



Short communication

Chondroitin sulfate attenuates formalin-induced persistent tactile allodynia



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ABSTRACT

We examined the effect of chondroitin sulfate (CS), a compound used in the treatment of osteoarthritis and joint pain, on the formalin-induced tactile allodynia in mice. A repeated oral administration of CS (300 mg/kg, b.i.d.) significantly ameliorated the formalin-induced tactile allodynia from day 10 after formalin injection. On day 14, the phosphorylation of spinal p38 MAPK and subsequent increase in c-Fos-immunoreactive dorsal lumbar neurons were attenuated by the repeated administration of CS. These findings suggest that CS attenuates formalin-induced tactile allodynia through the inhibition of p38 MAPK phosphorylation and subsequent up-regulation of c-Fos expression in the dorsal lumbar spinal cord.

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Chondroitin sulfate (CS) is a glycosaminoglycan with polysaccharide chains composed of an alternate sequence of D-glucuronic acid and D-N-acetyl galactosamine linked by $\beta(1 \rightarrow 3)$ and $\beta(1 \rightarrow 4)$ bonds, and is widely distributed in various connective tissues such as cartilage, skin, corpus vitreum and blood vessels. CS contributes to the viscoelastic property of the cartilage, and a reduced level of CS in the cartilage has been reported to be a risk factor for arthritic diseases in elderly people (1). CS is a compound categorized as a symptomatic slow-acting drug for the treatment of osteoarthritis (SYSADOA) because of its anti-inflammatory and chondroprotective properties (2). CS is also popular in North America as a dietary supplement intended to alleviate joint pain (3). In Japan, CS is available as an over-the-counter drug used against knee joint pain. Yet, the pain relief properties and mechanism of CS remain unclear, though it has been suggested that CS may play a role in pain relief through B₂ bradykinin receptor desensitization (4).

Thus, in the present study, we examined the effect of CS through oral administration on the formalin-induced persistent tactile allodynia in mice.

Male ddY mice weighing 22–24 g were used throughout the experiments. Mice were housed in cages with free access to food and water under conditions of constant temperature (22 ± 2 °C) and humidity ($55 \pm 5\%$), on a 12 h light–dark cycle (lights on: 7:00 to 19:00). Group of 12 mice for behavioral experiments, 7 mice for Western blotting, and 6–13 mice for immunohistochemical experiments were used in single experiments. All experiments were performed following the approval from the Ethics Committee of Animal Experiment at Tohoku Medical and Pharmaceutical University.

Formalin (Wako Pure Chemical Industries, Osaka, Japan) was prepared as a 2% solution (0.74% formaldehyde in saline) then 20 μ l were injected in the right hindpaw under the skin of the plantar surface using a 26-gauge microsyringe. CS (sodium chondroitin sulfate from swine cartilage supplied from Zeria Pharmaceutical Co., Tokyo, Japan) was dissolved in distilled water and orally administered twice daily starting 24 h after the formalin injection.

Tactile allodynia was determined by assessing paw withdrawal using the von Frey filament test. Mice were placed on a mesh floor inside a clear plastic cubicle for an acclimatization period of at least 30 min before the test. After adaptation to the testing environment, the von Frey filament was pressed perpendicularly against the plantar surface of the right hindpaw from beneath the mesh floor and held for 3 s with the filament slightly buckled. A positive response was noted if the paw was sharply withdrawn. Whenever

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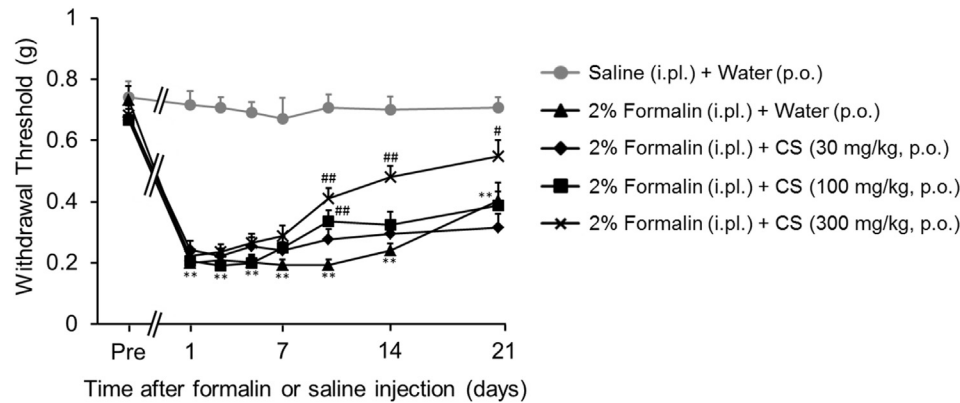


Fig. 1. Effect of CS on formalin-induced tactile allodynia in mice. The threshold for paw withdrawal to von Frey filaments was monitored during 21 days after the injection of formalin or saline. CS or water was orally administered twice daily starting 24 h after the formalin or saline injection. The threshold was measured before the first daily administration of CS or water. Values represent the means \pm S.E.M. for groups of 12 mice. Two-way ANOVA: group ($F_{4,55} = 98.11$, $p < 0.01$), time ($F_{7,385} = 73.40$, $p < 0.01$), group \times time ($F_{28,385} = 5.42$, $p < 0.01$). One-way ANOVA: pre, $F_{4,55} = 0.68$, $p = 0.61$; 1 day, $F_{4,55} = 54.81$, $p < 0.01$; 3 days, $F_{4,55} = 76.65$, $p < 0.01$; 5 days, $F_{4,55} = 52.44$, $p < 0.01$; 7 days, $F_{4,55} = 23.86$, $p < 0.01$; 10 days, $F_{4,55} = 33.79$, $p < 0.01$; 14 days, $F_{4,55} = 23.75$, $p < 0.01$; 21 days, $F_{4,55} = 10.91$, $p < 0.01$. ** $p < 0.01$ compared with the saline-water group and ## $p < 0.01$, # $p < 0.05$ compared with the formalin-water group.

a positive response to a stimulus occurred, the next smaller von Frey hair was applied whereas the pressure was increased one level in the absence of a withdrawal response. Ambulation was considered an ambiguous response, and in such cases the stimulus was repeated.

Confocal microscopy observation and Western blotting were carried out as previously described (5). For immunofluorescence double labeling, 40 μ m-thick slices were incubated with mouse anti-neuronal nuclei (NeuN) antibody (Millipore Co., MA, USA) and rabbit anti-c-Fos antibody (Cell Signaling Technology, MA, USA) overnight at 4 $^{\circ}$ C. These primary antibodies were diluted 1:200 and 1:100, respectively, with PBS containing 0.3% Triton-X and 1% normal goat serum (NGS; Invitrogen, CA, USA). Then, Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes, OR, USA) and Alexa Fluor 647-conjugated Fab fragment goat anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, PA, USA) (diluted 1:200 with PBS containing 0.3% Triton-X and 1% NGS) were allowed to react in the dark at room temperature for 2 h. Fluorescence was visualized on a D-Eclipse C1 microscope (Nikon, Tokyo, Japan). For Western blotting, we used rabbit antibodies against p38 MAPK (Cell Signaling Technology) or phospho-p38 MAPK (Cell Signaling Technology), followed by HRP-conjugated anti-rabbit IgG antibody (Cell Signaling Technology) as primary and secondary antibodies, respectively. The density of the corresponding bands was analyzed using Image-J 1.43u (National Institute of Health).

Data are expressed as means \pm SEM. Significant differences were analyzed by a one-way or two-way analysis of variance (ANOVA), followed by Fisher's PLSD test for multiple-comparisons. Student's t-test was used for comparisons between two groups. In all comparisons, $p < 0.05$ was considered statistically significant.

Clinical trials have reported a beneficial effect of CS, either alone (6) or in combination with glucosamine (7) on pain in patient with osteoarthritis. Although it is not a rapidly acting agent such as nonsteroidal anti-inflammatory drugs (NSAIDs), as a SYSADOA, CS contributes to reducing NSAIDs consumption in osteoarthritis patients. In this study, the repeated administration of CS (300 mg/kg, b.i.d.) significantly prevented the decrease in the ipsilateral paw withdrawal threshold from day 10 after formalin injection (Fig. 1). Thus, it took 9 days for CS to exhibit its anti-allodynia properties. One reason that may explain the delayed action is the fact that CS is poorly absorbed through the gastrointestinal tract because of its high molecular weight (5–50 kDa) and highly negative surface charge (8).

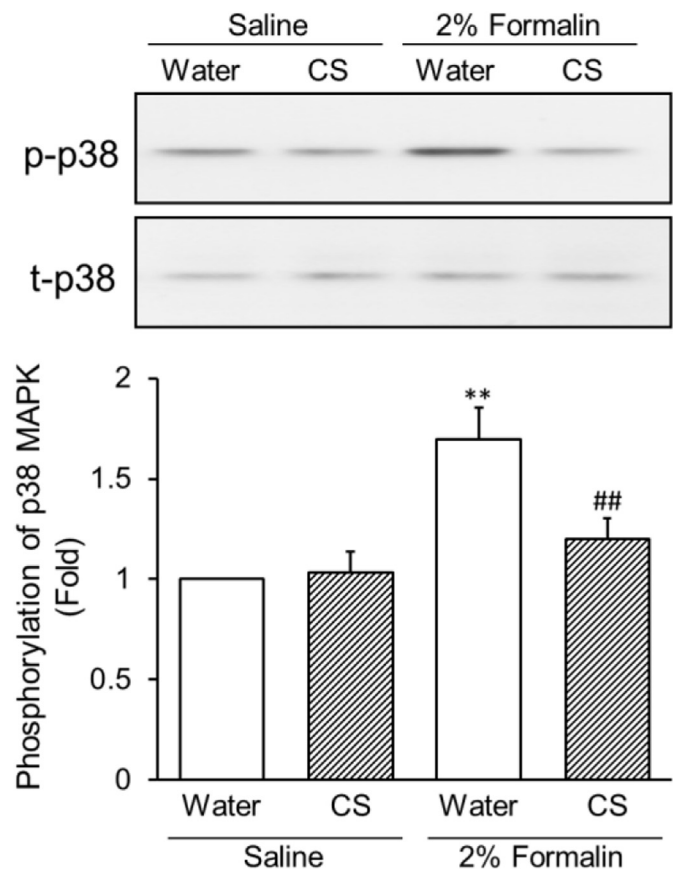


Fig. 2. Change in phospho-p38 MAPK levels in the lumbar spinal cord induced by formalin and the effect of CS treatment. CS (300 mg/kg) or water was orally administered twice daily starting 24 h after the formalin or saline injection, and spinal cord samples were collected on day 14. Top: representative Western blot showing phospho- and total-p38 MAPK. Bottom: relative quantification of phospho-p38 MAPK to total-p38 MAPK set as 1.0 in the saline/water group. Each value represents the means \pm S.E.M. of 7 mice in each group. Two-way ANOVA: group ($F_{1,12} = 12.23$, $p < 0.01$), treatment ($F_{1,12} = 6.75$, $p < 0.01$), group \times treatment ($F_{1,12} = 8.93$, $p < 0.05$). One-way ANOVA: $F_{3,24} = 8.94$, $p < 0.01$. ** $p < 0.01$ compared with the saline/water group, ## $p < 0.01$ compared with the formalin/water group.

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