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Effect of Sec-O-glucosylhamaudol on Mechanical Allodynia in a Rat Model of Postoperative Pain

조선대학교 대학원

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백서를 이용한 수술 후 통증 유발 모델에서
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정기태의 박사학위논문을 인준함

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ABSTRACT

백서를 이용한 수술 후 통증 유발 모델에서 Sec-O-glucosylhamaudol의 효과

정 기 태

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방풍(*Peucedanum japonicum Thunb.*,)은 한국, 중국, 일본, 필리핀 일대에 분포하는 다년생 식물로써, 전통적으로 진통효과가 있는 것으로 알려져 있다. 본 연구에서는 백서의 수술 후 통증 모델에서 sec-O-glucosylhamaudol (SOG)이 기계적 이질통에 미치는 영향에 대해 알아보고자 하였다. 백서에 척수강내 카테터를 삽입하고 1주일 후에 수술 후 통증 모델을 만들기 위해 백서를 마취한 후 우측 뒷발에 절개를 시행하였다. 2시간 후 발회피역치를 von Frey filament를 이용하여 측정하여 기계적 이질통의 발생을 확인한 후 SOG를 10 ㎍, 30 ㎍, 100 ㎍, 300 ㎍의 용량별로 척수강 내로 투여 후 발회복역치의 변화를 4시간동안 관찰하고, 용량-반응 관계와 50% 효과농도를 계산하였다. SOG와 아편제제 수용체와의 관계를 확인하기 위해 SOG 투여 10분 전 naloxone을 투여하여 발회복역치의 변화를 관찰하였다. 뒷발의 절개는 발회피역치를 의미있게 감소시켰으며, 척수강내로 투여한 SOG는 용량의존적으로 발회피역치를 증가시켜 진통효과가 있는 것을 확인하였다. 최대 효과는 투여 60분 후에 300 ㎍에서 나타났으며, 이때 예측되는 최대 효과는 85.35%였다. SOG의 진통효과는 naloxone을 전처치하였을 때 60분간 차단되었다. 이상의 연구 결과는 SOG는 수술 후 통증 모델에서 기계적 이질통을 효과적으로 줄여주는 진통효과가 있으며, 이는 아편제제 수용체를 통하여 발현되는 것으로 생각된다. 본 연구의 결과는 향후 SOG와 같은 천연물질을 이용한 진통제 개발을 위한 기초가 될 것으로 생각된다.

Ⅰ. INTRODUCTION

Even with the adequate use of medication, approximately 40% of patients experience moderate to severe postoperative pain [1]. Postoperative pain is a form of acute pain that is directly or indirectly involved in the occurrence of postoperative complications [2]. Surgical insults, such as incision tissue injuries, directly activate the peripheral nociceptors, resulting in an increase of central neuronal excitability, and leading to peripheral and central sensitization as a consequence [3]. Prolonged sensitization caused by inadequate analgesia may result in chronic postoperative pain or hyperalgesia and allodynia. These pathologic states may result in the decrease of the functions of the lung, heart, digestive system, and urological system, and can even cause psychological effects such as insomnia, depression, and anxiety in postoperative patients [2-4]. Accordingly, the effective mitigation of postoperative pain should be done as immediately as possible in order to reduce pain, promote recovery, and prevent complications [5]. Active postoperative pain management is an essential component of the care of surgical patients in the prevention of the development of chronic pain disorder. However, despite an increase in related scientific evidence, postoperative pain management remains insufficient because many patients still suffer from severe pain after surgery [5]. Thus, finding effective medications for postoperative analgesia is still necessary.

Substances derived from natural products have been used to treat pain disorders for a long time [6]. Discovering new chemical entities for postoperative pain from natural products can be an opportunity to improve the quality of life for the patient. The root of *Peucedanum japonicum*

Thunb., distributed throughout Japan, Philippines, China, and Korea [7], have been traditionally used as not only a food but also as an herbal medicine for cough, cold, headaches, and neuralgic disease [7, 8]. It has been known that substances derived from *Peucedanum* species show antiplatelet aggregation [8], antioxidant activity [9], anti-inflammatory activity [10], and inhibitory activity on COX-1 and COX-2 [11]. According to studies on the isolated constituents of *Peucedanum japonicum Thunb.,* sec-O-glucosylhamaudol (SOG) shows analgesic activity [11, 12]. In particular, a recent study showed that intrathecal SOG administration has an antinociceptive effect in a formalin test of rat model and shows the possibility of the involvement of SOG on opioid receptors [13].

Thus, I hypothesized that SOG exerts analgesic effects on postoperative incisional pain. I conducted the present study in order to evaluate the effect of SOG on mechanical allodynia in a rat model of postoperative pain in association with opioid receptors, and to set a clinical foundation for the use of SOG as a medication from natural products for postoperative pain control.

Ⅱ. MATERIALS and METHODS

1. Animals preparation and intrathecal catheterization

Following approval from the Institutional Animal Care and Use Committee of Chonnam National University (CIACUC2007-A0041), this study was conducted in compliance with guidelines from the International Association for the Study of Pain on ethical standards for the investigation of experimental pain in animals [14] (Fig. 1).

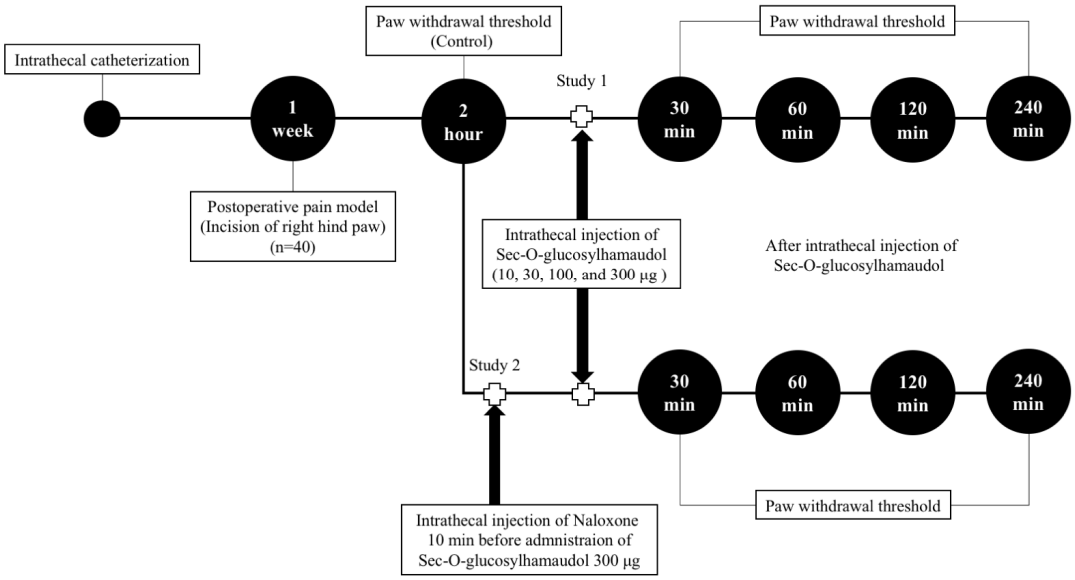

Fig. 1. Diagram illustrating the progress of the study protocol throughout the experiment.

Male Sprague-Dawley rats weighing 200-250 g were used for the

experiments (n=40). Rats were each housed in cages in the animal facility under constant temperature (22 ± 0.5 ℃), a 12-hour light/dark cycle, and food and water available *ad livitum*. For the administration of drugs, intrathecal catheterization was done with sevoflurane anesthesia [15]. Each rat was fixed in a stereotaxic apparatus, and a longitudinal incision was made on the atlanto-occipital membrane following sterile dressing for the exposure of cisterna magna. An intrathecal catheter (polyethylene-5) catheter was implanted via the cisterna magna and was advanced caudally by 8.5 cm so as to place the proximal portion of catheter on the level of lumbar enlargement. The distal portion of the catheter was secured to the skin of the head and anchored firmly by suture. The skin was then sutured with a 3-0 silk and the end of the externalized catheter was closed up with a 30-gauge stainless steel wire.

2. Postoperative pain model and behavioral study for the postincisional mechanical hyperalgesia

A week after intrathecal catheterization, rats were assessed for whether sensory abnormality had developed after intrathecal catheterization. Only rats without any neurological abnormality were used for this study. A postoperative pain model was applied according to the method outlined by Brennan et al. [16]. Anesthesia was conducted with sevoflurane, and the plantar surfaces of the left hind paws were prepared for incision. Following sterilization, a 1 cm longitudinal incision was made from 0.5 cm distal of the proximal edge of heel towards the toes. Skins and fasciae were incised in order to expose the plantaris muscle. The plantaris muscle was then

elevated and incised longitudinally with the muscle origin and insertion points kept intact. Gentle pressure was applied to control the bleeding and the incised skin was sutured with a 4-O silk. After dressing with povidone-iodine solution, rats were sent back to cages and recovered.

Two hours after the incision of the hind paw, an initial test was conducted in order to confirm the development of postoperative pain by assessing the occurrence of mechanical hyperalgesia and to measure the control value of postincisional paw withdrawal threshold (PWT). The PWT was measured in response to mechanical stimulation using von Frey filaments (Stoelting, Wood Dale, IL, USA). After acclimation in the laboratory environment for 30 min, the hind paws of the rats were accessed via apertures created in the mesh floor of the cage. Mechanical stimulation was applied to the plantar surface of the hind paw vertically for 5 s with a series of eight von Frey filaments (0.4, 0.7, 1.2, 2.0, 3.6, 5.5, 8.5, and 15 g). Abrupt withdrawal or a characteristic flinching response of the hindpaw after the stimulation of a filament was considered a positive response. The response was measured two times for each filament caliber with about a 3-min stimulation-free period in-between. The PWT was calculated by the up and down method [17]. The cut-off value was a negative response to 15 g and only rats showing marked mechanical allodynia (PWT < 5 g) were included for the study.

3. Drug preparations

Sec-O-glucosylhamaudol (purity >95%) was purchased from the Natural Product Bank (Gyeongsangbuk-do, Korea). Diluted solutions of SOG were prepared at 10, 30, 100, and 300 ㎍ after dissolving in 70% dimethylsulfoxide (DMSO).

4. Experimental design and administration intrathecal SOG

The total number of rats used was 40, with five to seven rats per dilution of SOG (Fig. 1). After confirmation of mechanical allodynia through achievement of the incisional pain model, intrathecal administration of SOG preparations (as 10 μl solutions) were conducted using a hand-driven, gear-operated syringe pump. Then, an additional 10 μl of 70% DMSO was administered to flush the SOG to the intrathecal space. Intrathecal administration of the experimental drug was randomly performed. The effects of intrathecal SOG of each dilution on the PWT were measured for four hours. The PWT measured 120 min after incision was regarded as the control post-incision threshold. A series of tests were then conducted prior to incision and 30, 60, 120, and 240 min after delivery of the drug.

5. Dose-responsiveness and ED50 of SOG

Dose-responsiveness and median effective analgesic dose (ED50) values were calculated. Dose-response data of the intrathecal SOG were calculated as the percentages of maximum possible effect (%MPE) as follows;

$$\%\mathrm{MPE} = \left[\frac{\text{post-drug PWT} - \text{pre-drug control PWT}}{\text{pre-incision PWT} - \text{post-incision control PWT}}\right] \times 100$$

The ED50 of SOG and its confidence interval were calculated using a standard linear regression analysis of a dose-response curve, according to the method outlined by Tallarida [18].

6. Reversal of the effect of intrathecal SOG by opioid antagonist

A further experiment was conducted in order to determine the involvement of SOG on the opioid receptor. A 10 μg dose of naloxone (Tocris Cookson, Avonmouth, UK) was administered intrathecally 10 min prior to intrathecal injection of SOG 300 ㎍, which showed a maximal antinociceptive effect. The PWT was measured in the same time sequence as that of the prior study.

7. Statistical analysis

Data are expressed as means ± SEM. Time response data are presented as PWT in g. Dose-response data are presented as %MPE. Behavioral experiments were analyzed by repeated measures two-way ANOVA followed by a post-hoc test with Tukey's test for multiple comparisons. Values with $p < 0.05$ were considered to be statistically significant as compared to vehicle treatment. Data of %MPE were analyzed by ANOVA followed by a post-hoc test with Bonferroni correction. Values with $p < 0.01$ were considered to be statistically significant as compared to vehicle treatment.

Ⅲ. RESULTS

1. Intrathecal SOG increased PWT in dose-dependent manner

Incision of the hind paw significantly decreased PWT to the mechanical stimulation, but PWT at the contralateral side (non-incised paw) was not changed during stimulation (Fig. 2).

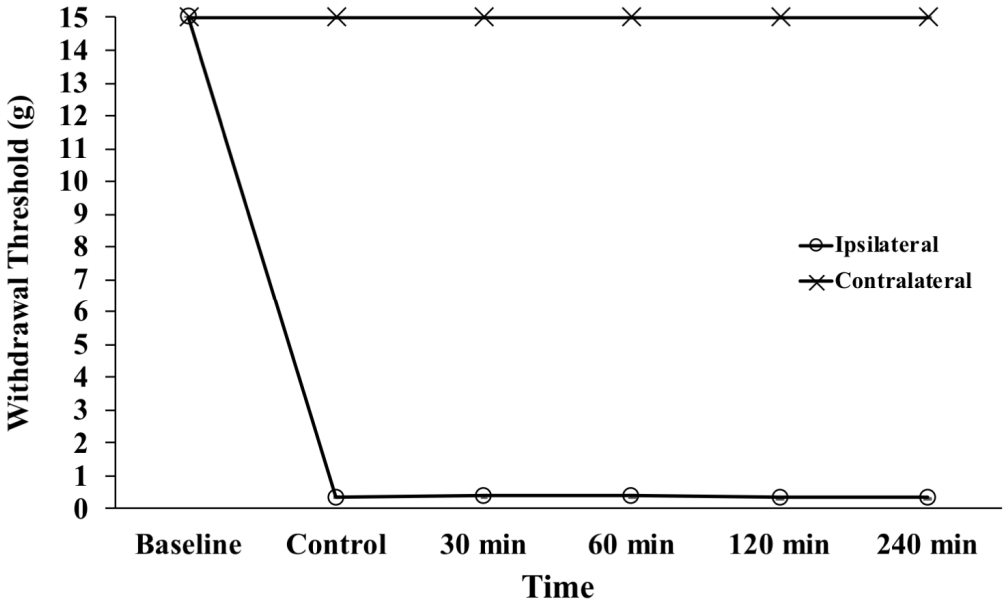

Fig. 2. Time course of paw withdrawal threshold after incision. Data are presented as the withdrawal threshold (g). Each line represents the mean ± SEM. Baseline is withdrawal threshold measured before paw incision. Control is 2 hours after paw incision.

The PWT decreased significantly two hours after incision and intrathecal SOG showed a significant increase of the PWT at the ipsilateral paw in a

dose-dependent manner (Fig. 3). Significant effects were shown from the dose of 30 ㎍ at 30 min after SOG administration (4.37 ± 0.52 g), but a constant effect was shown only in 100 ㎍ and 300 ㎍ throughout the observational periods (*P < 0.001 compared to the vehicle). The maximum effect was achieved in a dose of 300 ㎍ at 60 min after intrathecal SOG administration (12.85 ± 0.93 g).

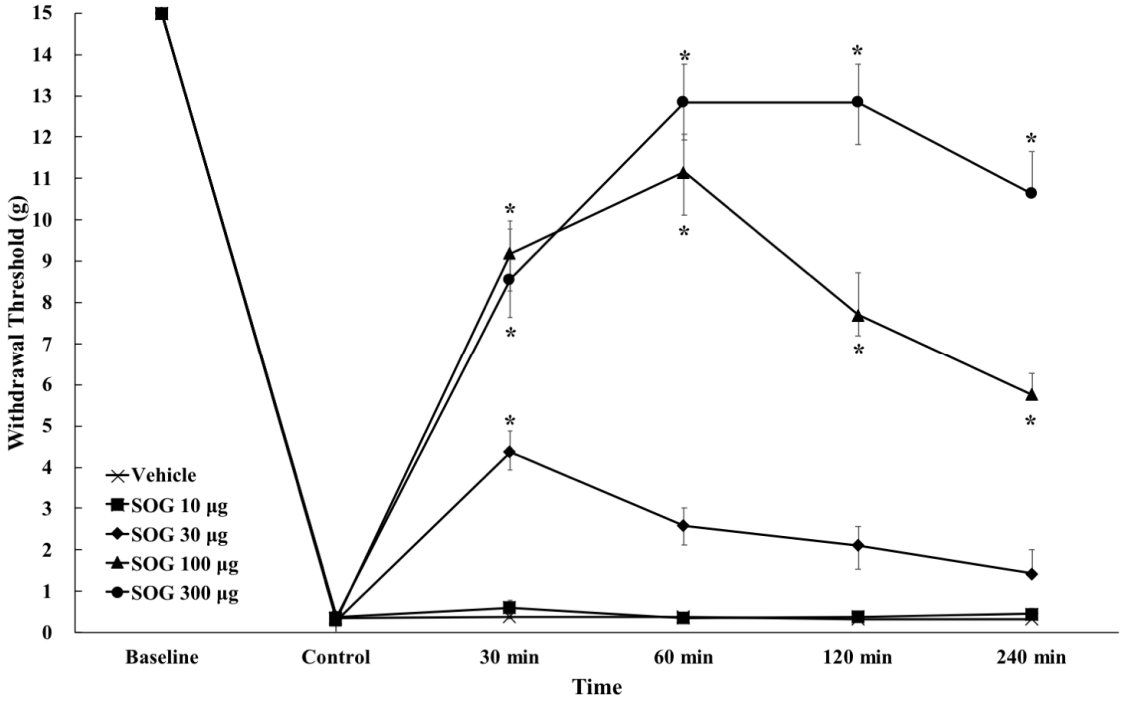

Fig. 3. Effects of intrathecal sec-O-glucosylhamaudol on paw withdrawal threshold after incision. SOG was administered immediately after measuring the control threshold 2 hours after paw incision. Intrathecal SOG administration showed a significant increase of the PWT in a dose-dependent manner. Data are presented as a withdrawal threshold (g). Each line represents the mean ± SEM of five to seven rats. Baseline is the withdrawal threshold measured before paw incision. Control is two hours after paw incision. Vehicle, 70% dimethyl sulfoxide; SOG,

sec-O-glucosylhamaudol. *P < 0.001 compared to the vehicle.

2. Maximal possible effects and ED50 of SOG

Maximum effects of each dose were seen at 30 min after intrathecal administration of SOG in doses of 10 ㎍ and 30 ㎍, while at 60 min in doses of 100 ㎍ and 300 ㎍. Maximal possible effects were calculated with each dose of SOG (Fig. 4). Intrathecal SOG administration showed a significant increase of %MPE in a dose-dependent manner. The %MPE in an intrathecal SOG administration dose of 300 ㎍ showed the greatest result, which was 83.35 ± 6.35%.

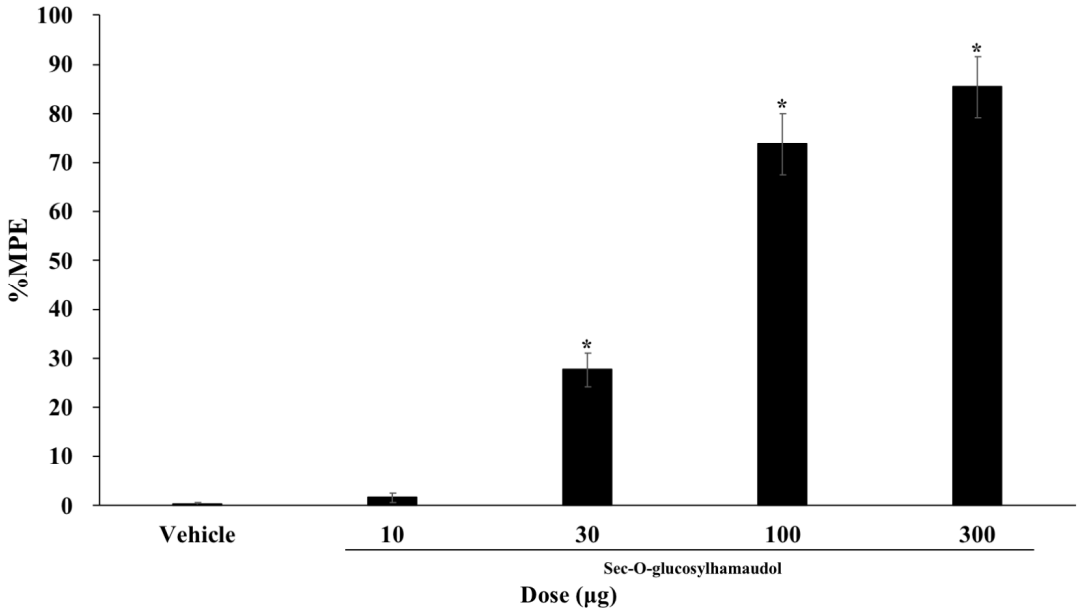

Fig. 4. Maximal possible effects of sec-O-glucosylhamaudol according to the dose. Data are presented as percentages of maximal possible effect (%MPE). Intrathecal SOG administration showed a significant increase of %MPE in a dose-dependent manner. Vehicle, 70% dimethyl sulfoxide; SOG,

sec-O-glucosylhamaudol. *P < 0.001 compared to the vehicle.

The maximal effects of these individual doses were used for analysis of standard linear regression in order to calculate ED50 values. The ED50 value (95% confidence intervals, CI) of intrathecal SOG was 191.3 (102.3 - 357.8) ㎍ with a slope (95% CI) of 50.74 (33.55 - 67.94).

3. Opioid antagonist reversed the antinociceptive effect of intrathecal SOG

Intrathecal administration of naloxone, an opioid antagonist, which was administered 10 min before the delivery of SOG, reversed the anti-nociceptive effect of the SOG (Fig. 5). Intrathecal administration of naloxone alone showed no antinociceptive effect after paw incision. Intrathecal administration of naloxone prior to the SOG 300 ㎍ decreased PWT (0.82 ± 0.52 g), which resembles the effect of vehicle alone until 60 min, but the inhibitory effect of naloxone against SOG vanished at 120 min after paw incision. The maximal PWT of naloxone + SOG 300 ㎍ showed 11.17 ± 0.81 g at 120min after paw incision, which was similar to the effect of SOG 300 ㎍ alone.

The %MPE in the administration of naloxone before delivery of SOG 300 ㎍ was 3.50 ± 3.50% at 60 min after incision, which was similar to the %MPE of vehicle or naloxone alone (Fig. 6). However, the %MPE increased to 73.95 ± 5.52% at 120 min, which was similar to the effect of SOG 300 ㎍ alone.

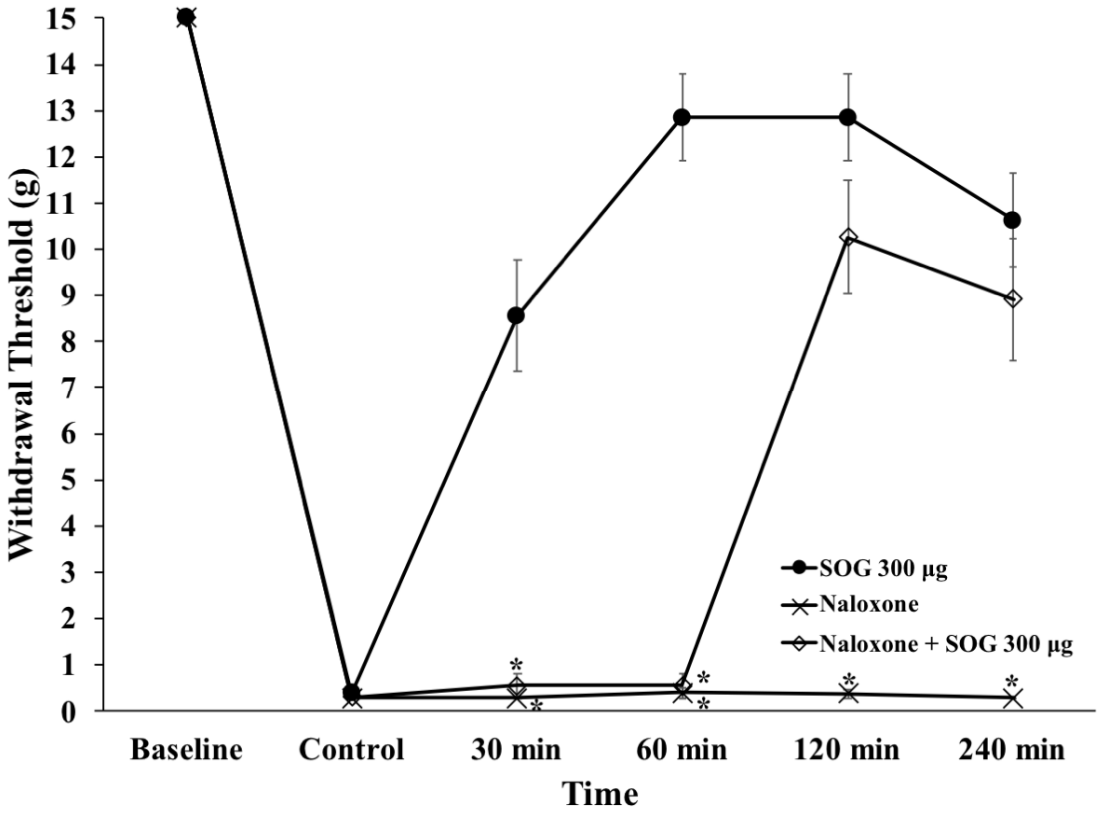

Fig. 5. The inhibitory effects of intrathecal naloxone against sec-O-glucosylhamaudol. Intrathecal administration of naloxone alone showed no antinociceptive effect. Intrathecal administration of naloxone 10 min before the delivery of SOG 300 ㎍ decreased PWT until 60 min. The inhibitory effect of naloxone against SOG vanished at 120 min. Data are presented as withdrawal threshold (g). Each line represents the mean ± SEM of five to seven rats. Baseline is the withdrawal threshold measured before paw incision. Control is two hours after paw incision. SOG; sec-O-glucosylhamaudol. *P < 0.001 compared to the SOG 300 ㎍.

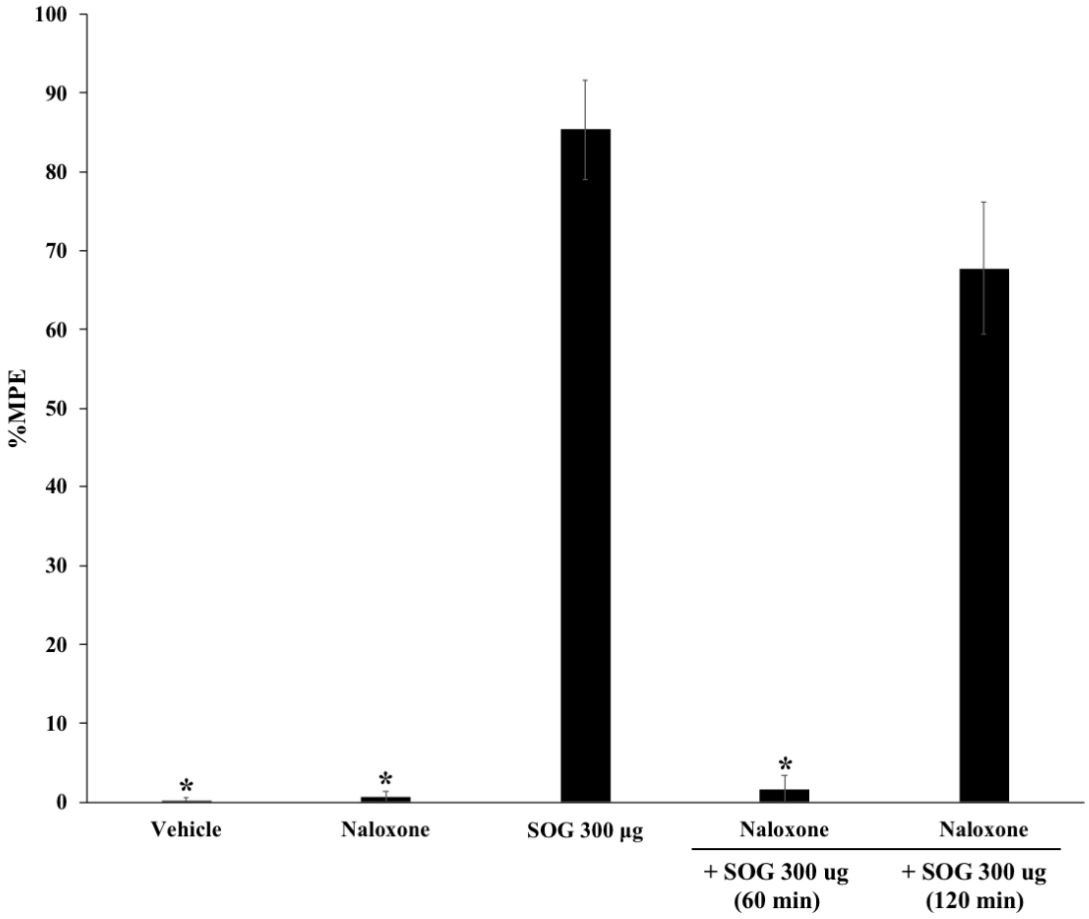

Fig. 6. Maximal possible effects of sec-O-glucosylhamaudol according to the inhibitory effects of intrathecal naloxone. Data are presented as percentages of maximal possible effect (%MPE). The %MPE in the administration of naloxone before delivery of SOG 300 ㎍ was decreased until 60 min, which was similar to the %MPE of vehicle or naloxone alone. The %MPE increased at 120 min, which was similar to the effect of SOG 300 ㎍ alone. Vehicle, 70% dimethyl sulfoxide; SOG, sec-O-glucosylhamaudol. *P < 0.001 compared to the SOG 300 ㎍.

Ⅳ. DISCUSSION

Effective analgesia after surgery not only has significant physiological benefits, but is also one of the most important factors for patients's recovery [19]. The surgical incisions lead to the activation and sensitization of peripheral nociceptors and spinal dorsal horn neurons through the biological response of releasing proinflammatory mediators at an incision site, and lead to pain sensation [5, 20]. Numerous studies on the pharmacological spinal modulation of the pain and hyperalgesia after incision have been conducted [5]. However, opioids that act on the central and peripheral receptors are still the main analgesic agent for postoperative pain management, but have limitations in clinical use because of their side effects such as nausea, vomiting, pruritus, urinary retention, and respiratory depression [19]. Thus, there are ongoing attempts to develop analgesic agents with reduced side effects by targeting putative opioid receptor splice variants or the receptor hetero-oligomers to separate the analgesic from undesirable effects [21]. Searching for a natural product as a promising analgesic candidate can be apart of that effort [22]. There is no doubt that natural products have provided key leads for drug discovery and the search for novel natural products with interesting bioactivity is an ongoing exercise [23]. As the number of studies on natural products increases, some flavonoids from medicinal plants showed the possibility that they can be promising candidates for new natural analgesic drugs [22]. Flavonoids have received increasing attention because of their bioactivity and their potential as anti-inflammatory and analgesic drugs. Recently, there was an interesting report that showed that micronized flavonoid fractions, made of a flavonoid fraction, reduced the severity of pain and intramuscular analgesic

requirement after hemorrhoidectomy [24].

As mentioned above, *Peucedanum japonicum Thunb.* have been traditionally used as analgesics for headaches or neuralgic disease [7, 8]. Substances derived from *Peucedanum* species show effects such as antiplatelet aggregation, antioxidant activity, anti-inflammatory activity, and COX-1 and COX-2 inhibition [8-11] which are the same bioactivity as chromone derivatives [23]. Chromones (4H-chomen-4-ones) are a group of naturally occurring compounds ubiquitous in nature, particularly in plants [25]. Classification in terms of chromone and flavonoid alkaloids is based on the part of the molecule to which the nitrogenous moiety is attached. Species of *Peucedanum* have constituents of coumarins, oils, chromones, flavonoids, and other acids [26]. SOG, one of the constituents of *Peucedanum* species, is known as a new chromone belonging to the flavonoids family [27], which have an analgesic and antiallodynic activity [11-13]. The mechanism of analgesic activity of SOG is still unknown because there are only few studies on the effects of SOG. Zheng et al. [11] isolated compounds from the *Peucedanum japonicum Thunb.* and revealed that SOG has an inhibitory effect on cyclooxygenase (COX) 1 and 2 by COX inhibition assay, but that SOG showed only a weak effect. Okuyama et al. [12] showed that oral administration of SOG 80 mg/kg significantly increased pain threshold in the tail pressure examination and in the test to detect neuropathic pain responses (modified Randall & Selitto test). They suggested that the effect of SOG acts on the opioid receptor of the central nervous system because the analgesic effect of SOG was reversed by the injection of naloxone. In particular, a recent study showed that intrathecal administration of SOG has an antinociceptive effect in a formalin test of rat model and the possibility

of the involvement of SOG on opioid receptors [13]. These results of previous studies are in agreement with my results. In the present study, intrathecal SOG showed strong antinociceptive effect for pain after incision in a dose-dependent manner. The maximal analgesic effect was achieved in a dose of 300 ㎍ at 60 min after intrathecal SOG administration (12.85 ± 0.93 g) and the %MPE was 83.35 ± 6.35%.

I hypothesized the potential mechanisms of antinociceptive effect of SOG according to the effect of flavonoids. First, SOG may acts as an anti-inflammatory drug on the central nervous system. There are numerous studies on anti-inflammatory of flavonoids, with many particularly focused on the inhibitory activities toward COX-1 and COX-2 [22, 25]. Thus, the COX inhibitory effect of flavonoids has been used as a basis for the synthesis of new anti-inflammatory agents [28]. One study on chromone extract *Saposhnikovia divaricata* which has SOG as one of the components, showed that it possesses potential anti-inflammatory, antiosteoarthritis, and antirheumatoid arthritis effects [29]. However, SOG showed only a weak inhibitory effect on both COX 1 and COX 2 according to the study of Zhenget al. [11]. Thus, the connection between antinociceptive effect of SOG on incisional pain and the inhibition of COX is small. However, anti-inflammatory actions related to lipoxygenase pathway or nitric oxide production inhibitors should be evaluated afterwards [25]. Second, antinociceptive effects of SOG are closely associated with opioid-related mechanisms themselves. In the present study, the maximal antinociceptive effect of SOG was significantly reverted by naloxone, which was administered 10 min before the delivery of SOG. Moreover, the reverse effect lasted only about an hour, which is in accord with the blocking effect

of the intrathecal naloxone on the drugs which has antinociceptive effect in involvement with opioid receptor [26]. These results are suggesting that opioid mechanisms are involved in the antinociceptive effects of SOG. Several studies have shown that flavonoids activate opioids system because of their structure-activity relationship with opioid receptors [27-30]. The actions of a flavonoids extract from H. perforatum may be mediated in part by opioid receptors especially at κ receptor [29], and a 3,3-dibromoflavanone, synthetic flavonoids, bind to MOR in the central nervous system and produce antinociception [30]. According to the literature, flavonoids act as and are opioid receptor ligands, and the stereochemistry of the C2 and C3 positions is important for antagonist activity and selectivity, thus structural modifications to the core structure can modulate intrinsic activity at opioid receptors [29]. Given these factors, further research into SOG could be a novel structural scaffold for the development of new drug targeting opioid receptors that only work on pain and avoiding adverse effects.

There are some limitations to this study. First, DMSO itself can have an antinociceptic effect [31]. The preparation of SOG was hydrophobic substances, thus DMSO had to be used as a solvent. However, 70% DMSO alone showed no antinociceptive effect. Second, further molecular work is needed to prove the hypothetic mechanisms of SOG. Further evaluations about anti-inflammatory effect and structural effect on opioid receptor should be done. Third, further evaluations about side-effects of SOG are needed for the clinical application because of its relationship with opioid receptors.

V. CONCLUSION

So far, the information about SOG on the antinociceptive effect and the pharmacological mechanisms has been very limited. Therefore, I evaluated the potential effect of SOG on the postoperative pain. I found that intrathecal administration of significantly decreased PWT in a dose-dependent manner and the maximal antinociceptive effects of SOG were significantly reverted until 60 min by naloxone. The maximum effect was achieved in a dose of 300 ㎍ at 60 min after intrathecal SOG administration (12.85 ± 0.93 g). The %MPE in an intrathecal SOG administration dose of 300 ㎍ which showed the greatest result was 83.35 ± 6.35%. Intrathecal administration of naloxone prior to the SOG 300 ㎍ decreased PWT (0.82 ± 0.52 g). The %MPE in an administration of naloxone before delivery of SOG 300 ㎍ was 3.50 ± 3.50% at 60 min after incision. These results strongly suggest that intrathecal SOG showed significant antinociceptive effect on the postoperative pain model through the involvement of opioid receptors. The current study can be a foundation for the development of new analgesics for postoperative pain control based on a natural product such as SOG.

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Legends for figures

Fig. 1. Diagram illustrating the progress of the study protocol throughout the experiment.

Fig. 2. Time course of paw withdrawal threshold after incision. Data are presented as the withdrawal threshold (g). Each line represents the mean ± SEM. Baseline is withdrawal threshold measured before paw incision. Control is 2 hours after paw incision.

Fig. 3. Effects of intrathecal sec-O-glucosylhamaudol on paw withdrawal threshold after incision. SOG was administered immediately after measuring the control threshold 2 hours after paw incision. Intrathecal SOG administration showed a significant increase of the PWT in a dose-dependent manner. Data are presented as a withdrawal threshold (g). Each line represents the mean ± SEM of five to seven rats. Baseline is the withdrawal threshold measured before paw incision. Control is two hours after paw incision. Vehicle, 70% dimethyl sulfoxide; SOG, sec-O-glucosylhamaudol. *P < 0.001 compared to the vehicle.

Fig. 4. Maximal possible effects of sec-O-glucosylhamaudol according to the dose. Data are presented as percentages of maximal possible effect (%MPE). Intrathecal SOG administration showed a significant increase of %MPE in a dose-dependent manner. Vehicle, 70% dimethyl sulfoxide; SOG, sec-O-glucosylhamaudol. *P < 0.001 compared to the vehicle.

Fig. 5. The inhibitory effects of intrathecal naloxone against

sec-O-glucosylhamaudol. Intrathecal administration of naloxone alone showed no antinociceptive effect. Intrathecal administration of naloxone 10 min before the delivery of SOG 300 ㎍ decreased PWT until 60 min. The inhibitory effect of naloxone against SOG vanished at 120 min. Data are presented as withdrawal threshold (g). Each line represents the mean ± SEM of five to seven rats. Baseline is the withdrawal threshold measured before paw incision. Control is two hours after paw incision. SOG; sec-O-glucosylhamaudol. *P < 0.001 compared to the SOG 300 ㎍.

Fig. 6. Maximal possible effects of sec-O-glucosylhamaudol according to the inhibitory effects of intrathecal naloxone. Data are presented as percentages of maximal possible effect (%MPE). The %MPE in the administration of naloxone before delivery of SOG 300 ㎍ was decreased until 60 min, which was similar to the %MPE of vehicle or naloxone alone. The %MPE increased at 120 min, which was similar to the effect of SOG 300 ㎍ alone. Vehicle, 70% dimethyl sulfoxide; SOG, sec-O-glucosylhamaudol. *P < 0.001 compared to the SOG 300 ㎍.