# Analgesic effects of 1,2,3,4,6-penta-O-galloyl-β-D-glucose in an animal model of lipopolysaccharide-induced pain

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**Abstract.** We examined the analgesic effects of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (β-PGG), a prototypical gallotannin, in an animal model of lipopolysaccharide (LPS)-induced pain. To evaluate the analgesic activity of β-PGG, we assessed the potential of β-PGG to inhibit the generation of nitric oxide (NO) in LPS-stressed RAW 264.7 cells, and found that β-PGG inhibits NO generation in a dose-dependent manner. Furthermore, the effects of β-PGG on the voluntary movements of LPS-exposed animals were evaluated. The results showed that the voluntary movements of animals were markedly recovered after β-PGG treatment. The mRNA expression of interleukin (IL)-1β (1.33±0.38-fold) and IL-6 (0.64±0.40-fold) in the brain tissue of β-PGG-treated animals markedly decreased compared with that observed in the control groups (3.86±0.91 and 2.45±1.12-fold, respectively) and in the other LPS-administered groups. The results showed that β-PGG has potential to alleviate pain, not only by decreasing cellular NO generation in RAW 264.7 cells but also by the recovery of voluntary movement lost owing to inflammatory pain. This suggests that β-PGG is comparable to ibuprofen, which was used as a positive control in this study. Collectively, these findings suggest that β-PGG is a valuable natural compound which possesses analgesic activity.

## Introduction

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage (1). Our understanding of pain has developed and evolved over recent years owing to the identification and investigation of various molecules and pathways including bradykinin, cyclooxygenases (COXs), nerve growth factor (NGF), interleukins (ILs), and other channels associated with pain (2-7). Owing to the improved knowledge of pain signaling mechanisms, novel therapeutic targets for the treatment of chronic pain have emerged. Nevertheless, effective therapies designed to prevent or treat both acute and chronic pain are limited in number.

Inflammation has been reported as a common symptom of acute and chronic pain as it is believed that pain is initiated by local inflammatory events (8,9). Inflammatory pain is typically treated with opioids or nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen, which act as COX inhibitors (10,11). However, side effects limit the use of these medications in both short- and long-term settings (12). Thus, the treatment of inflammatory pain is challenging, and effective, novel medications with fewer side effects are needed.

Many animal models and methods have been developed to measure pain intensity for different types of pain, including the hot plate test, the formalin test, acetic acid-induced writhing, the tail flick test and the von Frey hair test. These are reflex response-based tests which involve the application of physical or chemical stimuli to the abdomen, paw, or tail. Cho *et al* (13) devised a voluntary movement test to measure inflammatory- and neuropathic pain-related behavior in animals without the use of stimuli. In the present study, we administered lipopolysaccharide (LPS) to mice to induce systemic inflammatory pain and thereby established a mouse model of LPS-induced pain; we then evaluated their voluntary movements and performed biochemical analysis.

LPS is a major, bacterial Toll-like receptor 4 (TLR4) ligand that activates the innate immune response to infection. The administration of LPS not only induces pro-inflammatory cytokine production but also inhibits neurotrophic factor production (14). The response to systemic inflammatory pain induced by LPS administration involves the release of numerous pro-inflammatory cytokines such as IL-1β and IL-6. 1,2,3,4,6-Penta-O-galloyl-β-D-glucose (β-PGG), a prototypical gallotannin (Fig. 1A), is found in medicinal herbs such as *Rhus chinensis* Mill. (Fig. 1B) and *Paeonia suffruticosa* (Fig. 1C) (15). β-PGG has been demonstrated to exhibit antioxidant, anti-diabetic and anticancer effects (16-18). However, to the best of our knowledge, the analgesic effects of β-PGG in an animal model of LPS-induced pain have not been reported to date.

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In this study, we evaluated the analgesic effects of β-PGG on LPS-exposed RAW 264.7 cells and in an animal model of LPS-induced pain in order to determine whether β-PGG affects cellular nitric oxide (NO) generation, the voluntary movements of animals experiencing inflammatory pain and the mRNA expression of inflammation-related cytokines, each of which is associated with pain symptoms.

## Materials and methods

*Chemicals, reagents and cells*. All chemicals, solvents, reagents and standards used in the experiments were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solutions were freshly prepared with distilled water. RAW 264.7 cells were purchased from the American Type Culture Collection (no. TIB-71™; ATCC, Manassas, VA, USA).

*Cell viability*. Cell viability was assessed using the MTT assay as previously described (19). Briefly, RAW 264.7 cells were seeded in a 96-well flat-bottom microplate at a density of $2x10^4$ cells/well and incubated at 37˚C for 1 h. The cells were then treated with various concentrations of β-PGG (1-100 $\mu$M). After an additional 24 h incubation, 20 $\mu$l MTT [5 mg/ml in phosphate-buffered saline (PBS)] solution was added to each well, and the plate was incubated for a further 2 h. The absorbance was measured at 450 nm using a microplate reader (Victor3; PerkinElmer, Inc., Waltham, MA, USA).

*Measurement of cellular NO generation*. The concentration of NO in the medium was measured by Griess reagent as an indicator of NO production. Briefly, RAW 264.7 cells were seeded in a 96-well flat-bottom microplate at a density of $2x10^4$/well and incubated at 37˚C for 1 h. The cells were then exposed to LPS at 1 $\mu$g/ml and treated with various concentrations of β-PGG (1-100 $\mu$M). After an additional 24 h incubation, NO concentrations in the supernatants were measured by adding Griess reagent (20). The absorbance of the mixtures was determined using a microplate reader at a wavelength of 540 nm.

*Animals and care*. Male BALB/c mice aged 6 weeks and weighing 18-23 g (Samtako, Osan, Korea) were used in this study. The animals were housed in an air-conditioned room at a temperature of 22±1˚C and a humidity of 55±1% on a 12 h light/dark cycle. They were fed a standard commercial rodent pellet diet (Samtako) and water was provided *ad libitum*. The animal experiments complied with the in-house guidelines of Kyungpook National University Animal Care and Use Committee, and protocols (approval no. #KNU 2014-148) were in accordance with the guidelines of the Committee on Research and Ethical Issues of the International Association for the Study of Pain (21). All animals were permitted to adapt to the laboratory environment for at least 1 week prior to performing the experiments.

*Behavioral test to examine the effect of pain on voluntary movements*. Behavior was examined using a slightly modified method of Cobos *et al* (22). Briefly, a total of 35 animals were acclimated to the laboratory environment for at least 1 week prior to initiating the experiments and were randomized into 7 groups, each containing 5 animals: two saline-treated control groups (normal and negative); four positive control groups (ibuprofen, gabapentin, capsaicin, and ascorbic acid); and one treatment group (β-PGG). Animals received intraperitoneal injections of the samples daily for 3 days and the LPS (1 mg/kg) challenge was performed on the last day. Thirty minutes after the administration of LPS, the mice were subjected to behavioral tests for 60 min by placing each animal in a plastic cage (26x15 cm). We recorded the voluntary movement distances and cumulative voluntary movement distances covered by the animals at 10 min intervals over a 60 min period using Kinovea (http://www.kinovea.org/), an open-source motion tracking software program. At 120 min after LPS administration, the animals were euthanized by carbon dioxide inhalation. Brain tissues were removed and frozen for subsequent analyses.

*Reverse transcription polymerase chain reaction (RT-PCR)*. Total RNA was isolated from the brain tissues using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions (23). The RNA (1-10 $\mu$g) was reverse transcribed into first-strand cDNA using an RT-&GO Master Mix (MP Biomedicals, Santa Ana, CA, USA), and the product was used as the PCR template. RT-PCR was performed using a Takara PCR Thermal Cycler (Takara Bio, Otsu, Japan) and the following oligonucleotides primers were used: mouse IL-1β forward, 5'-AGAGCCCATCC TCTGTGACT-3' and reverse, 5'-CTCTGCTTGTGAGGTGC TGA-3'; mouse IL-6 forward, 5'-CACTTCACAAGTCGGAG GCT-3' and reverse, 5'-GCCACTCCTTCTGTGACTCC-3'. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control (forward, 5'-GGCAAATTCAACGGCACAGT-3' and reverse, 5'-CTCGT GGTTCACACCCATCA-3'). Genes for IL-1β, IL-6 and GAPDH were amplified with a denaturation step at 94˚C for 20 sec, an annealing step at 58˚C for 40 sec, and an extension step at 72˚C for 30 sec. The PCR products were electrophoresed at 100 V for 40 min on a 2% agarose gel in TBE buffer. The mRNA levels were normalized to the housekeeping gene, GAPDH.

*Statistical analysis*. The experiments were performed in triplicate and the results are expressed as the means ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance (ANOVA), using the program IBM SPSS statistics (23). When the data from the ANOVA were significant, the differences were analyzed by Tukey's HSD post hoc test or Duncan's test. The critical level for statistical significance was defined as $P<0.05$ or $P<0.01$.

## Results

*Cell viability and antioxidant potential of β-PGG*. The effects of various doses (1-100 $\mu$M) of β-PGG on the viability of RAW 264.7 cells were evaluated using an MTT assay in the present study. The results showed that the cell viability at various doses was 99.55±7.93%, 87.55±1.87%, 89.11±2.76%, 78.61±9.48%, and 61.31±4.51%, respectively. These findings indicated that there were no cytotoxic effects at doses up to 30 $\mu$M (Fig. 1D).

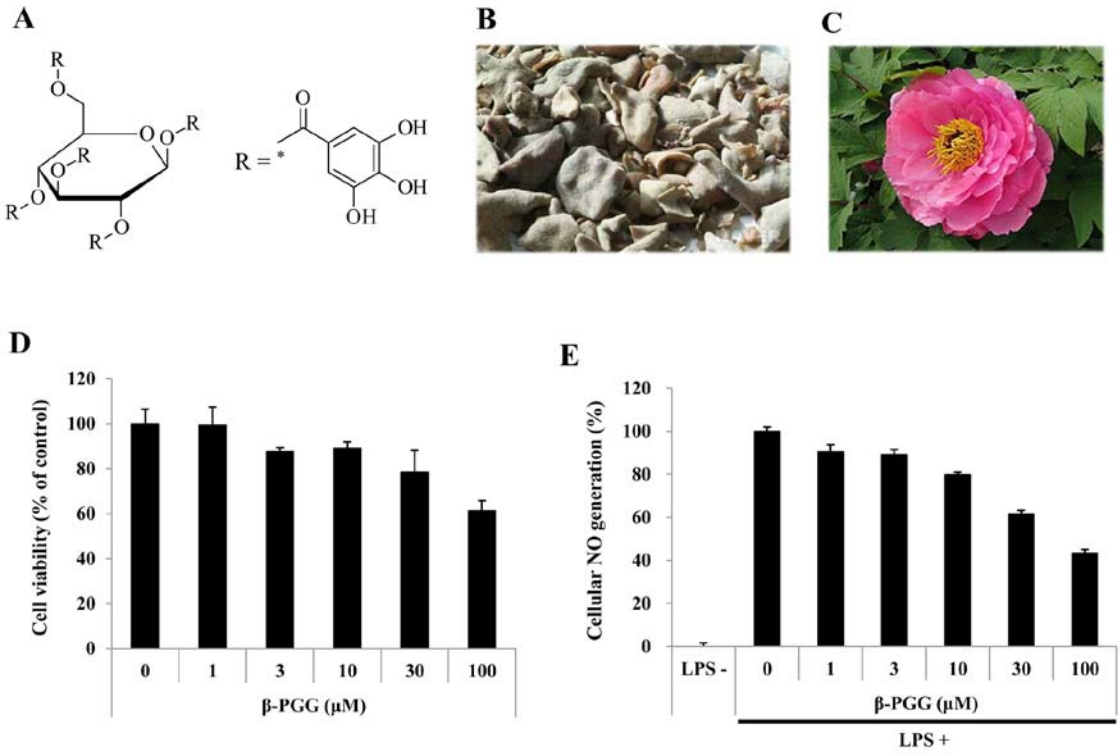


Figure 1. Basic profile of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (β-PGG). (A) Structure of β-PGG. (B) Nutgall tree seeds (*Rhus chinensis* Mill.). (C) Tree peony (*Paeonia sp.*). (D) Viability of RAW 264.7 cells following β-PGG treatment. RAW 264.7 cells were seeded at a density of $2x10^4$ cells/well (96-well plate) and treated with various concentrations of β-PGG. An MTT assay was then performed. (E) Inhibition of nitric oxide (NO) generation by β-PGG in RAW 264.7 cells. RAW 264.7 cells were seeded at a density of $2x10^4$ cells/well (96-well plate) and treated with various concentrations of β-PGG. The Griess assay was then performed.

*Inhibitory effect of β-PGG on cellular NO generation*. We then examined whether β-PGG had the potential to suppress NO generation in stressed RAW 264.7 cells. The amount of NO accumulated was used as an indicator of NO generation in the medium. Treatment with LPS (1 $\mu$g/ml) increased NO accumulation (114.80%) compared with that observed in the untreated control cells. Treatment with β-PGG at concentrations of 1, 3, 10, and 30 $\mu$M, decreased NO release by 9.62±3.29%, 10.86±2.36%, 20.25±1.38%, and 38.38±1.65%, respectively, compared with the stressed cells (Fig. 1E). As β-PGG did not induce cytotoxicity at these concentrations, the inhibition of NO generation may not be attributed to cytotoxicity. NO is a member of the reactive nitrogen species (RNS) family and the interaction of NO with reactive oxygen species (ROS) produces several types of RNS including NO, nitrogen dioxide, and peroxynitrite, which cause nitrosative stress and tissue damage at the cellular level (24). Thus, β-PGG induced a decrease in NO generation which indicates that β-PGG possesses anti-inflammatory potential.

*β-PGG recovers voluntary movements in an animal model of LPS-induced pain*. To determine the effect of pain on voluntary movements, each animal was placed in a plastic cage (26x15 cm) and the voluntary movements of the mice were subsequently recorded. When the saline-treated animals were placed in the plastic cage, they moved in a normal manner. The total distance traveled for 1 h was analyzed at 10 min intervals using Kinovea (http://www.kinovea.org/), an open-source motion tracking software program.

Fig. 2 shows representative motion tracking results of the saline-, LPS-, ibuprofen- and β-PGG-treated animals free to travel the plastic cage. We observed a marked difference in total voluntary movements between LPS- and ibuprofen-treated animals. We then evaluated whether ibuprofen (an NSAID), gabapentin (an NSAID), capsaicin [a ligand of transient receptor potential cation channel subfamily V member 1 (TRPV1)] and ascorbic acid (an antioxidant) could recover the voluntary movements. Ibuprofen (30 mg/kg), gabapentin (100 mg/kg), capsaicin (10 mg/kg), ascorbic acid (100 mg/kg) and β-PGG (10 mg/kg) were injected intraperitoneally to LPS-exposed animals 30 min before measuring the travelled distance. As shown in Fig. 3A, the distances travelled by LPS-exposed animals measured at 10-min intervals (0-10 min, 1259.20±392.77 cm; 10-20 min, 787.75±341.62 cm; 20-30 min, 528.04±202.52 cm; 30-40 min, 418.44±67.55 cm; 40-50 min, 353.12±62.43 cm; 50-60 min, 330.84±61.09 cm) significantly decreased after 20 min, compared with those of the animals treated with saline (0-10 min, 1722.91±415.83 cm; 10-20 min, 1592.70±295.27 cm; 20-30 min, 1487.65±263.81 cm; 30-40 min, 1395.34±330.80 cm; 40-50 min, 1150.06±489.36 cm; 50-60 min, 1091.26±434.85 cm), ibuprofen (0-10 min, 1494.47±666.93 cm; 10-20 min, 1365.26±388.63 cm; 20-30 min, 907.40±475.31 cm; 30-40 min, 616.64±265.66 cm; 40-50 min, 819.51±215.12 cm; 50-60 min, 626.84±207.32 cm) and β-PGG (0-10 min, 1434.79±623.69 cm; 10-20 min, 1265.63±658.47 cm; 20-30 min, 739.82±263.73 cm; 30-40 min, 546.70±173.29 cm; 40-50 min, 533.26±122.90 cm; 50-60 min, 486.00±183.54 cm), indicating that LPS-induced

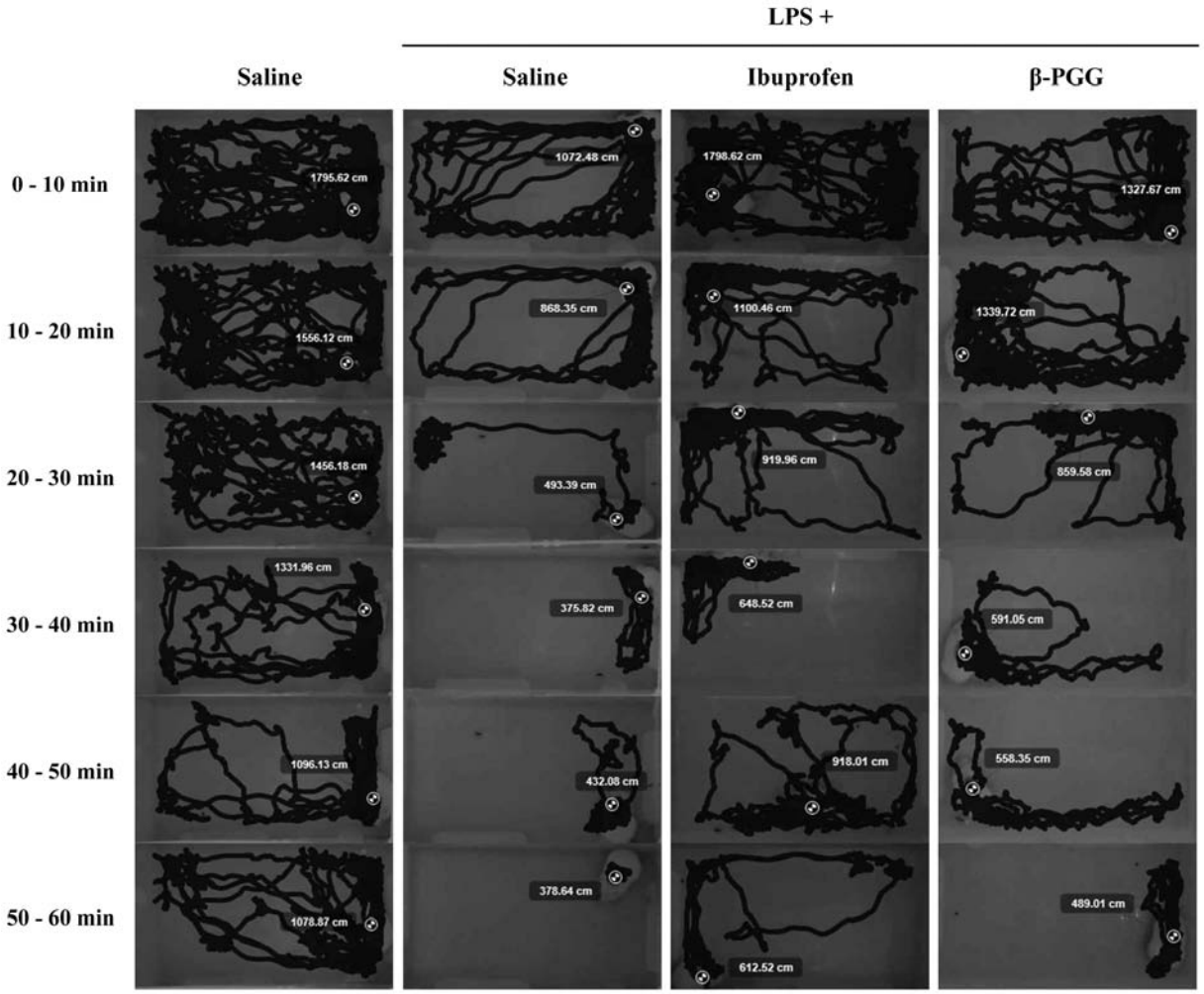


Figure 2. Representative motion tracking results of the voluntary movements of animals. A behavioral test to determine the effect of pain on voluntary movements was performed using a method of Cobos et al (22) as described in Materials and methods section.

pain effectively slowed motion, whereas β-PGG recovered 37.87% of voluntary movement (ibuprofen recovered 50.7%).

*Analysis of total distance travelled in an animal model of LPS-induced pain*. Measuring the cumulative distance travelled by animals for 60 min (Fig. 3B) showed that distance travelled by the animals treated with saline only (10 min, 1722.91±415.83 cm; 20 min, 3315.60±452.01 cm; 30 min, 4803.25±630.14 cm; 40 min, 6198.60±881.01 cm; 50 min, 7348.66±1014.98 cm; 60 min, 8439.91±1184.55 cm), as well as LPS-exposed animals treated with ibuprofen (10 min, 1494.47±666.93 cm; 20 min, 2859.73±1020.23 cm; 30 min, 3767.13±1189.96 cm; 40 min, 4383.77±1382.03 cm; 50 min, 5203.29±1525.56 cm; 60 min, 5830.13±1683.06 cm) and β-PGG (10 min, 1434.79±623.69 cm; 20 min, 2700.42±748.07 cm; 30 min, 3440.24±867.53 cm; 40 min, 3986.93±879.61 cm; 50 min, 4520.19±905.16 cm; 60 min, 5006.19±902.04 cm) increased steadily compared with the cumulative distances covered by LPS-exposed animals treated with saline (10 min, 1259.20±392.77 cm; 20 min, 2046.95±291.00 cm; 30 min, 2574.99±479.84 cm; 40 min, 2993.43±543.33 cm; 50 min, 3346.55±560.98 cm; 60 min, 3677.39±537.28 cm), gabapentin (10 min, 1057.10±667.47 cm; 20 min, 2034.88±619.42 cm; 30 min, 2933.02±714.53 cm; 40 min, 3328.61±749.11 cm; 50 min, 3622.07±692.24 cm; 60 min, 3930.24±622.75 cm), capsaicin (10 min, 1179.05±547.39 cm; 20 min, 1978.11±750.12 cm; 30 min, 2952.47±551.15 cm; 40 min, 3606.89±885.12 cm; 50 min, 4027.77±1097.88 cm; 60 min, 4319.74±1124.37 cm), and ascorbic acid (10 min, 1409.75±448.57 cm; 20 min, 2292.04±524.36 cm; 30 min, 2755.69±593.79 cm; 40 min, 3163.50±639.97 cm; 50 min, 3454.54±720.15 cm; 60 min, 3695.48±842.12 cm). As shown in Fig. 3C, the treatment of LPS-exposed animals with ibuprofen (69.08±19.94%) or β-PGG (59.32±10.69%) recovered the initial decrease in total voluntary movements compared with the saline plus LPS treatment group (43.57±6.37%) and saline alone group (100.00±14.04%). By contrast, voluntary movement in the gabapentin (46.57±7.38%), capsaicin (51.18±13.32%) and ascorbic acid (43.79±9.98%)-treated groups decreased in a similar manner to the saline plus LPS treatment group (43.57±6.37%). Thus, only β-PGG and ibuprofen recovered the reduction in total distance travelled due to LPS-induced pain.

*Gene expression of IL-1β and IL-6 by β-PGG*. We also examined whether the mRNA expression of IL-1β and IL-6 in brain tissues from LPS-exposed mice was altered by treatment with β-PGG or ibuprofen. We isolated brain tissues from mice treated with β-PGG or ibuprofen, and then compared the gene expression profiles to those of the control using RT-PCR. As shown in Fig. 4A and B, LPS exposure (1 mg/kg) increased the

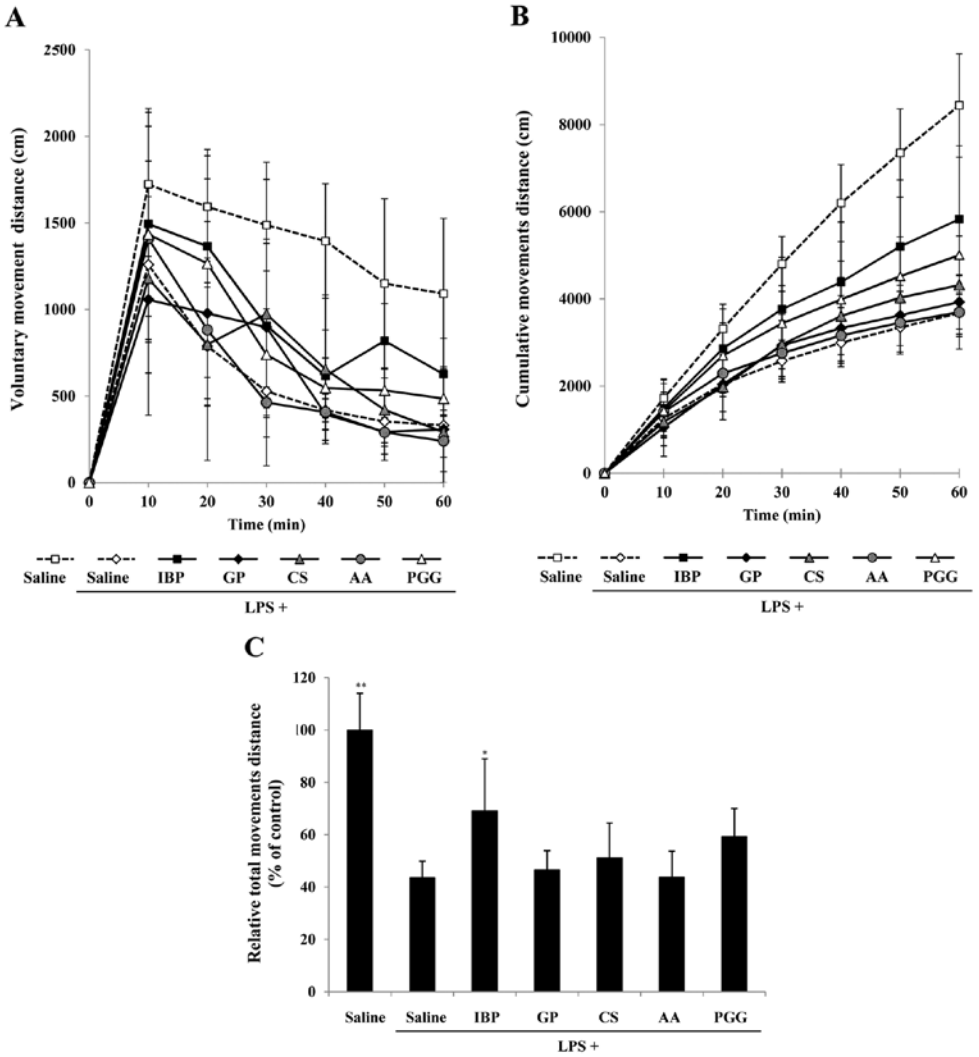


Figure 3. Analgesic effects of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (β-PGG) demonstrated by measuring voluntary movement distances, cumulative voluntary movement distances, and relative total voluntary movement distances covered by animals over a 60 min period. (A) Voluntary movements of animals were measured for 60 min after lipopolysaccharide (LPS) administration and analyzed at 10 min intervals. (B) Cumulative voluntary movement distances covered by animals were measured and analyzed for 60 min after LPS administration. (C) Total movement distances covered by animals measured for 60 min after LPS administration. $^{*}$P<0.05 and $^{**}$P<0.01, relative to the saline- and LPS-treated group, using one-way ANOVA followed by Tukey's post hoc test.

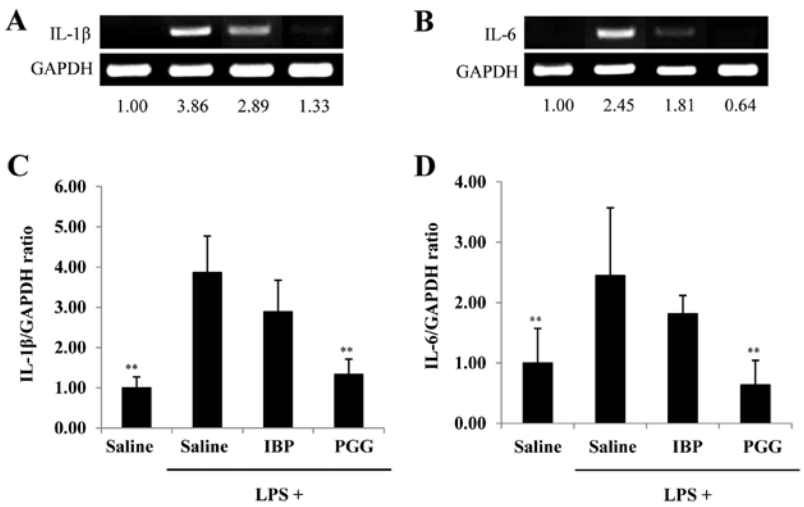


Figure 4. Analysis of the mRNA expression of interleukin (IL)-1β and IL-6 in the brain tissues from LPS-exposed mice following treatment with 1,2,3,4,6-penta-O-galloyl-β-D-glucose (β-PGG) . (A and B) Male BALB/c mice aged 6 weeks and weighing 18-23 g were administered lipopolysaccharide (LPS) and thereafter treated with saline, ibuprofen or β-PGG for 3 days. The brain was excised from each animal and mRNA levels of (A) IL-1β and (B) IL-6 were evaluated using RT-PCR. Comparison of the relative mRNA expression of (C) IL-1β and (D) IL-6 in the brain following treatment with β-PGG as shown in (A and B). $^{**}$P<0.05, compared with the saline and LPS-treated groups, using a one-way ANOVA followed by Tukey's post hoc test.

mRNA expression of IL-1 and IL-6. β-PGG treatment significantly decreased the mRNA levels of IL-1β (1.33±0.38-fold) and IL-6 (0.64±0.40-fold) compared with those in the the LPS plus saline group (3.86±0.91 and 2.45±1.12-fold) and the ibuprofen (2.89±0.78 and 1.81±0.30-fold) group, respectively.

## Discussion

Previous studies have investigated the antioxidant, anti-diabetic and anti-inflammatory effects as well as the anticancer potential of β-PGG (25-29). However, to date, no studies have evaluated the effects of β-PGG in a mouse model LPS-induced pain, to the best of our knowledge. The results of the present study suggest that β-PGG possesses analgesic potential comparable to other known analgesic compounds, namely ibuprofen, gabapentin, capsaicin and ascorbic acid.

Antioxidants, among other nutritional substances, aid in the eliminiation of potentially harmful free radicals from the body. We examined whether β-PGG was capable of eliminating free radicals in immune cells. We treated RAW 264.7 cells with β-PGG and using the ORAC assay measured how much β-PGG scavenges free radicals (data not shown). The ORAC assay utilizes an AAPH-derived peroxyl radical that mimics the lipid peroxyl radicals involved in the lipid peroxidation chain reaction *in vivo*. The inhibition of peroxyl radical-induced oxidation of a fluorescent probe, fluorescein, by antioxidants was serially monitored. β-PGG exhibited potent antioxidant activity, which is possibly conferred by the hydroxyl groups in its structure. This suggests that β-PGG has the potential to eliminate such radicals during numerous antioxidative events and in several antioxidant pathways. Chronic pain is associated with oxidative stress processes, thus the presence of free radicals may play a role in the development and persistence of pain. It follows that antioxidants such as vitamins C and E, selenium and β-carotene, those present in fruits, vegetables, green tea, and red wine, may be used as therapy for acute pain, fibromyalgia, dysmenorrhea, diabetic neuropathy, osteoarthritis, and recurrent pancreatitis. We therefore hypothesized that various food ingredient(s) exhibit analgesic activity. This hypothesis can be proven using an animal model of LPS-induced pain.

Pain has long been defined as an unpleasant sensory and emotional experience which is associated with actual or potential tissue damage as well as with major stresses on both the mind and the body. Generally, pain intensity has been measured with one of several different tests: the hot plate test, the formalin test, acetic acid-induced writhing, the tail flick test, or the von Frey hair test, among others. We selected a model of LPS-induced pain for use in this study as the model is easy and simple to establish and it generates reproducible data.

Fig. 3 shows the voluntary movement distances covered by mice subjected to LPS-induced pain. Previous research has shown that voluntary movement distances provide measurements of some types of chronic pain including inflammatory and neuropathic pain (13). We examined voluntary movement in an animal model of LPS-induced pain, and the results showed that β-PGG reduced the effect of inflammation on voluntary movement and recovered the reduction in movement distances lost owing to LPS-induced pain. The findings of the present study suggest that β-PGG possesses analgesic potential and is superior in terms of movement distance recovery compared with other compounds including gabapentin, capsaicin and ascorbic acid. Although all compounds used in this study are used as analgesic drugs, they have different pharmacokinetic activities in the mammalian body. For example, gabapentin increases GABA biosynthesis, glutamate decarboxylase and branched chain aminotransferase, and is used for the treatment of neuropathic pain (30). Capsaicin binds to a TRPV1, and produces similar sensations to excessive heat (31). Ibuprofen is an NSAID used for relieving pain by reducing inflammation, and inhibits COX-2 which is involved in mediating fever, inflammation, and pain (32). The cytokines IL-1β and IL-6 are associated with the molecular inflammation of specified tissues, marking them as potential biomarkers of local inflammatory events. We evaluated changes in the mRNA expression of both IL-1β and IL-6 using RT-PCR and found that β-PGG decreased the mRNA expression of both IL-1β and IL-6, suggesting that β-PGG exhibits anti-inflammatory effects. We also found that β-PGG was more effective at reducing IL-1β and IL-6 levels than ibuprofen, but that it was not more effective at reducing NGF (data not shown). We anticipate that NGF levels will markedly decrease during pain exposure; however, we are unable to prove this currently. Furthermore, the molecular targets which are involved with the pain signaling pathways remain unknown, and further studies are warranted since chronic pain can cause severe depression and emotional distress, potentially leading to suicide.

Notably, as β-PGG possesses anti-diabetic activity, we can investigate ways to alleviate the acute or chronic pain associated with diabetes if we use dual effects (analgesic and anti-diabetic) of this excellent food ingredient/biomaterial. Our study has several limitations, including the fact that we only investigated the analgesic effects of β-PGG in inflammatory pain and not in other various types of pain such as nociceptive and neuropathic pain. Future studies of the pain-related biomarkers expressed by β-PGG, if any, in an animal model of pain and other analgesic models are warranted, and should seek to determine the clinical viability of β-PGG as a curative or preventive therapy in the management of acute and chronic pain.

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