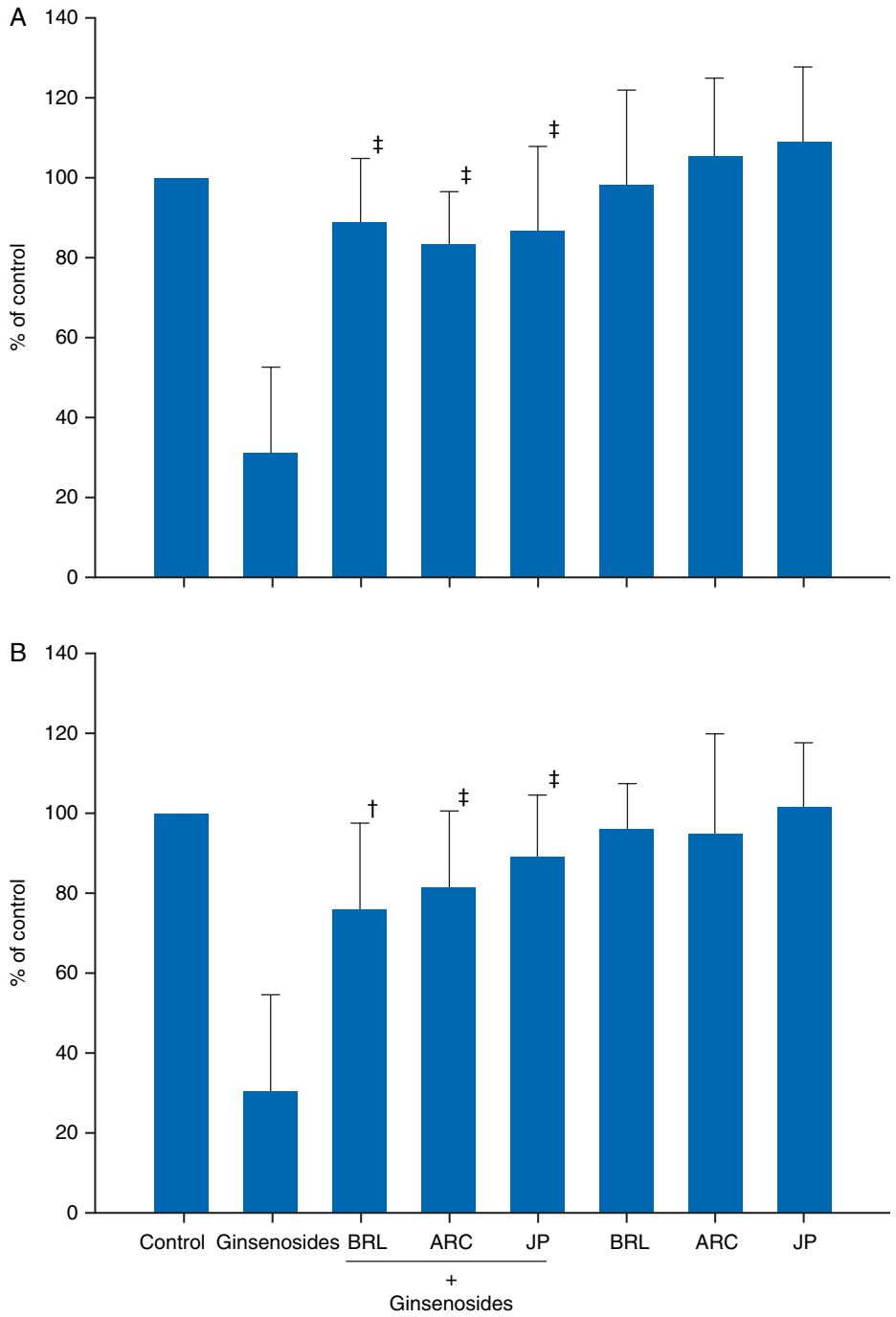

Fig 4 The antagonistic effects of intrathecal BRL 44408 (100 μg), ARC 239 (1 μg), and JP 1302 (0.01 μg) for the antinociceptive action of intrathecal ginsenosides (300 μg) during Phase 1 (A) and Phase 2 (B) in the formalin test. BRL 44408, ARC 239, and JP 1302 were given 10 min before ginsenosides administration, and the formalin test was performed 10 min after ginsenosides delivery. Data are presented as a percentage of the control. All BRL 44408, ARC 239, and JP 1302 reversed the effect of ginsenosides in both phases. Each bar represents mean (SD) of 5–6 rats. $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$, *vs* ginsenosides.

of intrathecal Korean red ginseng was attenuated by a non-selective α-2 antagonist, yohimbine, in the formalin test.[11] Furthermore, in the present study, α-2A (BRL 44408), -B (ARC 239), and -C (JP 1302) adrenoceptor antagonists decreased the effect of ginsenosides at the spinal level. Furthermore, BRL 44408, ARC 239, and JP 1302 display relative selectivity for α-2A, α-2B, and α-2C adrenoceptors, respectively.[22–24] Moreover, immunohistochemical and *in situ* hybridization studies indicate that α-2 adrenoceptors exist in the dorsal horn of the spinal cord, an important area in the modulation

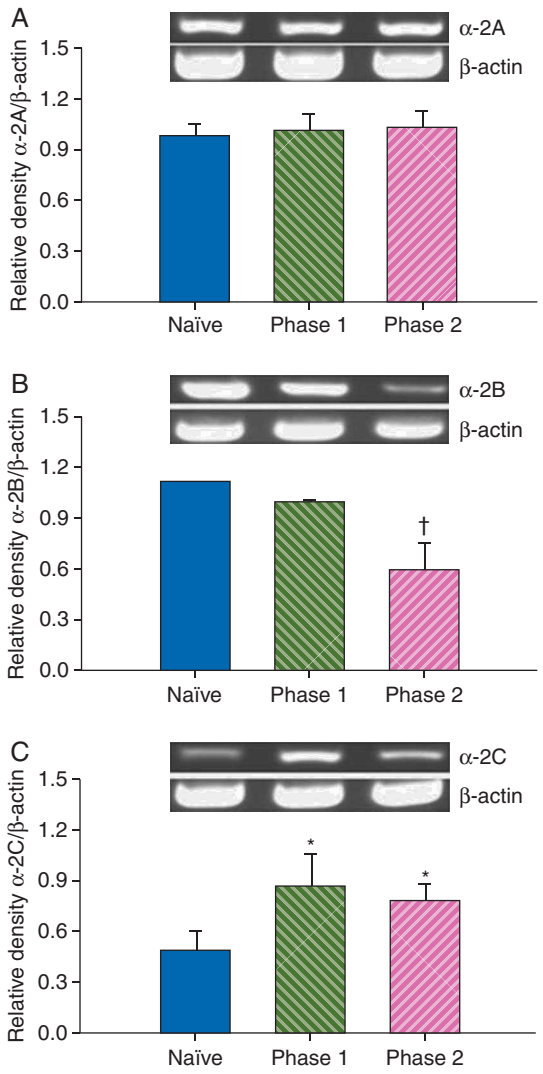

Fig 5 α-2 adrenoceptor subtype mRNA expression in the spinal cord of naïve and formalin-injected rats. The 500, 312, 456, and 425 bp RT–PCR products correspond to the mRNA of β-actin, α-2A (A), -B (B), and -C (C) adrenoceptors, respectively. No difference between naïve and formalin-injected rats was noted in the level of α-2A adrenoceptor mRNA expression. After formalin injection, the mRNA expression of α-2C adrenoceptor was elevated in phases 1 and 2, but α-2B adrenoceptor mRNA expression was lower in phase 2. Each bar represents means (SD) of six rats. *$P<0.05$, †$P<0.01$, *vs* naïve.

of nociception.[30 31] In particular, studies examining the expression of α-2A adrenoceptor mRNA in the spinal cord neurones found labelling in the intermediolateral cell column in the thoracic cord and throughout the dorsal horn, including in superficial layers.[32 33] Moreover, α-2B adrenoceptor mRNA was identified in the superficial dorsal horn of adult rats.[33 34] Additionally, mRNA labelling for α-2C adrenoceptor is detected in the dorsal horn.[32 33] Also, in this study, all α-2A, -B, and -C adrenoceptor mRNA was expressed in the spinal cord. Thus, together, these findings suggest that α-2 adrenoceptors may play an important role in the action of ginsenosides at the spinal level. Furthermore, α-2A, -B, and -C adrenoceptor subtypes are apparently all involved in the activity of ginsenosides.

In the present study, intrathecal clonidine suppressed the flinching response during phases 1 and 2 in the formalin test, consistent with previous studies.[16 17] Pharmacologically, intrathecal clonidine can directly act on spinal α-2 adrenoceptors and exhibit an antinociception.[18] Interestingly, RT–PCR in this study revealed that α-2 adrenoceptor mRNA was expressed differently in the spinal cord, according to the subtype, after formalin injection. The α-2A adrenoceptor mRNA level was little changed. On the other hand, α-2B adrenoceptor mRNA expression was found to be decreased in phase 2, but not in phase 1. However, the α-2C adrenoceptor mRNA level was increased in both phases. These findings suggest that drugs acting on α-2C adrenoceptors, rather than on α-2A or -B adrenoceptors, could be more powerful analgesics in acute pain and the facilitated pain state.

According to an isobolographic analysis, intrathecal ginsenosides additively interacted with clonidine during phases 1 and 2 in the formalin test. These observations indicate that spinal ginsenosides do not augment the antinociceptive action of clonidine alone in the nociceptive state evoked by the formalin injection. The present study demonstrates, for the first time, an additive interaction between ginsenosides and clonidine at the spinal level.

A synergistic interaction is considered likely if basically different mechanisms contribute jointly to the observed actions of the two drugs at a given endpoint, such as antihyperalgesia. However, a synergistic interaction may not be expected if the mechanisms of action of one drug are involved in those of another drug. A previous study has shown that Korean red ginseng suppressed the flinching response in both phases of the formalin test, and this antinociceptive effect was attenuated by an α-2 antagonist in the spinal cord,[11] indicating a pivotal role of α-2 adrenoceptors in the antinociceptive action of ginsenosides at the spinal level. Moreover, the current study showed that the antinociceptive effect of ginsenosides was reversed by all α-2 adrenoceptor subtype antagonists in the spinal cord. These findings suggest that the antinociception of ginsenosides may be mediated by spinal α-2 adrenoceptors. Thus, ginsenosides and clonidine may have common pharmacological sites of action. On the basis of such observations, ginsenosides may not interact with clonidine in a synergistic fashion. A previous study also observed an additive interaction between intrathecal adenosine and MK 801 or NBQX.[35] Another factor that might affect the drug interactions is the stimulus intensity of nociception. It was previously reported that morphine interacts synergistically with pentobarbital at a low intensity stimulus, while interacting additively with a higher intensity stimulus.[36] The extent of antinociception produced was greater with a lower stimulus intensity.[37] Thus, a synergistic relationship might be observed with an injection of a lower formalin concentration, which is believed to be a milder stimulus.

In summary, intrathecal ginsenosides and clonidine alleviate formalin-induced acute pain and the facilitated state. Intrathecal ginsenosides also interact with clonidine in an additive manner. Additionally, spinal α-2A, -B, and -C subtypes of adrenoceptors, which exist in the spinal cord, are involved in the antinociception of intrathecal ginsenosides.

Conflict of interest

None declared.

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