Formulation and Analgesic Effect of Sodium Hyaluronate and Magnesium Sulfate Combination in Rats Following Intra-articular Injection

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The objective of this study was to formulate hyaluronate (HA) combined with magnesium sulfate (MS) for intra-articular (IA) injection, to provide rapid and profound analgesia in the treatment of osteoarthritis (OA). The injectable HA/MS combination was optimized with respect to the kinds and amounts of buffering agent (10 mM histidine, pH 7.0) and stabilizer (10 mM sodium citrate), by evaluating changes in the appearance, pH, and intrinsic viscosity at 60°C. Increasing concentrations of sodium citrate significantly inhibited the decline in intrinsic viscosity, denoting the depolymerization of HA, preserving over 91% of initial value. In an efficacy study in iodoacetate-induced OA rats, treatment with the HA/MS combination (1/2% w/v) significantly reduced joint swelling and pain, compared to the vehicle- and HA-treated groups ($p < 0.05$) at 7 days postdosing. Thus, IA administration of the combination of HA, a slow-acting symptom-modifying agent, with the inorganic analgesic is a potentially effective therapeutic approach for rapid pain relief in the OA knee.

Keywords: Sodium hyaluronate, Magnesium sulfate, Intrinsic viscosity, Intra-articular injection, Analgesic effect, Anti-inflammatory effect

Introduction

Intra-articular (IA) administration of hyaluronic acid (HA) is one of the currently available pharmacological modalities to alleviate pain and regain physical function in patients with osteoarthritis (OA), along with oral analgesics or nonsteroidal anti-inflammatory drugs (NSAIDs) and IA corticosteroids.[1,2] HA, a natural linear anionic polysaccharide, is the principle constituent of synovial fluid and the extracellular matrix of connective tissues. In osteoarthritic knees, the elastoviscosity of HA is remarkably lower than that in healthy knees, due to oxidative depolymerization of the polysaccharide.[3,4] Thus, viscosupplementation has been recommended as a relatively safe treatment option, reducing pain and inflammation in the synovium.[5] However, HA is a slow-acting antinociceptive agent and thus, commonly prescribed with oral NSAIDs or IA corticosteroids to relieve pain more rapidly.[2] IA coadministration of HA with steroids has an early impact on pain relief in the knee, but its frequent use requires caution because of the increased incidence of articular infections, cartilage breakdown, and loss of elasticity of the articular cartilage.[6,7]

IA administration of magnesium ion (Mg^{2+}), the highly abundant cation in the body, has been shown to exert antinociceptive effects in animals and humans with chronic pain.[8–12] Several studies have shown that IA injection of magnesium sulfate (MS) is effective for postoperative analgesia, as compared to saline in arthroscopic knee.[8,9] In addition, the combination of MS with bupivacaine produces better analgesic effects in postoperative pain-management, as compared to the local anesthetic alone.[13] The action-mechanism for the analgesic effect of Mg^{2+} is unclear; however, blockage of the *N*-methyl-*D*-aspartate (NMDA) receptor, which is highly expressed in the peripheral terminal of articular fibers within the joint, impedes the initiation of central sensitization to nociceptive stimuli.[14] Moreover, an *in vivo* toxicity study in normal rats indicated no significant difference in tissue irritation and cartilage degeneration between normal saline-treated and high-concentration MS (10% w/v)-treated groups,[15] suggestive of the absence of serious adverse effects from IA administration of MS.

Therefore, IA injection of HA combined with MS could be a therapeutic option for patients with OA, with the more rapid pain relief. However, preparation of a stable liquid formulation is a challenge, because HA is extremely susceptible to oxidative degradation and Mg^{2+} forms insoluble precipitates with inorganic salts. Herein, the objective of this study was to formulate a stable injectable combination to provide more rapid pain relief than HA alone in OA patients. A liquid formulation of HA/MS was prepared using different kinds of buffering agents (phosphate, sulfate, acetate, carbonate, and histidine) and stabilizers (mannitol and sodium citrate) and their physicochemical stabilities were evaluated based on the appearance, pH, and intrinsic viscosity ([η]). Moreover, the analgesic and anti-

inflammatory effects of IA HA combined with MS, vs. each drug alone, were assessed in a rat model of OA.

Experimental

Materials. HA powder with molecular weight ranging from 1500 to 2500 kDa was acquired from Humedix Co., Ltd (Sungnam, Korea). MS·7H_2O, monosodium phosphate, sodium acetate, bicarbonate, L-histidine, mannitol and sodium citrate, and monosodium iodoacetate (MIA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of reagent grade and were used with no further pretreatment.

Preparation of HA/MS Formulations. The exact compositions of each formula are listed in Table 1. The formulations were prepared on a large scale (50-fold) for further experiments. First, different kinds of buffering agents (phosphate, sulfate, acetate, carbonate, and histidine), stabilizers (mannitol and sodium citrate), and sodium chloride were dissolved in 50 mL of distilled water at room temperature. Approximately 2.04 g of MS·7H_2O powder (1.0 g as MS) was added to the vehicle solution and stirred for 30 min. The transparent solution was sterilized using a syringe filter with 0.22 μm pore-size. The pH of the aqueous solution was adjusted to 7.0 by adding 0.1 N sodium hydroxide or hydrochloric acid dropwise. Subsequently, HA powder (500 mg) was slowly added to the solution and dispersed using an overhead stirrer equipped with a paddle-shaped propeller (Eurostar 20, Ika Works Inc., Wilmington, NC, USA), until a homogeneous viscous gel was formed (about 14 h) at room temperature. The prepared samples were stored in a refrigerator until tested.

Physicochemical Characterization of HA/MS Combinations. Each formulation was evaluated for appearance, pH, osmolality, and [η]. The appearance (transparency or homogeneity) of the sample contained in a 45° angle. Acidity and osmolality were measured using a pH meter (S220, Mettler-Toledo LLC, Columbus, OH, USA) and an osmometer (Micro-osmometer 210, Fiske Associates, Norwood, MA, USA), respectively. The [η] values of the samples were determined based on the method described in the European Pharmacopoeia.[16] Briefly, samples were serially diluted with normal saline to the concentration of 0.04, 0.09, 0.14, and 0.19 g/mL, respectively, at 4°C. Relative viscosity [η_r] of each dilution was calculated by comparing the rate of flow in the viscometer with that of normal saline. The specifications of the viscometer were as follows: viscometer constant, about 0.005 mm^2/s^2; kinematic viscosity range, 1–5 mm^2/s^2; internal diameter of tube, 0.53 mm; volume of bulb, 5.6 mL; and internal diameter of tube, 2.8–3.2 mm. [η] was then estimated by linear least-squares regression analysis using the Martin equation:

$$\log\left(\frac{\eta_r - 1}{c}\right) = \log[\eta] + k[\eta]c$$

where, η_r is the relative viscosity of each diluent, c is the concentration of HA in each diluent (kg/m^3), [η] is the intrinsic viscosity of formulation, and k is the interaction constant. A representative graph is shown in Figure 1. The decimal antilogarithm of the y-axis intercept is the [η] value expressed in m^3/kg.

Stability Test. Stability tests were carried out at two different conditions: stress (60°C) and accelerated condition (25°C/60% RH). Sample were transferred to dark glass vials after preparation and stored under stress condition for 5 days and accelerated condition for 12 weeks, respectively. At predetermined time, samples were evaluated for appearance, pH, osmolality, and [η].

Animals and Induction of OA in Rats. Male Sprague–Dawley rats (200–250 g; 6–7 weeks) were provided from Samtako (Kyungki-do, Korea) and kept under specific pathogen-free conditions. Chow and water were available *ad libitum*. The animal experiment was carried out in accordance with the NIH "Principles of Laboratory Animal Care" guidelines; and the protocols were approved by the Dankook University Institutional Animal Care and Use Committee (Chungnam, Korea). After shaving and

Table 1. Compositions of the injectable HA/MS formulations.

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Compositions										
HA (mg)	10	10	10	10	10	10	10	10	10	10
MS (mg)	20	20	20	20	20	20	20	20	20	20
Sodium dihydrogen phosphate (mM)	—	10	—	—	—	—	—	—	—	—
Sodium bisulfate (mM)	—	—	10	—	—	—	—	—	—	—
Sodium acetate (mM)	—	—	—	10	—	—	—	—	—	—
Sodium carbonate (mM)	—	—	—	—	10	—	—	—	—	—
Histidine (mM)	—	—	—	—	—	10	10	10	10	10
Mannitol (mM)	—	—	—	—	—	—	10	—	—	—
Sodium citrate (mM)	—	—	—	—	—	—	—	5	10	20
Distilled water (mL)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Note: The pH of the solution was adjusted to 7.0.

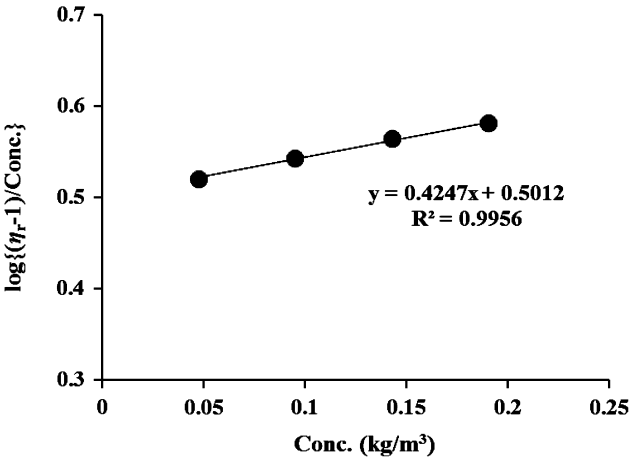

Figure 1. Representative graph of the ratio of specific viscosity ($\eta_r - 1$) to concentration against the concentration of the serial dilutions to determine the [η] of HA in the formulations. The decimal antilogarithm of the intercept denotes [η] value expressed in m^3/kg, according to the Martin equation.

sterilizing, under anesthesia with 10% chloral hydrate at a dose of 4 mL/kg, male SD rats were interarticularly administered with 3 mg MIA (30 μL in saline) into the right knee; whereas, normal saline was treated in the right knee of the sham group. In all groups, the left knee was untreated. A substantial swelling in the right knee was observed in rats for 3 days after MIA injection as described earlier.[17]

Drug Treatment. Three days after MIA injection (defined as day 3), the animals were divided into five groups by a stratified randomization scheme. Each group of eight rats received a single IA injection of each sample using an insulin syringe (31G) on day 3: (1) sham group treated with normal saline (50 μL); (2) MIA-treated group treated with vehicle; (3) treated with HA alone (1% w/v); (4) treated with MS alone (2% w/v); and (5) treated with HA/MS combination (1/2% w/v), respectively.

Joint Swelling Measurement. The severity of inflammation in the OA joint was estimated by evaluating the diameter of the hind limb of knee joint.[12] The diameters of the right and left knee joint were measured with digital electronic calipers (Mitutoyo, UK) at 3- and 7-days postdosing (defined as day 6 and day 10, respectively). The difference in diameter between the knees, representing asymmetry of knee diameters, was calculated by subtracting the width of the normal left knee from that of the OA-induced right knee.

Pain Assessment. OA was usually typified by unequal weight distribution between each hind paw.[18] Thus, a weight-bearing apparatus (Incapacitance tester; Linton Instrumentation, Norfolk, UK) was utilized to measure the difference in the weight distribution between the osteoarthritic right and the normal left hind limbs at 3- and 7-days postdosing. Animals were placed in a plexiglass booth with each hind paw on a separate force plate. Under unmoving position, the force delivered on each plate was recorded. A total three readings were taken for each rat and the average value was calculated. The study was carried out in a single-blinded condition, and % weight distribution of the right limb was estimated using the following formula: % weight of the right limb = 100 × (right limb weight/[left limb weight + right limb weight]).

Statistical Analysis. Statistical significance was analyzed using the Student's *t*-test; and $p < 0.05$ was considered as significant unless otherwise indicated.

Results and Discussion

Formulation of Injectable HA/MS Combinations. In formulating the HA/MS combination, the concentration of HA in solution was set to 1% w/v, which is effective in alleviating OA joint pain based on the results of clinical trials.[19,20] In addition, 0.7–5.0% w/v MS is known to reduce the intensity and/or duration of postoperative discomfort after knee surgery.[13,21] The median effective concentration of Mg^{2+} required to block NMDA receptors ranges between 400 and 600 μM.[22] Thus, the concentration of MS was targeted to 2% w/v, which is ≥270-fold higher than the median effective concentration, following IA injection. In order to lessen the irritation and/or perturbation in joint tissue, the osmolality and pH of all the formulations were adjusted to 290–340 mOsm/kg and 6.5–7.5, respectively.

Table 2. Physicochemical stability of the injectable HA/MS formulations with different buffering agents at 60°C.

	Initial		After 5 days at 60°C	
	Appearance	pH	Appearance	pH
F1	Transparent	7.0	Transparent	7.8
F2	Transparent	7.3	Precipitated	—[a]
F3	Transparent	7.1	Precipitated	—
F4	Transparent	7.2	Transparent	7.8
F5	Transparent	7.2	Transparent	7.7
F6	Transparent	7.2	Transparent	7.1

Note: Data on acidity are represented as mean values (n = 3). All standard deviations were less than 10% of the mean values.
[a] This indicates "not determined."

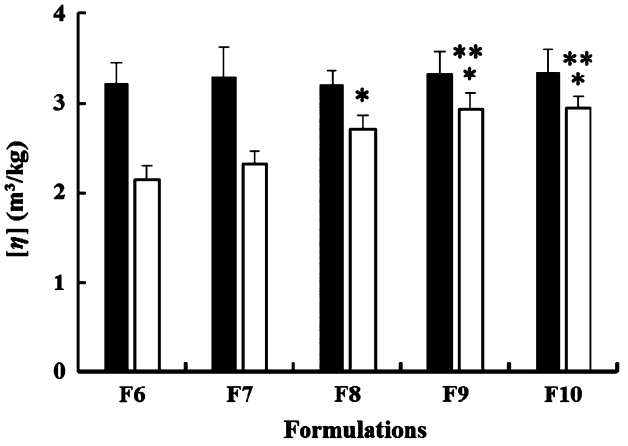

Figure 2. Effect of pharmaceutical excipients on the [η] of the injectable HA/MS formulations after 5 days under 60°C. Note: Data represent mean ± SD (n = 3) and statistical analysis was carried out using the Student's t-test; $^{*}p < 0.05$ vs. F6 and $^{**}p < 0.05$ vs. F7.

At first, the buffering agent in the injectable HA/MS solution was selected by evaluating its effect on the appearance and pH of the formulations during the storage period (Table 2). In the F1 and F2 formulas, insoluble white-colored precipitates were formed after 5-days storage at 60°C, as Mg^{2+} formed insoluble precipitates with phosphate and sulfate salts. The acetate buffer (pKa value of 4.8) and bicarbonate buffer (pKa values of 6.3 and 10.3) showed no effective buffering capacity against pH 7.0 medium. On the other hand, in the F5 formulation containing histidine as buffering agent, the pH change was <0.1 and no change in appearance was observed under harsh condition. Histidine has pKa values of 1.8, 6.0, and 9.17, and is known to exhibit excellent buffering capacity at neutral pH (5.5–7.4),[23] due to its protonated amine group as well as anionic carboxylic acids; hence, histidine was selected for further preparation of HA/MS injectable formulae.

HA and its derivatives are extremely susceptible to pH, oxygen, storage temperature, and other additives.[24–26] In acidic condition, hydrolysis reaction occurs on the glucuronic acid and the hemiacetal ring moieties in the polymer. Hydrolysis also occurs on the *N*-acetylglucosamine residue in basic solution, following first-order reaction kinetics.[24] Moreover, oxygen-derived free radicals promote the degradation process of HA, thereby decreasing the mean molecular weight of the anionic polymer.[27] The oxidative-reductive depolymerization reaction causes the random destruction of unit monosaccharides by oxygen-derived free radicals, followed by secondary hydrolytic cleavage of the glycosidic substituents. Thus, to protect the liable polysaccharide from hydrolysis and/or oxidative depolymerization, the pH of the formulations was adjusted to the neutral condition. In addition, two pharmaceutical excipients with free radical scavenging activities[28,29] were included in the formulations and the effect on [η] of the HA/MS solution was evaluated. The [η] value has been used as the standard method for indirectly estimating the average molecular weight (M_w) of the polymer based on the Mark–Houwink equation. Log [η] vs. log M_w shows a linear relationship for an unbranched/linear polymer and oxidative depolymerization of the polysaccharide shifts the [η] to lower values.[30,31]

As shown in Figure 2, the storage of HA/MS solution with no stabilizers (F6) at 60°C markedly decreased the molecular weight of the polysaccharide, with approximately 35% decrease in [η] value for 5 days. The addition of mannitol to the injectable combination (F7) facilitated the inhibition of oxidative degradation of HA due to its antioxidant activity, but a 27% decline in [η] value was observed. In a previous study, mannitol was reported to effectively protect the liable polysaccharide from oxygen free radical-mediated degradation.[32] However, in this study, the benefit of combining HA and mannitol was not significant, possibly due to the quantity of the antioxidant in the formulation. In the previous study, the HA solution contained 200 mM mannitol, whereas, the amount of the antioxidant used in our study was only 10 mM. In contrast, the degradation of HA was reduced in the presence of sodium citrate; and the [η] values of F8 (sodium citrate 5 mM), F9 (10 mM), and F10 (20 mM) were 2.70, 2.94, and 2.95 m^3/kg, respectively. Increases in the concentration of the oxygen scavenger resulted in dose-dependent inhibition of the depolymerization process that reached a plateau with10 mM of sodium citrate (F9), showing over 91% of initial [η] value after 5 days; whereas, no significant differences in the [η] value were detected between F9 and F10. Thus, 10 mM sodium citrate was sufficient to stabilize HA in the liquid formulation.

The physicochemical stability of the optimized F9 formula was determined based on the appearance, pH, osmolality, and [η] under accelerated conditions (25°C, 60% RH). The appearance, pH, and osmolality showed no significant changes after storage for 12 weeks, indicative of suitability for IA injection (Table 3). Moreover, the [η] value showed no differences pre- and post-storage, indicating that sodium citrate inhibited HA degradation. Thus, the proposed HA/MS formulation has appropriate physicochemical stability for further investigations.

Efficacy Test in OA-induced Rats. A MIA-induced OA model was used to assess the symptom-modifying effects of IA HA/MS combination. The IA injection of MIA was reported to cause the damage of cartilage proteoglycans, followed by serious weakening of the cartilage and lesions in the subchondral bone and cartilage.[33,34] Fernihough *et al.* (2004) reported that IA injection of MIA in rats induced histological change in the joint, and mechanical allodynia and thermal hyperalgesia, showing similar response to conventional analgesics with OA patients.[17]

As shown in Figure 3(a), IA injection (3 mg/30 μL) of MIA caused a significant increase in the width of the right knee joint at 3 days (approximately 2 mm), compared to

Table 3. Physicochemical stability of the injectable HA/MS formulation (F9) under accelerated storage condition (25°C/60% R.H.) after 12 weeks.

Appearance	Initial Transparent	After 12 weeks Transparent
pH	7.14 ± 0.01	7.10 ± 0.02
[η] (m^3/kg)	3.33 ± 0.24	3.28 ± 0.27
Osmolality (mOsm/kg)	310 ± 2.5	308 ± 1.1

Note: Data are represented as mean ± SD (n = 3).

the sham group ($p < 0.05$). The swelling of the knee joint was gradually decreased (approximately 15%) at 7 days after IA injection of vehicle. Compared to vehicle-treated group, IA administration of HA, MS, and HA/MS combination caused a significant reduction in the swelling of the hind limb knee postdosing. In particular, the HA/MS-treated group showed the greatest decrease in the right knee swelling, *i.e.*, >57 and 74% at 3 and 7 days postdosing, respectively, indicating a significant improvement as compared to the vehicle- and HA-treated groups ($p < 0.05$) at 7 days postdosing (defined day 10 in Figure 3).

The weight distribution of the OA-induced hind paw and normal hind paw was used as an indicator for estimating the analgesic effect of the HA/MS combination. Weight distribution on the right hind paw was decreased after IA injection of MIA, as compared to the sham group (Figure 3 (b)), due to the joint pain caused by cartilage destruction.[35] The vehicle group showed no remarkable changes in weight distribution over the entire post-MIA injection-period. Compared to the vehicle group, the MS and HA/MS-treated groups exhibited increased weight-bearing on the right hind paw at 7 days postdosing ($p < 0.05$). The HA/MS-treated group exhibited more rapid and profound

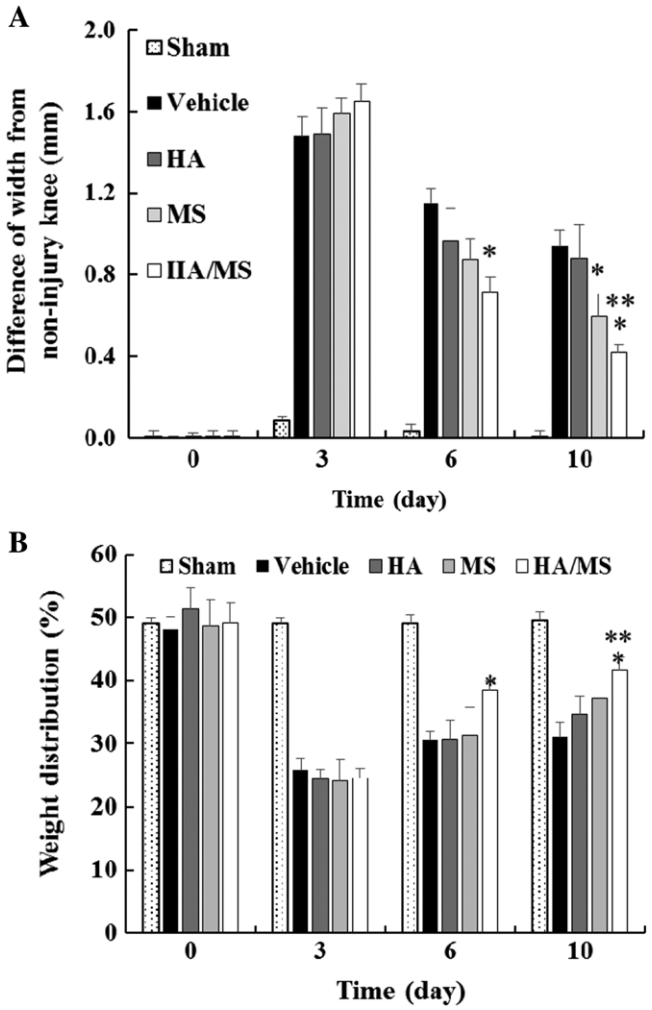

Figure 3. Changes in knee swelling (a) and weight distribution of each hind paw (b) in OA-induced rats following IA injection of HA/MS combination: sham group treated with normal saline (sham); MIA-treated groups treated with vehicle (vehicle); HA (HA); 2% w/v MS (MS); and 1/2% w/v HA/MS (HA/MS). (a) The anti-inflammatory effect was estimated by determining the difference in diameter between the OA-induced right and normal left knees. (b) Weight distribution (%) on the right hind paw was calculated by the following formula: % weight of the right limb = 100 × (right limb weight/[left limb weight + right limb weight]). Error bar represents SD (n = 8). Statistical analysis was carried out using the Student's t-test; *$p < 0.05$ vs. vehicle-treated group; **$p < 0.05$ vs. HA-treated group.

restoration in weight-bearing, as compared to the HA-treated group, at 3 and 7 days postdosing in OA-induced rats. This preclinical result revealed that both HA and MS are effective agents in alleviating symptoms of OA such as pain and inflammation; and the combination of HA with MS could provide synergistic and/or additive effects in the OA knee, with different mechanisms of action.

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Conclusion

An injectable HA/MS formula was successfully prepared by employing histidine and sodium citrate as buffering agent and stabilizer, respectively. In the presence of sodium citrate, the degradation of HA was significantly lessened, retaining over 91% of initial [η] value after 5 days under harsh condition. HA combined with MS (1/2% w/v) showed synergistic and/or additive effects in alleviating pain and inflammation in the knee joint of OA-induced rats. Thus, the novel HA/MS combination is a potential alternative therapy for rapid and effective pain relief in OA patients.

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Graphical abstract

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