Sodium humate accelerates cutaneous wound healing by activating TGF-β/Smads signaling pathway in rats

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Abstract  Sodium humate (HA-Na) has been topically used as a wound healing and anti-inflammatory agent in folk medicine. In the present study, HA-Na was investigated for cutaneous wound healing in Sprague–Dawley rats. HA-Na solution (1.0%, w/v) was topically administered to rats undergoing excision wound models. Healing was assessed with a recombinant bovine basic fibroblast growth factor for external use as positive control. Wound healing rates were calculated on Day 3, 6, 9, 14 and 21 after injury, and tissues were also harvested after the same intervals for histological analysis. In addition, tissue hydroxyproline levels were measured. Furthermore, mRNA levels and protein expressions of transforming growth factor-β1, 2, 3 (TGF-β1, 2, 3) were determined by RT-PCR and western blot. Protein expression levels of Smad-2, -3, -4 and -7 were also detected by western blot. Our study demonstrates that HA-Na has the capacity to promote wound healing in rats via accelerated wound contraction and increased hydroxyproline content. More importantly, these wound healing effects of HA-Na might be mediated through the TGF-β/Smad signaling pathway. HA-Na may be an effective agent for enhanced wound healing.

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1. Introduction

Over the past several years, research on medicinal properties of natural products has increased significantly, and a large body of evidence suggests extracts from peat and sapropel may represent a source of novel compounds with medicinal properties. Humic substances have been used traditionally in folk medicine for treatment of a variety of disorders, including colitis and Alzheimer's disease. Humic acid originates from a class of humic substances via chemical and/or biochemical degradation. It is not a single acid; rather, it is a complex mixture of many different acids containing carboxyl and phenolate groups. Humic acid has been utilized in traditional Chinese medicine (TCM) and possesses various pharmacological properties. Sodium humate (HA-Na) is a salt of humic acid and has been used therapeutically for centuries as part of traditional systems of medicine in many countries. Especially, HA-Na has been traditionally applied for the medicinal practice over thousands of years in China. HA-Na, a potent anti-inflammatory, anti-microbial, antivirus, antitumor and anti-ulcer drug, has been used as a treatment for immunoregulation, endocrine regulation and other diseases.

Wound healing is a complicated process involving different phases of inflammation, new tissue formation and remodeling. These phases are not distinct, but form a continuous wound healing process. Wound healing is a response to injury aiming at reconstructing damaged tissue and requires precise coordination of connective tissue repair, re-epithelialization, and angiogenesis. In many developed country, several growth factors have been used for wound healing. Currently, patients are treated by three growth factors: platelet-derived growth factor-BB (PDGF-BB), basic fibroblast growth factor (bFGF), and granulocyte-macrophage colony-stimulating factor (GM-CSF), but the cost of this therapy is high in developing countries. Burn trauma and wounds are still a major problem in developing countries, often having severe complications and involving high costs for therapy. The use of TCM in treating burns and wounds is an important mode to improve healing, as well as to reduce the financial burden. In China, several plants and herbs have been experimentally used as traditional medicine to treat skin disorders and wound injuries. HA-Na is also used as anti-inflammatory, hemostatic, antidiarrheal, and anti-rheumatic agents and it has been a unique drug treatment of trauma in many Chinese hospitals for many years. At present, however, controlled studies of the effect of HA-Na on wound healing are lacking.

The wound healing activity of HA-Na was presently determined in rats to explore the efficacy and possible mechanisms of action to rationalize its traditional use.

2. Materials and methods

2.1. Drugs and reagents

HA-Na sample was purchased from Institute of Coal Chemistry, Chinese Academy of Sciences (Taiyuan, China). The components of HA-Na consist of 14.22% water (air dry basis), 23.34% burning residue (dry basis), 73.33% humic acid (dry basis) and 4.48% water soluble substances (dry basis) according to analysis report of the product. Recombinant bovine basic fibroblast growth factor (rb-bFGF) for external use was purchased from Zuhai YiSheng biological pharmaceutical Co., Ltd. (Zhuai, China). Hydroxyproline detection kit (alkaline hydrolysis method) was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Gel preparation kit (Tris–HCl, SDS, TEMED, AP), phenylmethane-sulfonyl fluoride, glycine, nitrocellulose membrane, primary/secondary antibodies and western blot ECL chromogenic detection kit were purchased from Wuhan boster biological engineering Co., Ltd. (Wuhan, China). CD31 primary antibody was from Bioss biological engineering Co., Ltd. (Beijing, China). Biotin-streptavidin HRP detection systems were purchased ZSGB biological engineering Co., Ltd. (Beijing, China). All other chemicals and reagents were of analytical grade.

2.2. Experimental animals

Male Sprague–Dawley rats (SPF, weight 200–250 g) were purchased from the Experimental Animal Center of Xi'an Jiaotong University School of Medicine (Xi'an, China). The animals were housed under controlled conditions (12 h/12 h light/dark cycle, 22–28 °C and 60–70%–70% humidity), fed with normal mice chow and water ad libitum. All animals were acclimated for at least 3 days prior to the first treatment. The rats were anesthetized before and during infliction of the experimental wounds. The surgical interventions were carried out under sterile conditions using 10% chloral hydrate anesthesia (300 mg/kg, i.p.). All experimental procedures carried out in this study were approved by the Ethics Committee of School of Medicine, Xi'an Jiaotong University and were in accordance with the US Guidelines for the Care and Use of Laboratory Animals (NIH publication #85-23, revised in 1985).

2.3. Wound creation and treatment

The rats were weighed before the experiment and anesthetized prior to and during infliction of the experimental wounds. The rats were inflicted with excision wounds as described by Arunachalam et al. and Ganeshkumar et al. Briefly, the rats were anesthetized, the dorsal fur of the animal was shaved with an electric clipper and the area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. The depth of the wound was outlined by inserting a flexible device into the wound, making sure it is lined up perpendicular to the edge of the wound. A full thickness of the excision wound of 500 mm² and 2 mm depth was created along the markings using toothed forceps, a surgical blade and pointed scissors. The rats were randomly divided into 3 groups. Group 1 was non-treated as a control group; group 2 was treated with 0.5 mL/cm² rb-bFGF (4200 IU/mL) and served as a reference standard (positive control); group 3 was treated topically with an equal amount of 1.0% (w/v) HA-Na solution. Standard wound cleansing was performed daily for all the groups prior to the application of HA-Na or rb-bFGF. HA-Na or rb-bFGF were topically applied twice daily for 14 days. Epithelialization was considered to have occurred when the eschar fell off without leaving any residual raw wound. Any rat showing signs of infection was excluded and sacrificed with a 10% chloral hydrate overdose.

The wound closure rate was assessed by tracing the wound on Day 3, 6, 9, 14 and 21 post-wounding with transparency paper and a permanent marker. The wound areas recorded were measured using a graph paper. Time to wound closure was defined when the wound bed was completely re-epithelialized and filled with new tissue. The wound healing activity of HA-Na was presently determined in rats to explore the efficacy and possible mechanisms of action to rationalize its traditional use.
changes in the wound size were expressed as percentage contraction of the original wound size (Day 0). The wound healing rate was calculated with a formula as following:

\[
\text{Percentage wound contraction} = \left( \frac{A_n - A_0}{A_0} \right) \times 100 \tag{1}
\]

where \(A_n\) is the wound area on Day \(n\), and \(A_0\) is the wound area on Day 0. For histopathological studies and biochemical analyses, the wounds together with a uniform perimeter of surrounding tissue (excluding any underlying muscle and extraneous tissue) were harvested on Day 3, 6, 9, 14 and 21 following wounding. A portion of harvested tissue was immediately stored in liquid nitrogen for further analysis and another portion was fixed in 4% formaldehyde for histopathological study.

2.4. Histopathological evaluation

Tissues from the wound site after treatment with HA-Na solution, rb-bFGF and untreated control were removed after sacrifice and fixed in 4% formaldehyde, dehydrated through a series of alcohol, cleared in xylene and embedded in paraffin wax. Five µm thick sections, which were harvested on Day 6 and 14 post-wounding, stained with hematoxylin–eosin (HE) and Masson's trichrome (MT), and observed and photographed under light microscopy. Immunohistochemical staining for CD31 was carried out on control group, bFGF group, and 1.0% HA-Na group postoperatively on Day 6 and 14. Brown dots present in images of endothelial cells indicated positive staining. Six random, unreplicated fields were selected for observation (400 ×). The number of newly grown microvessels (brown staining) was calculated using Image-Pro Plus 6.0 according to the conversion of each vision field area of 0.1885 mm\(^2\) being equal to 1 mm\(^2\). An average of 6 replicates was recorded as the microvessel density (MVD)\(^{18}\).

2.5. Hydroxyproline assay

Tissue hydroxyproline levels were measured on Day 3, 6, 9, 14 and 21 post-injury using hydroxyproline detection kit according to the manufacturer's protocol.

2.6. RNA extraction and RT-PCR analysis

The expression patterns of TGF-β1, TGF-β2 and TGF-β3 of wounded tissue on the 6th and 14th days post-wounding were analyzed by RT-PCR. The total RNA content was isolated according to the manufacturer's instruction (guanidine thiocyanate phenol, chloroform procedure, total RNA isolation kit, FSATGEN biological technology Co., Ltd., Shanghai, China). Reverse transcription reactions were performed in an GeneAmp PCR System (Applied Biosystems, CA, USA) following manufacturer's guidelines (Revert Aid TM First Strand cDNA Synthesis Kit, Canada). Custom primers were created to TGF-β1 (F5'-CTGAACCAAGGAGCGAATAC-3', R5'-AACCCAGTCTTCTCATAAAGTC-3'), TGF-β2 (F5'-AGGCAGAAGTCCAGGCTTTTCT-3', R5'-TGGCTCTTTATTTGGGATGATG-3'), TGF-β3 (S5'-GAAAGGTGGAGGACCATAG-3', R5'-GAGGACACATTGAAGCGAAG-3') and GAPDH (F5'-GCTTTCCATCATGTGTTGAAAGTGA-3', R5'-GTTCCGTTTGAAACGGGATTG-3) and obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Initial denaturing was performed at 94 °C for 3 min; then 40 cycles at 94 °C for 30 s (denaturing), at 53 °C for TGF-β1, TGF-β2, TGF-β3 and GAPDH for 30 s (annealing) and at 72 °C for 1 min (extension) and a further extension at 72 °C for 5 min were carried out. After amplification, the RT-PCR products were electrophoresed on 2% agarose gel containing geltidian bromide. Images were captured and subjected to densitometric analysis using Bio-Rad Gel Doc™ XR gel documentation system equipped with Image-ProPlus Software. The values were expressed as band intensity of the target gene (TGF-β1/TGF-β2/TGF-β3) relative to the level of the reference mRNA for the housekeeping gene (Gapdh). Three independent experiments were carried out to study mRNA levels.

2.7. Western blot analysis

The harvested wound tissues were homogenized in a lysis buffer containing 1% Triton X-100, 1% deoxycholate, 0.1% SDS and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 12,000 rpm for 10 min at 4 °C (Micro-21R, Thermo Scientific, USA). The protein concentration was estimated by the NanoDrop1000 (ND-1000, NanoDrop, USA). Equal amount of protein was loaded on to the 10% SDS-PAGE with 5% stacking gel wells and electrophoresed at 80 V for 25 min, and then 120 V for 45 min; thereafter, proteins were transblotted onto the NC membrane. After blocking with blocking solution, the membranes were incubated with TGF-β1, 2, 3, Smad-2, 3, 4, 7 and β-actin primary antibodies (1:400) for overnight followed by 1 h incubation with secondary antibodies (1:5000) on rocking shaker (ZHWY-100B, Shanghai zhicheng, China) at 37 °C. The desired proteins were detected by western blot ECL chromogenic detection kit. Optical densities of the bands were scanned and quantified with the Syngene Gene Tools (Syngene Corp., Cambridge, UK). Three independent experiments were carried out to study protein expression.

2.8. Statistical analysis

The results are expressed as mean ± SEM. Data were statistically analyzed using one way analysis of variance (ANOVA) followed by the Dunnett test. A \(P\)-value < 0.05 was considered statistically significant as compared to control group.

3. Results

3.1. Wound contraction and epithelialization period

The wound appeared clean and free of exudates throughout the study in all the animal groups. Granulation tissue was evident at the wound edges from Day 3 post-injury in all groups. The results of wound contraction and epithelialization period after topical administration of the HA-Na solution are reported in Table 1 and Fig. 1. Wound contraction following 1.0% (w/v) HA-Na treatment was significantly greater (\(P<0.01\)) on Day 6, 9 and 14 as compared to the respective control group. The epithelialization period was also higher on the 14th day in subjects treated with 1.0% HA-Na solution.

3.2. Histopathology

Well organized wound healing processes were observed following treatment with either HA-Na solution or rb-bFGF. In haematoxylin and eosin stained sections, wounds from the 1.0% (w/v) HA-Na solution group showed more fibroblasts, collagen fibers, and robust dermal blood vessel formation, with surrounding dense and thick mesenchymal matrix deposition, as compared with control treatments. The appearance of the tissues from the HA-
Na treatment group was quite similar as those of the positive control group on the 6th and 14th days after wounding. Poor collagen fibers and epidermis were present in wounded tissues from the control group. Histopathological sections of the 6th day post-wounding showed new skin thickening, granulation tissue hyperplasia, and small blood vessels in the HA-Na and positive control groups. Tissues from the control group showed inflammation, thin skin, and significantly fewer numbers of fibroblasts as compared with the HA-Na group (Fig. 2A).

Histopathological sections showed only a few fibroblasts in the HA-Na and positive control groups on the 14th day post-wounding, with newly-formed collagen in alignment. Cell differentiation and wound healing were also apparent. Control wound tissue showed small numbers of inflammatory cells with increased numbers of fibroblasts forming the epidermal layer. In these tissues, the new collagen appeared to be disorganized. Masson's trichrome staining showed clear and distinctly high macrophage and fibroblast density in 1.0% HA-Na solution treatment. In control group, the arrangement of collagen bundles was loosely packed and wounds were only moderately cellular with fibroblasts (Fig. 2A).

On the 6th and 14th days after surgery, CD31-positive expression was significantly higher in the HA-Na group than in the control and positive control groups. CD31-positive expression was confined within the scaffold and the contacting zone of the subcutaneous tissue. Moreover, after 14 days in each group, different levels of epidermalization appeared and the cells in the epidermis were highly aggregated (Fig. 2A). MVD was higher in the HA-Na group, when compared to control and positive control groups on the 6th and 14th days (Fig. 2B).

### 3.3. Hydroxyproline content

Collagen is present in wound granulation tissue and is produced by new fibroblasts

The hydroxyproline content of tissue could reflect the collagen synthesis. Hydroxyproline content was significantly increased in the HA-Na treated group as compared to the control group. Although the hydroxyproline content of rb-bFGF treated group was slightly increased as compared to HA-Na group, the level was not statistically significant (Fig. 3).

### 3.4. Levels of mRNA and protein expression of TGF-β1, TGF-β2 and TGF-β3

The mRNA levels of Tgf-β1, Tgf-β2 and Tgf-β3 were semi-quantitatively measured using RT-PCR analysis and expressed as densitometric band intensities of the target gene relative to the level of the reference mRNA for the housekeeping gene Gapdh. Results showed that mRNA expression of Tgf-β1 on the 6th day after wound creation was significantly increased in HA-Na group vs. control, but mRNA expression of Tgf-β1 on the 14th day was decreased as compared to control group. Moreover, mRNA expressions of Tgf-β2 and Tgf-β3 6 days after wound creation had no obvious change in the HA-Na group, whereas mRNA expressions of Tgf-β2 and Tgf-β3 on the 14th day was increased as compared to control group (Fig. 4). We further observed effects of HA-Na on protein expressions of TGF-β1, TGF-β2 and TGF-β3 after wound creation by western blot analysis, and found that protein expression patterns were the same as the mRNA

| Day | Control group (%) | bFGF group (%) | 1.0% HA-Na group (%) |
|-----|------------------|----------------|---------------------|
| 3   | 10.43±1.21       | 12.67±1.53     | 14.22±1.18          |
| 6   | 30.22±2.98       | 34.23±2.16     | 40.16±3.22†         |
| 9   | 72.33±2.40       | 75.42±1.17     | 88.11±1.68†         |
| 14  | 88.41±3.24       | 95.36±2.51†    | 98.76±1.26†         |
| 21  | 100              | 100            | 100                 |

Epithelialization time (days) 17.16±2.87 14.33±1.03‡ 14.16±1.47‡

Values are mean±SEM, n=8 in each group.

**P<0.01 vs. control group.
patterns for Tgf-β1, Tgf-β2 and Tgf-β3 on the 6th and 14th days (Fig. 5).

3.5. Protein expression patterns of Smad-2, -3, -4 and -7

Since intracellular Smad proteins transduce the extracellular TGF-β signals to the fibroblast nucleus for collagen production, the expression of Smad-2, -3, -4 and -7 on the 6th and 14th days was evaluated in wound granulation tissues. Western blot analysis revealed that expressions of Smad-2, -3 and Smad-4 were increased in the HA-Na group as compared with controls. However, Smad-7 protein levels diminished (Fig. 6).

4. Discussion

HA-Na has been topically used as a wound-healing and anti-inflammatory agent in folk medicine. In the present study, HA-Na was investigated for cutaneous wound healing in rats to accurately elucidate its traditional value. Our study demonstrated that HA-Na has the capacity to promote wound healing in rats via accelerated wound contraction, elevations in hydroxyproline content. More importantly, the wound healing effects of HA-Na might be mediated through the TGF-β/Smad signaling pathway. Therefore, our results substantiate the utility of HA-Na as a wound-healing agent.

Wound contraction is the centripetal movement of the healing edges of a full-thickness wound and healing progress can be assessed by the periodic measurement of contracting wound tissue. The wound contraction values of HA-Na treated group were significantly enhanced as compared to control group. Topical application of HA-Na remarkably accelerated the wound
contraction, and histopathological examination supported the above finding. Additionally, the histological study demonstrated that the HA-Na treated group had more rapid re-epithelization and angiogenesis, compared with the control group. Moreover, bFGF has also been studied for its role in tissue repair, most commonly in healing of excisional skin wounds. In the present study, compared with the positive group (bFGF group), topical application of HA-Na significantly promoted wound contraction and re-epithelization, further suggesting that HA-Na is better than the traditional drug treatment. In fact, in the preliminary experiment, the indicated concentrations of HA-Na (0.5%, 1% and 2%) accelerated the wound contraction in a concentration-dependent manner (data not shown). Therefore, HA-Na (1%) was topically administrated in this study.

Collagen, a principal component of connective tissue, plays a key role in the healing of wounds and provides a structural framework for the regenerating tissue and the healing process depends on the regulated production, deposition and their

Figure 4  Effects of HA-Na on mRNA levels of Tgf-β1, Tgf-β2 and Tgf-β3. (A)–(C) Representative RT-PCR analysis of Tgf-β1, Tgf-β2 and Tgf-β3 mRNA in rats wound tissue on the 6th and 14th days. Gapdh was co-amplified as an internal standard. The values were normalized to Gapdh expression and represented as band intensity of Tgf-β1, Tgf-β2 and Tgf-β3 (target gene)/Gapdh. Values are expressed as mean ± SEM (n = 8). **P<0.01 vs. control group.

Figure 5  Effects of HA-Na on protein expressions of TGF-β1, TGF-β2 and TGF-β3. (A) and (B) Protein expressions and quantitation data of TGF-β1, TGF-β2 and TGF-β3 in rats wound tissues on the 6th and 14th days detected by western blot. The values were normalized to β-actin expression and represented as band intensity ratio of TGF-β1, TGF-β2 and TGF-β3 (target protein)/β-actin. Values are mean ± SEM (n = 8). *P<0.05, **P<0.01 vs. control group.
subsequent maturation. In this study, topical application of HA-Na evidently enhanced the granulation tissue to contain more collagen, fibroblasts and less inflammatory cells. Our data also revealed that HA-Na dramatically increased collagen formation, because the hydroxyproline content of granulation tissue in HA-Na treated animal group was obviously higher as compared to control group. In our opinion, the wound contraction and collagen synthesis promoting activity of HA-Na could be due to the accelerated the wound healing activities.

During the stages of wound healing, multiple cell types and growth factors are required to complete a variety of cellular activities. TGF-β is a key active molecule in wound healing and scar formation. Three subtypes of TGF-β (TGF-β1–3) play critical roles within different phases of wound healing. These proteins are of particular important in the late stage of wound healing. In our experiment, HA-Na up-regulated mRNA expression of TGF-β1 on Day 6 after wounding and down-regulated mRNA expression of TGF-β1 on Day 14. It is well established that TGF-β1 functions as a wound-healing promoting factor, and excess production of this protein may lead to over-healing outcomes, such as hypertrophic scarring and keloid. This fact may help to understand the down-regulation of TGF-β1 in the

Figure 6 Effects of HA-Na on protein expressions of Smad-2, -3, -4 and -7. (A) and (B) Protein expressions and quantitation data of Smad-2, Smad-3, Smad-4 and Smad-7 in rats wound tissues on the 6th and 14th days detected by western blot. The values were normalized to β-actin expression and represented as band intensity ratio of Smad-2, -3, -4 and -7 (target protein)/β-actin. Values are mean ± SEM (n=8). *P<0.05, **P<0.01 vs. control group.
Sodium humate accelerates cutaneous wound healing by activating TGF-β1/Smads signaling pathway in rats

HA-Na group at Day 14. Our study also showed that HA-Na induces mRNA expressions of TGF-β2 and TGF-β3 on Day 14. Moreover, we further observed effects of HA-Na on protein expressions of TGF-β1, TGF-β2 and TGF-β3 after wound creation by western blot analysis, and protein examination supported the above findings, suggesting that the wound healing effect of HA-Na might be mediated through TGF-β signaling pathway.

The TGF-β-mediated signaling pathway is believed to be closely associated with wound healing and scar formation30. Smad proteins function as intracellular signaling mediators of TGF-β family members30. The present study detected that HA-Na topical application up-regulated expression of Smad-2, -3 and -4 proteins, whereas the expression of Smad-7, the inhibitory protein, was down-regulated. The diminished level of inhibitory Smad-7 also amplified the effect of HA-Na in wound healing, further suggesting that HA-Na might activate TGF-β1/Smad signaling pathway. So far, this study has used excisional wounds generated in rats followed by measurement of TGF-β1/Smad signaling pathway components. The present results found that the wound healing promoting activity of HA-Na could be associated with the TGF-β1/Smad signaling pathway. However, further studies using TGF-β isoform-specific neutralizing antibodies and adenoviral delivery of Smad-2, Smad-3, and/or Smad-4 at the stages of wound healing in rat model are possible avenues of future research to further elucidate the regulation of HA-Na on TGF-β1/Smad signaling pathway.

Targeting the TGF-β1/Smad signaling pathway using therapeutic agents to improve wound healing and/or reduce scarring has been successful in pre-clinical studies. CAT-192 is a human monoclonal antibody that neutralizes TGF-β1 and was shown to improve corneal wound healing in bovine organ cultures by promoting re-epithelialization31. As an example, peptide 144 (P144) is a 14-mer peptide from human betaglycan that was designed as a TGF-β1 inhibitor and has shown efficacy in reducing fibrosis in different animal models12,32. In addition, Smad-3 antisense oligonucleotides accelerated wound healing and reduced scarring in a mouse excisional wound model33. Since the role of TGF-β1/Smad signaling pathway in wound healing has been well characterized, it is feasible to explore the effect of HA-Na on wound healing and the molecular mechanisms underlying the TGF-β1/Smad signaling pathway.

HA-Na, an anti-inflammatory, anti-microbial, and anti-ulcer drug, is thought to have the broadest spectrum of effects. The use of HA-Na in treating burns and wounds is an especially important mode to improve healing, as well as to reduce the cost of therapy. Although the present study is promising, it remains to be seen whether HA-Na will be of clinical use, as HA-Na have yet been screened in phase I clinical trials for safety and tolerability.

5. Conclusions

HA-Na has the capacity to promote wound healing in rat model via accelerated wound contraction, higher hyaluronic acid content and improved the level of tissue healing. More importantly, the wound healing effect of HA-Na might be mediated through the TGF-β1/Smad signaling pathway. HA-Na may be used as a future novel wound-healing agent.

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