Research Article

The Effects of Xiangqing Anodyne Spray on Treating Acute Soft-Tissue Injury Mainly Depend on Suppressing Activations of AKT and p38 Pathways

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Objectives. In the present study we try to elucidate the mechanism of Xiangqing anodyne spray (XQAS) effects on acute soft-tissue injury (STI).

Methods. Acute STI model was established by hammer blow in the rat hind leg muscle. Within 8 hours, instantly after modeling and per 2-hour interval repeated topical applications with or without XQAS, CP or IH ethanol extracts spray (CPS and IHS) were performed, respectively; muscle swelling rate and inflammation-related biochemical parameters, muscle histological observation, and mRNA and protein expression were then examined.

Results. XQAS dose-dependently suppressed STI-caused muscle swelling, proinflammatory mediator productions, and oxidative stress as well as severe pathological changes in the injured muscle tissue. Moreover, CPS mainly by blocking p38 activation while IHS majorly by blocking AKT activation led to cytoplasmic IκBα degradation with NF-κBp65 translocated into the nucleus. There are synergistic effects between CP and IH components in the XQAS on preventing from acute STI with suppressing IκBα degradation, NF-κBp65 translocation, and subsequent inflammation and oxidative stress-related abnormality.

Conclusion. Marked effects of XQAS on treating acute STI are ascribed to strong anti-inflammatory and antioxidative actions with a reasonable combination of CP active components, blocking p38-NF-κB pathway activated, and IH active components, blocking AKT-NF-κB pathway activated.

1. Introduction

There are many ancient archives for treatment of soft-tissue injury (STI) using Traditional Chinese Medicine. STI care can be traced back to early civilizations, and many of these treatments were based on the use of herbal remedies. The report showed that approximately one-third of all traditional medicines in use are for the treatment of wounds and soft-tissue disorders, compared to only 1%–3% of modern drugs [1]. Xiangqing anodyne spray (XQAS) is a spray formulation by topical administration, which is a mixture of two ethanol extracts that were extracted, respectively, from equal-mass crude of two Traditional Chinese Medicines, Cynanchum paniculatum (CP) and Illicium henryi (IH), with additional penetration enhancers. In previous study, we have found that XQAS effects on treating neuralgia and postherpetic neuralgia in clinical application were prominent and superior to the ethanol extract of IH or CP treatment alone [2, 3]. In the modern medicine, the ethanol or water-soluble extracts of CP have been demonstrated to relieve various pains, such as rheumatic arthralgia, lumbago, pain due to traumatic injuries, abdominal pain, and toothache, which were also used to treat skin diseases such as eczema, rubella, and neurodermatitis [4]; the water-soluble extracts of IH have also been applied clinically as an analgesic agent by intramuscular injection in China [5]. We found that paeonol and quercetin may be the major active compounds in CP and IH (see Section 2), respectively. Paeonol effects on anti-inflammatory, antioxidant, and cardiovascular protective activities have been demonstrated [6], while antioxidant properties of...
quercetin associated with anti-inflammatory effects and so forth were also reported [7, 8].

The acute STI, majorly showing skeletal muscle injury, is mainly traumatic aseptic inflammation. When it occurs, the pathological changes include local tissue necrosis, blood capillary dilation, inflammatory cell infiltration with release of inflammatory mediators, and tissue edema [9]. Further, the secondary lesion growth is related to progressive microcirculatory and inflammatory reaction. Direct trauma to microvessels results in membrane damage, endothelial cell swelling, widely vascular hemorrhage, and uncontrolled clot formation, leading to additional local ischemia, which, in turn, induces further degradation of membrane phospholipids, accumulation of free radicals, and inflammation-induced oxidative stress [10, 11]. As the extension of injury process, severe muscle fibers degeneration and necrosis following marked collagen fiber hyperplasia, massive inflammatory cell infiltrates, and interstitial ecchymosis will emerge in the site of damaged tissue to create irreversible structural changes [12]. Hence, early diagnosis and treatment with rapid attenuating tissue swelling and inhibiting inflammation can improve functional results and diminish structural damage in the early and acute period of skeletal muscle injury, which is necessary for treatment of STI [13]. According to the mechanism of inflammatory production, nuclear factor-κB (NF-κB) directly participates in inflammation reaction [14], while both MAPK and AKT signaling activation, in turn, activates transcription factors, such as activator protein-1 and NF-κB, which promotes the gene and protein expressions of inflammation-correlated cytokines [14, 15].

Our previous study has indicated that repeated topical administration with XQAS in the STI site, which was modeled by hammer blow in the hind leg of rat, showed rapidly therapeutic effects on soft-tissue swelling and inflammatory mediators production, while repeated XQAS treatment for four days significantly suppressed STI-caused skeletal muscle necrosis and fibrosis, effectively recovering impaired muscle tissue in a dose-dependent manner. There is a synergistic effect between CP and IH in the XQAS [16]. XQAS effects were associated with suppression of activated NF-κB p65 gene expression in the impaired muscle tissue. However, the molecular mechanism of XQAS effects, especially the synergistic effect between CP and IH on rapidly treating STI, remains unclear. This study tries to elucidate the mechanism of prominent efficacy of XQAS on treating acute STI and the reason why the efficacy is superior to CP or IH alone. An acute STI model with mass-drop injury method, according to Stratton et al. introduction [17], was constructed in this study.

2. Material and Methods

2.1. Preparation of Xiangqing Anodyne Spray (XQAS). XQAS was prepared as previously described [16]. Briefly, the root barks of Cynanchum paniculatum (CP) or Illicium henryi (IH) were extracted by 75% ethanol, and penetration enhancers, which include Azone, peppermint oil, and PEG 400 borneol in the 75% ethanol, were added. The final XQAS contains 0.50 g (crude drug) CP and 0.50 g (crude drug) IH per milliliter, which contains 0.858 mg paenol and 7.33 mg quercetin per milliliter. XQAS was examined by high performance liquid chromatography analysis. Using the same method, the Cynanchum paniculatum spray (CPS) and Illicium henryi spray (IHS) were prepared, containing 1.0 g (crude drug)/mL CP or IH, respectively.

2.2. Animals. The animal experiment was performed in accordance with China state regulations on animal experimentation and approved by Animal Experimental Ethical Center of Southeast University (protocol number 20120023). Male Sprague-Dawley rats (250 to 300 g) were supplied by Suzhou Industrial Park, Matt Ireland Ltd. All animals were maintained on a standard 12 h light-dark cycle, in a temperature-controlled environment (24 ± 2°C), with free access to water and chow. They were acclimatized for at least one week before initiating the experiments.

2.3. Acute Closed Soft-Tissue Injury (STI) Induction. Like the previously described method [16], the rats were anesthetized by ethyl ether, the inner hind thigh was epilated with depilatory Na2S solvent in advance and fixed in lateral, and the thigh muscle was hit with a cylindrical hammer (200 g in weight, 1.0 cm in top diameter, and 1.5 cm in bottom diameter) by free falling vertically from inside of a hard smooth plastic tube (75 cm in length and 1.5 cm in inner diameter), only resulting in closed STI but not to cause femoral fracture, which was established by a drop-mass method, similar to the model first described by Stratton et al. [17].

2.4. Animal Experiments. The rats were randomly divided into six groups (n = 8 per group): control (Ctrl), model (Mod), high-dose XQAS treatment (HXQAS), low-dose XQAS treatment (LXQAS), CPS treatment (CPS), and IHS treatment (IHS). Except for Ctrl group, other group rats were subject to two-time blows to hind leg muscle to model acute STI. After modeling, the rats were instantly applied topically with 150 μL/time penetration enhancers in the Ctrl and Mod group, XQAS in the HXQAS (containing 0.50 g (crude drug)/mL both CP and IH, resp.) and LXQAS (containing 0.25 g (crude drug)/mL both CP and IH, resp.) group, CPS (containing 1.0 g (crude drug)/mL CP), or IHS (containing 1.0 g (crude drug)/mL IH), respectively. Next, topical application was repeated with a 2-hour interval in a duration of 8 h after modeling, and the circumference located in the injured thigh muscle was measured at 0 (before modeling), 1, 2, 3, 4, 6, and 8 h; muscle swelling rate (MSR) was then calculated by MSR (%) = (S/S0 – 1) × 100%, where S0 represents the circumference at 0 h, while S represents the circumference at the different time point after making the model) [18]. At the end of experiment, rats were sacrificed after anesthesia with urethane (1.0 g/kg), and the injured thigh muscle in each rat was then divided into two parts: one was stored at −80°C to be used to measure biochemical parameters and to detect gene or protein expressions; the other was fixed in neutral buffered formalin to be used to observe the morphological change.

2.5. Biochemical Analysis of the Muscle Tissue. The muscle tissue was homogenized in 0.02 M phosphate buffered solution (PBS, pH 7.4). The parameter was measured according
to the protocols of respective kits. The levels of interleukin-
1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and
prostaglandin-E2 (PGE-2) were measured by ELISA kits
provided by Abcam (HK) Ltd. The contents of myeloperoxidase
(MPO), nitric oxide (NO), malondialdehyde (MDA), and
superoxide dismutase (SOD) were measured by spectropho-
tometer kits, respectively, provided by Nanjing Jiancheng
Bioengineering Institute.

2.6. Reverse Transcription-Polymerase Chain Reaction (RT-
PCR) Analysis. The muscle tissue in six of the eight rats in
each group was randomly chosen for RT-PCR assay. Total
RNA was isolated from tissues using RNAiso plus Isolation
Reagent (TAKARA, Otsu, Shiga, Japan). Total RNA solution
was first reverse transcribed and then immediately ampli-
ﬁed in a GeneAmp PCR system (Eppendorf). Primers used were

- NF-κB p65 (forward-GGG ACT ATG ACT TGA ATG CG;
reverse-CAG GCT AGG GTG AGC GTA T), IL-1β (forward-
GAT GAC GAC CTA GTG TT; reverse-CTT CTT CTT
TTG GTA TTG TT), TNF-α (forward-TCC AGG CCG
TTG CCT ATG T; reverse-GAG CGT GGT GGC CCC),
and GAPDH (forward-ATG TAT CCG TGG ATC TG;
reverse-GAG ATT CGA GAG AAG GG). The gel was
photographed by GeneGenius automatic gel imaging and
analysis system (Syngene, Cambridge, UK) and the bands
on the ﬁlm were scanned by densitometry for quantitation. To
exclude variations due to RNA quantity and quality, the data
for all genes were adjusted to GAPDH.

2.7. Western-Blot Analysis. Six randomly chosen muscle
camples from each group were homogenized in lysis buffer
(150 mM NaCl, 10 mM HEPES, pH 7.9, 1 mM EDTA,
0.6% NP-40, 0.5 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL
aprotinin, and 10 μg/mL trypsin inhibitor). Samples were
then sonicated and incubated on ice for 15 minutes. Protein
was extracted by Cytosol and Nucleus Protein Extraction Kit
(KeyGEN Bio TECH). Protein concentration was determined
by Bradford’s method. Samples were separated by SDS-
PAGE gel and electrophoretically transferred to nitrocellulose
membrane. Nonspeciﬁc binding sites were blocked with
TBS with 0.1% Tween-20), membranes were
washed with TBST (TBS with 0.1% Tween-20), membranes were
incubated with secondary antibody for 2 h at room temper-
ature, which was conjugated to horseradish peroxidase (Santa
Cruz Biotechnology, Inc., Santa Cruz, CA). Immunopositive
bands were visualized by a chemiluminescent method (ECL,
Tanon-5200).

2.8. Histological Observation of Muscle Tissue. After ﬁxation
in neutral buffered formalin, the muscle was sectioned and
processed routinely for hematoxylin-eosin (HE) staining
for qualitative histological analysis. All histopathological
examinations were performed by a trained pathologist. The
muscle injury, including mainly muscle ﬁbers degeneration
and necrosis, interstitial ecchymosis, and inﬂammatory cell
inﬁltrates, in each sample was assessed using the method
reported by Bunn et al. [13].

2.9. Statistical Analysis. Results were expressed as mean ±
standard deviation (SD). For statistical signiﬁcance, except
the MRS differences between groups which were tested using
analysis of covariance (the baseline value in the control as a
covariate), the other data were tested using one-way analysis
of variance (ANOVA) followed by LSD’s multiple comparison
test. Differences were considered signiﬁcant at P < 0.05
and extremely signiﬁcant at P < 0.01.

3. Results

3.1. The Effects of XQAS on Muscle Swelling Rate (MSR) in
Acute STI Model. The MSR in the hit hind leg of rats quickly
elevated at 1 h after hitting and reached top extent at 2 h,
which were in the top value always at 2 h to 8 h after hitting.
XQAS treatment dose-dependently attenuated MSR with
signiﬁcant inhibition on MSR starting from 1 h after hitting
in the HXQAS and LQAS group, respectively. Either CPS or
IHS treatment also attenuated MSR similarly to XQAS. How-
ever, the effect of HXQAS was obviously superior to both CPS
effect at 2 h, 3 h, 4 h, and 6 h after hitting and IHS effect at 3 h,
4 h, and 6 h after hitting, exhibiting an effect with “one plus
one more than two,” suggesting a synergistic effect between
CP and IH on inhibition of hit-induced acute traumatic
swelling (Figure 1).

3.2. Evaluation of XQAS on Anti-Inﬂammatory Reaction and
Antioxidative Stress in the Acute STI Model Induced by
Hitting Hind Leg Muscle in Rats

3.2.1. Effects of XQAS on mRNA Expressions and Levels of
TNF-α and IL-1β in the Injured Muscle Tissues. As inﬂam-
nation is the most important reaction when acute STI occurs
[19], we found that the mRNA expressions and levels of TNF-
α and IL-1β in the damaged muscle tissue were markedly
increased at 8 h after hitting hind leg muscle. XQAS treatment
dose-dependently inhibited the overexpression of TNF-α and
IL-1β mRNA and both levels increased in the acute injured
muscle tissue. These effects were also observed in both CPS-
and IHS-treated group but were signiﬁcantly less than the
HMQAS effects, indicating a synergistic effect between CP
and IH (Figures 2(a) and 2(b))

3.2.2. Effects of XQAS on Expressions of COX-2 and iNOS and
Levels of PGE-2 and NO in the Injured Muscle Tissue. The
overexpressions of COX-2 and iNOS with increased PGE-
2 and NO levels in the damaged muscle tissue were also
exhibited. These abnormalities were suppressed markedly by
XQAS treatment in a dose-dependent manner or by either
CPS or IHS treatment, while the more signiﬁcant suppression
was found in the HXQAS-treated group compared to the
CPS- or IHS-treated group, also suggesting a synergistic effect
between both (Figures 2(c) and 2(d)).
3.3.1. Effects of XQAS on Activation of AKT, p38, and JNK

Activation of MAPK (p-38, JNK, etc.) and AKT signaling cascadedly activate their downstream NF-κB by promoting IkBα degradation and NF-κB gene expression [14]. In the present study, the levels of NF-κB gene expression significantly increased, followed by NF-κB expressions increased both in endochylma and in nucleus, while XQAS, CPS, and IHS treatment can inhibit NF-κB expressions abnormal increase in different degrees. The levels of total AKT (t-AKT), total p38 (t-p38), and total JNK (t-JNK) expression were significantly increased, suggesting AKT, p38, and JNK were activated. XQAS treatment dose-dependently inhibited the activations of both p-AKT and p-p38 in the acute STI model. Notably, CPS treatment masterly suppressed p38 activated but almost did not change AKT activated with only a decreased tendency in phosphorylated AKT expression. However, IHS treatment, showing a contrary action with CPS, masterly suppressed AKT activated but almost did not change p38 activated with very little decrease in phosphorylated p38 expression. In addition, comparing the effects of XQAS, CPS, and IHS with an equal-dose treatment on suppressing both AKT and p38 activated, the XQAS effect was less than the CPS on suppressing p38 activated and was also less than the IHS on suppressing AKT activated. Treatment with XQAS, CPS, or IHS did not improve p-JNK activated in the injured muscle (Figure 3(d)).

3.4. Histopathologic Evaluation of Acute Injured Muscle Tissue with or without XQAS, CPS, and IHS Treatment.

In the Ctrl group, no marked injury was exhibited in the muscle tissue, while in the model group, severe muscle tissue injury was exhibited with large areas of muscle fibers disruption, muscle cells necrosis and significant vascular damage leading to red blood cell accumulation in the interstitial space, and considerable inflammatory cells infiltration in the interstitial space. In the HXQAS group, injured muscle was significantly improved with greater reduced muscle fibers disruption and necrosis and significantly decreased interstitial ecchymosed following a few inflammatory cells infiltration. In the LXQAS group, the muscle-injured improvement was less than that in the HXQAS group, with a marked interstitial ecchymosis, muscle cell necrosis, and inflammatory cells infiltration.
Figure 2: Continued.
In the CPS or IHS group, the injured muscle was also improved significantly but less than that in the HXQAS group. Representative histopathological findings in each group are shown in Figure 4.

4. Discussion

The early recovery phase of STI is characterized by the overlapping processes of inflammation and following occurrence of secondary damage [4]. Within the injured muscle tissue there is leukocyte infiltration and local production of various pro- and anti-inflammatory cytokines, which are crucial for initiating the breakdown and the subsequent removal of damaged muscle fragments, but overexpression of proinflammatory cytokines can be infaust to subsequent healing [24]. In our present study, it was found that the proinflammatory cytokines and their gene overexpressions in the acute STI muscle tissue were notably suppressed by XQAS treatment, which demonstrates that XQAS has an efficacy to inhibiting inflammatory injuries in the acute closed STI. XQAS had quick treatment effects on acute STI, and the active components contained in the XQAS, which compose XQAS formulation, have a synergistic effect on treating acute STI.

Free radical generation is known to accelerate progression of muscle pathologies [20]. Oxidative stress in skeletal muscle is increased during acute STI and is ascribed to increases in enzyme-initiated oxidant production and neutrophil-derived myeloperoxidase (MPO) activity [21]. In the present study, XQAS displayed a strong effect on reduction of MPO activity as well as attenuation of inflammatory cell infiltration in the interstitial space of injured muscle. Meanwhile, increased MDA level and decreased SOD activity, both of which are markers of oxidative stress [22], were significantly reversed by XQAS in a dose-dependent manner. Cyclooxygenase (COX), an enzyme that converts arachidonic acid to PGs, has been found to have two isofoms, namely, COX-1 and COX-2. COX-2 is responsible for production of large amounts of proinflammatory PGs, especially PGE-2, at the inflammatory site [25]. PGE-2 has an effect on dilating blood vessels, resulting in fever and pain. It can also work synergistically with other inflammatory mediators to exacerbate inflammatory response [26]. NO, which is produced by iNOS activation, can regulate contractile function of skeletal muscle [27] and also lead to vasodilatation to increase perfusion, which is essential for remodeling the damaged muscle but results in tissue edema [28]. XQAS can also suppress STI-caused upregulations of COX-2 and iNOS expression with overproductions of PGE-2 and NO in a dose-dependent manner. In addition, the effects of treatment with CPS and IHS alone on improving aforementioned abnormality in the injured muscle tissue were less than the XQAS effects and there is a synergistic effect between CP and IH components in the XQAS.

NF-κB is a pivotal factor that transfers inflammatory signals from cytoplasm into the nucleus and induces a series of inflammatory responses in the cell [29]. During inactive state, NF-κB subunit p65/p50 is combined with its multiple inhibitors, IκBα such as IκBα, in the cytoplasm. IκBα can be phosphorylated by IκB kinase (IKKα and IκKβ) resulting in separation between IκB and NF-κB and IκB degradation via ubiquitination; the nuclear localization signal of NF-κB located in the p65 is then unmasked. Next, the NF-κB complex would translocate into the nucleus after separation with IκBα [14]. NF-κB activity can be regulated by MAPK and AKT pathway activation through promoting degradation of IκBα. The MAPK family consists of extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 [30]. p38 not only leads to IκBα phosphorylation but also leads to nuclear p65 phosphorylation at Ser276 via activating mitogen- and stress-activated protein kinase-1 (MSK-1) [31]. However, JNK mainly mediates NF-κB signaling via effects on c-Jun and c-Fos [32]. AKT mediates NF-κB activity mainly through inducing IκKβ phosphorylation and then leads to IκBα ubiquitinated degradation [33]. An important aspect of the significance of NF-κB p65/p50 in inflammatory reaction
Figure 3: Effects of XQAS on inflammation-related signaling pathway with IκBα expression in cytosol (a), NF-κB p65 mRNA expression (b), NF-κB p65 expression in cytosol extraction (CE) and nuclei extraction (NE) (c), and expressions of total AKT (t-AKT), Ser\(^{473}\) phosphorylation of AKT (p-AKT), total p38 (t-p38), Thr\(^{180}/\text{Tyr}^{182}\) phosphorylation of p38 (p-p38), total JNK (t-JNK), and Thr\(^{183}/\text{Tyr}^{185}\) phosphorylation of JNK (p-JNK) in the control (Ctrl), acute STI model (Mod), high-dose XQAS-treated (HXQAS), low-dose XQAS-treated (LXQAS), CPS-treated (CPS), and IHS-treated (IHS) group after modeling with or without repeated drug treatment for 8 h. The data were shown as mean ± SD (n = 6 per group). *P < 0.05; **P < 0.01.
Figure 4: Representative histopathological micrographs (×200, HE staining) in the control (Ctrl), acute STI model (Mod), high-dose XQAS-treated (HXQAS), low-dose XQAS-treated (LXQAS), CPS-treated (CPS), and IHS-treated (IHS) group after modeling with or without repeated drug treatment for 8 h. Normal muscle tissue in Ctrl; severe muscle fibers disruption and necrosis, significant red blood cell accumulation in the interstitial space, and inflammatory cells infiltration in the Mod; very significant pathological improvement in injured muscle with markedly reduced area of the disruption and necrosis, decreased interstitial ecchymosed and inflammatory cells infiltration in the HXQAS group, and still marked muscle tissue injury but improved with marked muscle fibers disruption and necrosis, red blood cell accumulation in the interstitial space, and inflammatory cells infiltration in the LXQAS, CPS, and IHS group were shown.

stems from it both being activated by and inducing the expression of inflammatory cytokines [14].

A recent study has indicated that blockade of MAPK-NF-κB pathway activation protects mice from tissue injury by reducing the production of proinflammatory cytokines [34]. Suppression of AKT activation has also been shown to reduce LPS-induced inflammatory responses in the human endothelial cells [35]. From our results, we found that XQAS by suppressing activations of both AKT and p38 pathway quickly abolished activation of NF-κB p65. NF-κB gene expression and its translocation from cytosol to nucleus were enhanced owing to IκBα degraded in the acute injured muscle, while XQAS treatment led to a strong suppression on inflammatory cascade to acute STI. XQAS combining characteristics of CPS and IHS, where CPS merely suppressed p38 activation while IHS suppressed AKT activation majorly, achieved a super effectiveness on preventing IκB degraded and NF-κB translocation into nucleus activated by STI. There is a synergistic effect between CPS and IHS active components in the XQAS. The critical super effectiveness of XQAS on injured muscle may be effective suppression on IκBα degradation to lead to abolishment of NF-κB translocation from cytosol to nucleus, resulting in a strong suppression on transcriptional activation of NF-κB itself and target with inflammation-related genes and on subsequent muscle necrosis, blood stasis in the interstitial space, inflammatory cells infiltration,
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5. Conclusion

XQAS is a quite reasonable formulation with a combination of active components of *Cynanchum paniculatum* (CP) and *Illicium henryi* (IH) in treating acute STI. The mechanism of impressive effects of XQAS, at least majorly, is due to its anti-inflamatory and antioxidative activities with a synergistic effect between active components of CP and IH, in which the CP components suppress STI-induced p38 phosphorylated activation with intranuclear NF-κB p65 phosphorylation, while the IH components suppress STI-induced AKT phosphorylated activation; subsequently, IκB degradation in the cytoplasm, which is promoted by both p38 and AKT pathways activation, is blocked to lead to decrease in NF-κB p65/p50 translocated into the nucleus after separation with IκB; ultimately, NF-κB p65/p50 actions on transcriptional activation of NF-κB itself and target with inflammation-related genes, all of which are enhanced by both p38 and AKT pathways activation, are abolished. The mechanism process was shown in Figure 5.
Competing Interests
The authors declare that they have no competing interests regarding this paper.

Authors’ Contributions
Shudong Wang, Tao Li, and Wei Qu equally contributed to this work.

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