

Wogonoside alleviates inflammation induced by traumatic spinal cord injury by suppressing NF- κ B and NLRP3 inflammasome activation

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Abstract. Wogonoside possesses anti-oxidative, anti-inflammatory, anti-allergy and anti-tumor properties. The aim of the present study was to evaluate whether wogonoside alleviates spinal cord injury (SCI)-induced inflammation via nuclear factor (NF)- κ B and nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) inflammasome activation. Sprague-Dawley rats were positioned in the jaws of a calibrated aneurysm clip with a closing pressure of 55 g. The jaws were placed on the dorsal and ventral surfaces of the spinal cord and left in place for 1 min. SCI rats were treated with 12, 25 and 50 mg/kg wogonoside. Following this, the locomotor function was assessed using the Basso Beattie Bresnahan scale. The water content of the spinal cord was measured, tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β and IL-6 levels were assessed and western blot analysis was performed to evaluate the expressions of NF- κ B and NLRP3. Wogonoside was demonstrated to significantly ameliorate the SCI-induced reduction in Basso Beattie Bresnahan score ($P<0.01$) and significantly reduce the water content of the spinal cord in rats with SCI-induced inflammation ($P<0.01$). Results also indicated that treatment with wogonoside significantly reduced the levels of IL-1 β , TNF- α and IL-6 in rats with SCI-induced inflammation ($P<0.01$), potentially via the phosphorylation of NF- κ B inhibitor α . Furthermore, treatment with wogonoside

inhibited the expressions of toll-like receptor 4, NLRP3 and caspase-1 protein in SCI model rats ($P<0.01$). In conclusion, the results of the present study suggest that wogonoside alleviates SCI-induced inflammation by suppressing NF- κ B and NLRP3 inflammasome activation.

Introduction

Spinal cord injury (SCI) is a common type of trauma and cause of disability in China (1). The underlying mechanisms that cause secondary neuron injury following primary spinal cord injury are complex (2). It has been reported that experimental pharmacological agents are unable to act on multiple injury mechanisms at the same time, and inhibition of secondary injuries while ignoring their favorable aspects to the body, such as pain reduction and inflammation inhibition, may cause detrimental side-effects (3). Consequently, there is currently a lack of effective and safe clinical treatments for SCI.

SCI typically induces severe pathological nerve damage with limited functional recovery (4). The initial mechanical injury may destroy neurons and neuroglia, while even greater destructive effects are caused by delayed secondary pathological damage (5). Secondary injury may manifest as neuron and/or glial cell apoptosis, increased permeability of the blood-spinal cord barrier and a complicated neuro-inflammatory response, which may endure for months or years after injury (6).

Wogonoside (Fig. 1) has been reported to possess extensive pharmacological activities. In addition to anti-inflammatory, anti-allergy, anti-viral and antipyretic properties, recent studies have demonstrated that wogonoside possesses anti-oxidative and anti-tumor effects, along with cardiovascular protective effects (7,8). Wogonoside is derived from *Scutellaria baicalensis*, and has exhibited efficacy as an anti-thrombotic, antihypertensive and antitumor therapeutic, and in the treatment of coronary heart disease (9). The aim of the present study was to determine whether wogonoside alleviated inflammation induced by activation of nuclear factor (NF)- κ B and the nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) inflammasome in an SCI rat model.

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Materials and methods

Animals and surgery. Adult male Sprague-Dawley (SD; age, 9-11 weeks; $n=50$) rats weighing 230-250 g were obtained from the Animal Resource Center of the Yantai Affiliated Hospital of Binzhou Medical University (Yantai, China) for use in the present study. Rats were housed at an ambient temperature of $22\pm1^{\circ}\text{C}$ under a 12-h light/dark cycle, and 50-70% relative humidity. Pellet rat chow and tap water were available *ad libitum*. A rat model of SCI was induced as described previously (10). Briefly, rats were anesthetized intravenous injection (i.v.) with 35 mg/kg pentobarbital (all from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany.) and underwent a laminectomy, during which the T8 and T9 vertebral peduncles were removed. The jaws of a calibrated aneurysm clip with a closing pressure of 55 g were placed on the dorsal and ventral surfaces of the spinal cord and left in place for 1 min to induce SCI. All experimental procedures were approved by the Ethics Committee of the Yantai Affiliated Hospital of Binzhou Medical University.

Experimental groups and procedures. All SD rats were underwent SCI surgery and were randomly divided into four groups: i) Control group (control; $n=10$), which underwent sham surgery (the operational area was exposed but no trauma was induced) and were administered with physiological saline (1 ml/kg, i.p.); ii) SCI model group (SCI model; $n=10$), which underwent SCI surgery and were administered with physiological saline (1 ml/kg, i.p.); iii) 12 mg/kg wogonoside group ($n=10$), administered with 12 mg/kg wogonoside (i.p.) for 10 days; iv) 25 mg/kg wogonoside group ($n=10$), administered with 25 mg/kg wogonoside (i.p.) for 10 days; and v) 50 mg/kg wogonoside group ($n=10$), administered with 50 mg/kg wogonoside for 10 days (i.p.; Sigma-Aldrich; Merck KGaA).

Evaluation of locomotor function. Locomotor function was assessed by two researchers, using the Basso Beattie Bresnahan (BBB) locomotor rating scale, as described previously (11). A score of 0 indicates complete paralysis and a score of 21 indicates normal locomotion.

Evaluation of spinal cord contusion volume after SCI. Rats were sacrificed using decapitation following treatment with wogonoside (day 10) under anesthesia (35 mg/kg pentobarbital). Spinal cord contusions were assessed by calculating the water content of the spinal cord. Injured spinal cords were dried at -80°C for 48 h and the volume of spinal cord contusions was calculated using the following formula: Water content of spinal cord (%) = (wet weight - dry weight)/wet weight \times 100.

Evaluation of serum oxidative stress and inflammation. Blood (500 μl) was harvested and serum was collected after centrifugation at $2,000 \times g$ for 10 min at 4°C . Serum tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6 levels were analyzed using respective commercial immunoassay kits (cat. no. RTA00, RLB00 and R6000B, respectively; R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's protocol.

Western blot analysis. Following sacrifice, spinal cord tissues were harvested and homogenized in RIPA lysis buffer

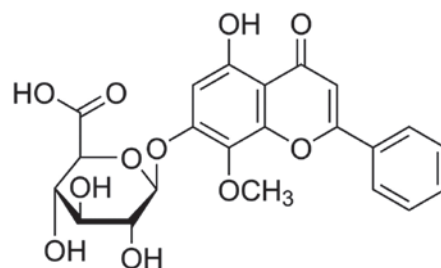


Figure 1. Chemical structure of wogonoside.

(Beyotime Institute of Biotechnology, Haimen China). The homogenate was centrifuged at $12,000 \times g$ for 20 min at 4°C , and quantification of protein in the supernatant was performed using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). A total of 50 μg protein was loaded per lane, separated by 10% SDS-PAGE and electrotransferred onto nitrocellulose membranes. Membranes were blocked with 5% skim milk powder in TBST for 1 h at 37°C and incubated with anti-TLR4 (sc-10741, 1:200; Santa Cruz Biotechnology, Inc.), anti-NF- κB p65 (cat. no. sc-109; 1:2,000; Santa Cruz Biotechnology, Inc.), anti-phosphorylated (p)-inhibitor of NF- κB (cat. no. 2859; I κB ; 1:200; Cell Signaling Technology, Inc.), anti-NLRP3 (cat. no. sc-66846; 1:300; Santa Cruz Biotechnology, Inc.), anti-caspase-1 (cat. no. sc-514; 1:300; Santa Cruz Biotechnology, Inc.) and anti- β -actin (cat. no. sc-7210; 1:500; Sangon Biotech Co., Ltd., Shanghai, China) at 4°C overnight. Membranes were subsequently incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (cat. no. 14708; 1:5,000; Cell Signaling Technology, Inc.) at 37°C for 1 h. The optical densities of immunopositive bands were visualized with BeyoECL Star (Beyotime Institute of Biotechnology) and determined using GeneTools software, version 4.1 (SynGene, Ltd., Cambridge, UK).

Statistical analysis. All data are presented as the mean \pm standard deviation, assessed using SPSS, version 17.0 (SPSS, Inc., Chicago, IL, USA). Wilcoxon signed-rank tests were performed to evaluate differences in between the injured and control groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Wogonoside improves BBB score in rats with SCI-induced inflammation. BBB scores were calculated to analyze the effect of wogonoside on SCI-induced inflammation in rats. The BBB scores of rats in the SCI model group were significantly reduced compared with the control group ($P<0.01$; Fig. 2). Treatment with 12.5 mg/kg wogonoside for 10 days significantly alleviated the SCI-induced reduction in BBB score ($P<0.01$), as did treatment with 25 or 50 mg/kg wogonoside for 5 days or more ($P<0.01$; Fig. 2).

Wogonoside reduces the volume of spinal cord contusions in rats with SCI-induced inflammation. The volume of spinal cord contusions in rats with SCI-induced inflammation was assessed, and was observed to be significantly increased when compared with controls ($P<0.01$; Fig. 3). In turn, treatment with

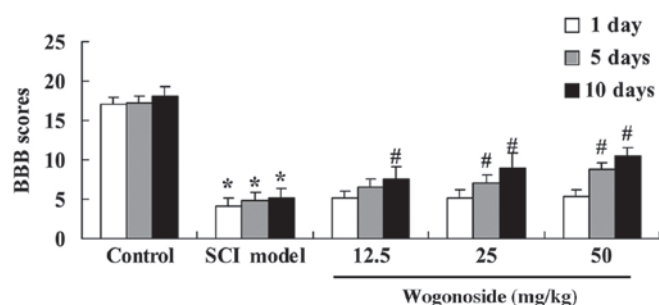


Figure 2. Wogonoside improves BBB score in SCI model rats. *P<0.01 vs. control group; #P<0.01 vs. SCI model group. BBB, Basso Beattie Bresnahan; SCI, spinal cord injury.

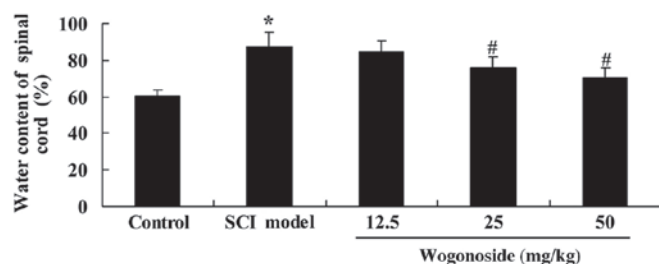


Figure 3. Wogonoside reduces the volume of SCI-induced contusions in rats. *P<0.01 vs. control group; #P<0.01 vs. SCI model group. SCI, spinal cord injury.

25 or 50 mg/kg wogonoside significantly reduced the increased volume of spinal cord contusions in SCI rats (P<0.01; Fig. 3).

Wogonoside suppresses SCI-induced upregulation of IL-1 β , TNF- α and IL-6 in rats. To further elucidate the anti-inflammatory effects of wogonoside, ELISA was performed to measure the levels of IL-1 β , TNF- α and IL-6 in SCI model rats. IL-1 β , TNF- α and IL-6 were significantly upregulated in the SCI model group when compared with the control group (P<0.01; Fig. 4). No significant differences were observed in the levels of inflammatory cytokines between the 12.5 mg/kg wogonoside and model groups; however, treatment with 25 or 50 mg/kg wogonoside significantly reduced the levels of IL-1 β , TNF- α and IL-6 compared with the model group (P<0.01; Fig. 4).

Wogonoside alleviates NF- κ B and NLRP3 overexpression, and increases the activation of I κ B expression in SCI model rats. Western blotting was used to evaluate the effect of wogonoside on NF- κ B, phosphorylated (p)-inhibitor of NF- κ B (p-I κ B) and NLRP3 expression (Fig. 5A). A significant increase in the protein expression of NF- κ B and NLRP3, and inhibition of p-I κ B expression were observed in the SCI model group when compared with the control group (P<0.01; Fig. 5B-D). Treatment with 12.5 mg/kg wogonoside had no significant effect, whereas 25 or 50 mg/kg wogonoside significantly reduced NF- κ B and NLRP3 expression, and increased p-I κ B expression compared with the SCI model group (P<0.01; Fig. 5B-D).

Wogonoside ameliorates caspase-1 and TLR4 overexpression in SCI model rats. The effect of wogonoside on caspase-1 expression in SCI rats was assessed using western blotting (Fig. 6A). SCI was demonstrated to significantly increase

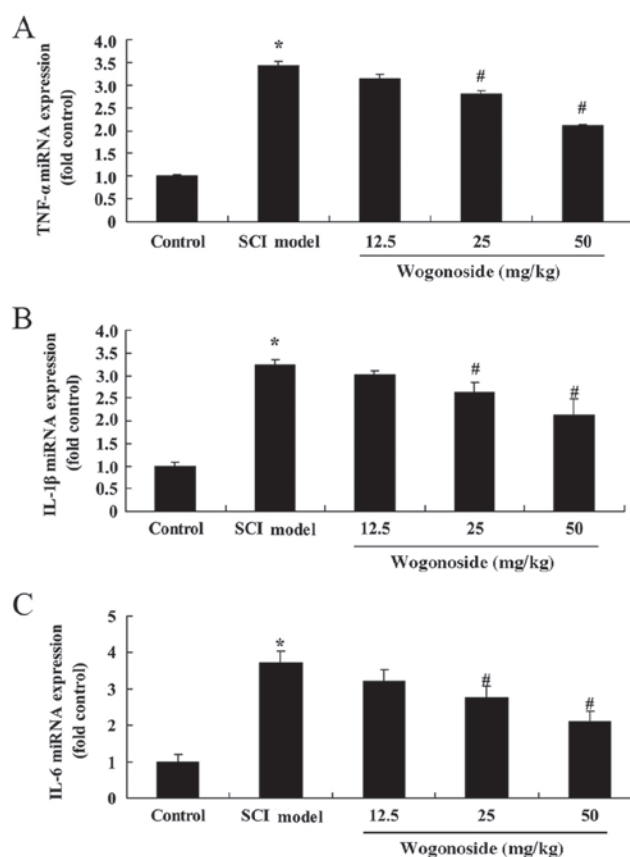


Figure 4. Wogonoside suppresses SCI-induced upregulation of (A) TNF- α , (B) IL-1 β and (C) IL-6 in rats. *P<0.01 vs. control group; #P<0.01 vs. SCI model group. SCI, spinal cord injury; IL, interleukin; TNF, tumor necrosis factor.

the protein expression of caspase-1 and TLR4, relative to the control group (P<0.01; Fig. 6B and C). Treatment with 12.5 mg/kg wogonoside induced no significant difference in caspase-1 or TLR4 expression, whereas 25 or 50 mg/kg wogonoside significantly reduced the levels of caspase-1 in SCI rats (P<0.01; Fig. 6B and C).

Discussion

SCI is a traumatic central nervous system injury, and frequently leads to varying degrees of anesthesia and loss of motor function in the limb below the plane of injury, making it a leading cause of disability in the field of orthopedics (12). Increasing urbanization has led to an increase in injuries caused by traffic accidents, falling accidents and professional accidents for engineers and construction workers (13). Over the last 10 years, the morbidity of SCI in China and other countries has increased (14). SCI-induced functional paralysis causes physiological and psychological trauma to patients, and is a cause of medical and economic stress for families and society (14). In the present study, treatment with wogonoside was demonstrated to significantly increase the BBB score and decrease the volume of spinal cord contusions in rats with SCI-induced inflammation.

Cytokines may be classified into two major types; proinflammatory factors and anti-inflammatory factors. Proinflammatory factors are expressed to a high-level in conditions of oxidative stress, which may further increase the level of oxidative stress (15).

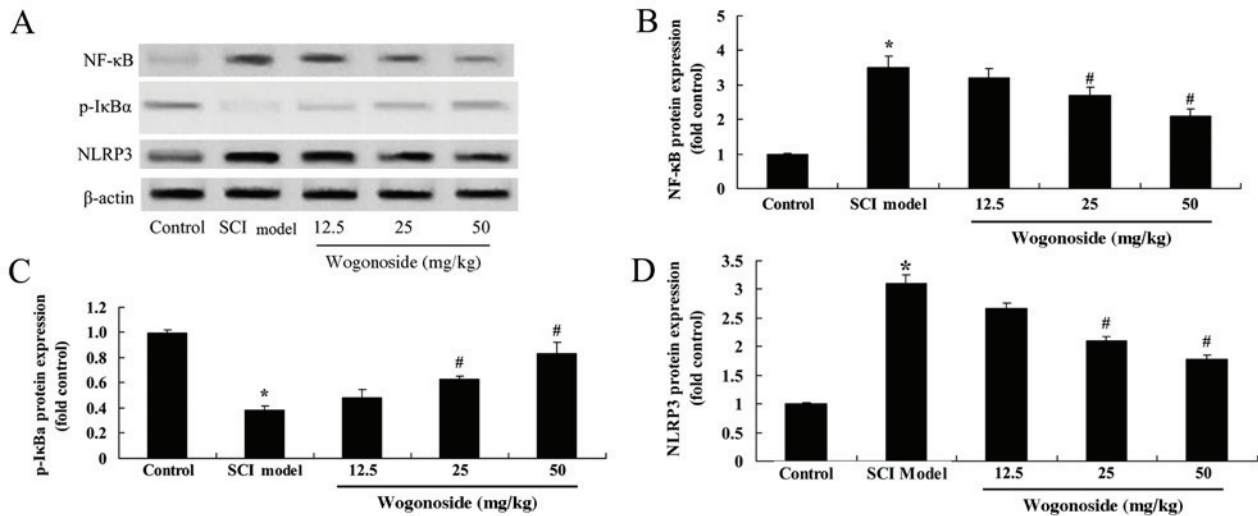


Figure 5. Wogonoside suppresses SCI-induced upregulation of NF- κ B, p-I κ B and NLRP3 in rats. (A) Western blot analysis with β -actin as a control. Statistical analysis of (B) NF- κ B, (C) p-I κ B and (D) NLRP3 protein expression in an SCI rat model. * $P < 0.01$ vs. control group; # $P < 0.01$ vs. SCI model group. SCI, spinal cord injury; NF, nuclear factor; NF- κ B, nuclear factor- κ B; p-I κ B; NLRP3, nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3.

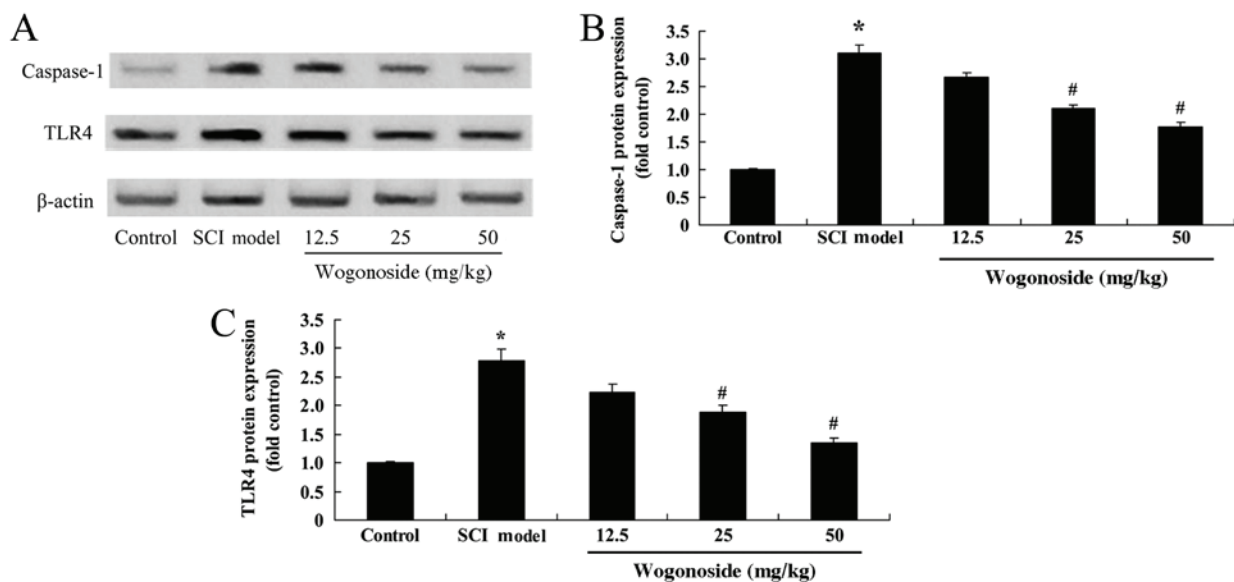


Figure 6. Wogonoside suppresses SCI-induced overexpression of caspase-1 and TLR4 in rats. (A) Western blotting and (B and C) statistical analysis of caspase-1 and TLR4 protein expression in an SCI rat model. * $P < 0.01$ vs. control group; # $P < 0.01$ vs. SCI model group. SCI, spinal cord injury.

During oxidative stress, the upregulated proinflammatory factors include TNF- α , IL-1 β , IL-2, IL-6 and IL-12, and NF- κ B is also activated (16,17). In the present study, treatment with 25 or 50 mg/kg of wogonoside significantly inhibited the upregulation in IL-1 β , TNF- α and IL-6 in SCI model rats. Yang *et al* (18) suggested that wogonoside induces anti-inflammatory effects in RAW264.7 cells, which indicated that wogonoside possessed anti-inflammation effect in SCI.

Interactions between lipopolysaccharides (LPS) and TLR4 induce the activation of transcription factors, including NF- κ B and activator protein-1, which ultimately induces the expression of proinflammatory and anti-inflammatory factors (19). NF- κ B is a multi-functional nuclear transcription factor, and is associated with the LPS-induced inflammatory response (19). In the cytoplasm, I κ B binds with inactive NF- κ B, which covers the

nuclear localization sequence of NF- κ B and thus prevents the activation and nuclear translocation of NF- κ B (20). I κ B degradation is therefore required for the activation of NF- κ B. I κ B is regulated by I κ B kinase, which catalyzes phosphorylation of the 42' serine residue in I κ B as a signal for degradation, which induces rapid degradation of I κ B (typically within several min) and exposes the nuclear localization sequence of NF- κ B (21). The nuclear localization sequence of NF- κ B is disclosed and NF- κ B is activated and translocated to nucleus (22). Once there, NF- κ B binds with the κ B locus in the NF- κ B reactive gene to regulate gene transcription and downstream cellular processes, including cell growth, differentiation, adhesion, apoptosis and the inflammatory response (22). Excessive phosphorylation of I κ B results in a loss of NF- κ B inhibition (23). In the present study, it was demonstrated that treatment with 25 or

50 mg/kg of wogonoside significantly inhibited inflammation through the suppression of NF- κ B/I κ B in an SCI rat model. Similarly, Zhang *et al* (7) reported that wogonoside ameliorates LPS-induced acute lung injury in mice through the suppression of TLR4-mediated NF- κ B signaling.

The NLRP3 inflammasome promotes the secretion of IL-1 β , IL-18 and IL-33, and the production of these factors is critical in the control of pathological infection (24). However, excessive cytokine production is harmful to the body (24), and therefore activation of the NLRP3 inflammasome must be strictly regulated to maintain hemostasis (25). Similarly, Sun *et al* (26) demonstrated that wogonoside protects against dextran sulfate sodium-induced colitis by suppressing NF- κ B and NLRP3 inflammasome activation in mice.

In conclusion, the present study demonstrated that wogonoside alleviated the reduced BBB scores and increased spinal cord contusion volume of SCI model rats. Furthermore, it was observed that wogonoside ameliorated inflammation via the NF- κ B/I κ B and NLRP3/caspase-1/TLR4 pathways. The present study was limited as it only assessed the *in vivo* aspect, therefore *in vitro* or clinical models should be assessed in future studies. The results of the present study suggest that wogonoside is a potential novel treatment for SCI trauma, and may have clinical applications in ameliorating the associated inflammation.

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