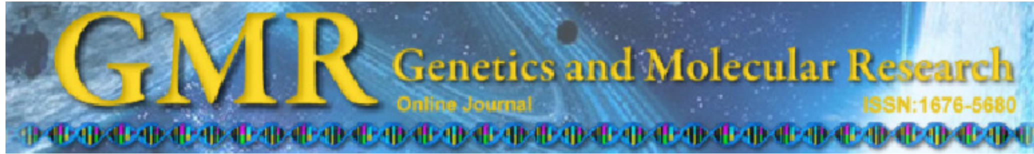

A potential indicator of denervated muscle atrophy: the ratio of myostatin to follistatin in peripheral blood

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ABSTRACT. Myostatin is a secreted negative regulator of muscle mass, and follistatin antagonizes the function of several members of the TGF-β family, including myostatin. Previously, myostatin expression was found to be closely associated with atrophy of the gastrocnemius muscle, showing a linear correlation, after sciatic nerve injury. In this study, we investigated the possibility of myostatin being an indicator of denervated muscle atrophy. ELISA was used to detect the concentration of myostatin and follistatin in sera collected from individual rats at different times after sciatic nerve crush. A strong correlation was shown between the expression level of secreted myostatin in circulation and the wet weight ratio of the gastrocnemius muscle. The ratio of follistatin/ myostatin could be used to monitor the progress of target muscle

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atrophy and recovery. Our study provides a potential serological test to detect denervated muscle atrophy for clinical purposes.

Key words: Myostatin; Follistatin; Denervated muscular atrophy; Serological test

INTRODUCTION

Myostatin (Mstn), also called growth differentiation factor 8 (GDF8), is a transforming growth factor β protein family member, specifically expressed in developing and adult skeletal muscle, serving as a negative regulator of muscle mass (McPherron et al., 1997; Lee and McPherron, 2001). Like other TGF-β family proteins, Myostatin is primarily produced as a precursor protein containing a signal peptide, an N-terminal propeptide and a C-terminal domain that harbors the active peptide. During myostatin maturation, its N-terminal is cleaved by proteases to release the active C-terminal dimer. When secreted into the circulatory system, the N-terminal propeptide remains bound noncovalently to the C-terminal dimer, which maintains myostatin in a latent, inactive form. When latent myostatin arrives at the target cell of skeletal muscle, it releases the active C-terminal dimer, which binds to its receptor called activin type II, to activate the downstream signal pathway (Thies et al., 2001; Wolfman et al., 2003). Myostatin acts as a modulator to control the normal size of skeletal muscle. Animals lacking myostatin naturally or artificially show large muscles. It has been reported that skeletal muscle atrophy is associated with an increased expression of myostatin (Whittemore et al., 2003; Lee et al., 2010).

In our previous study, we found that myostatin expression is closely associated with atrophy of the gastrocnemius muscle in a linear fashion after sciatic nerve injury (transection or crush) in the rats, and that the expression level of myostatin promotes the progression of muscle atrophy after nerve injury and nerve regeneration. Therefore, myostatin could be considered a marker of muscle atrophy and nerve regeneration (Zhang et al., 2006; Liu et al., 2007). A benefit of myostatin being a protein secreted into the bloodstream is that it is easy to detect the alteration of myostatin expression level. Here, we investigated the possibility of myostatin being an indicator of denervated muscle atrophy.

Meanwhile, we considered that the concentration of circulating myostatin in individuals is variable, since it is sometimes influenced by age, gender or hormone levels of the patient. Therefore, a certain value of myostatin in one's blood is not sufficient to determine the progression of muscle atrophy. We looked for another gene that could directly reflect the function of myostatin in the body, where their relationship could be followed.

Follistatin, known as activin-binding protein, was initially identified as a protein that inhibits follicle-stimulating hormone (FSH) secretion from the anterior pituitary (Ueno et al., 1987), and now it has been determined that follistatin antagonizes the function of several members of the TGF-β family that are secreted signaling factors, including myostatin (Amthor et al., 2004). Lee and McPherron (2001) addressed this inhibition of myostatin, either by using knockout mice or by increasing the amount of follistatin, resulting in greatly increased muscle mass. Amthor et al. reported that follistatin and myostatin interact directly with high affinity *in vitro*, and found that follistatin complexes with myostatin and could antagonize myostatin-mediated inhibition of myogenesis *in vivo* (Amthor et al., 2004). To date, numerous studies

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have reported the ability of follistatin to antagonize myostatin both *in vitro* and *in vivo* (Gilson et al., 2009; Rodino-Klapac et al., 2009; Diel et al., 2010; Lee et al., 2010). Previously, it was found that the lack of follistatin resulted in muscle mass loss (Matzuk et al., 1995), whereas over-expression of the follistatin gene led to excess muscle growth. Thompson et al. clearly revealed that the structure of follistatin protein has the characteristic to bind activin A and other BMP ligands, such as myostatin. Particularly, the N-terminal domain of follistatin occupies the type I receptor binding site, and follistatin domain 1 and follistatin domain 2 could block the type II receptor binding site (Thompson et al., 2005). In this respect, follistatin may be regarded as a potent blocker of myostatin *in vivo*. Thus, in this study, we also wanted to detect alterations in follistatin with myostatin in blood simultaneously.

Skeletal muscle atrophy, characterized by a profound reduction in skeletal muscle mass and functional capacity, occurs as a result of disuse (e.g., immobilization, denervation, muscle unloading), aging, starvation, and a number of disease states (e.g., cachexia). Atrophy of the target skeletal muscle induced by denervation after peripheral nerve injures is common, but recovery is often incomplete. Early and accurate diagnosis is critical for proper treatment and successful control of muscle atrophy. Now, in clinical trial, the methods to detect the dystrophic degree of denervated skeletal muscle mainly utilize the electromyogram (EMG), the difference in perimeter of the affected and unaffected limbs, the level of serum creatine kinase (CK), or biopsy of the affected muscle, and so forth. Among these, EMG and perimeter difference are functional indices. Serum CK is an enzyme that catalyzes the conversion of creatine and consumes ATP to create phosphocreatine and ADP, and it is expressed in various tissues and cell types (Wallimann et al., 1992). These methods provide nonspecific indices for target skeletal muscle. Although the method of muscle biospy and pathological examination is the gold standard for the diagnosis of muscular dystrophy, it could offer a new injury to the patient. Therefore, a novel non- invasive and effective method for the diagnosis of skeletal muscle atrophy with high sensitivity and specificity is worth pursuing. Based on a good understanding of the myostatin gene, we investigated the possibility of myostatin serving as a novel indicator in a clinical test. Therefore, in this study, we used the gastrocnemius muscle atrophy model caused by rat sciatic nerve crush. Following the progression of muscle atrophy and recovery, we determined the sciatic nerve functional index (SFI) (Dinh et al., 2009) and took blood samples for each rat at different postoperative time points. The blood was collected to detect the expression of myostatin and follistatin using ELISA methods.

MATERIAL AND METHODS

Animals and reagents

Thirty-six male Sprague Dawley (SD) rats (180-200 g), were provided by the Experimental Animal Center of Nantong University. Rat myostatin (MSTN) and follistatin (FST) ELISA kits were from R&D Company, USA.

Preparation of animal model

The surgical procedure of sciatic nerve crush was performed according to a modification of the pervious reported method (Liu et al., 2007). Briefly, the SD rats were anesthetized

by an injection of complex narcotics (3 mg/kg), and the sciatic nerve was identified and lifted through an incision on the lateral aspect of the mid-thigh of the left hind leg. The left lateral sciatic nerve was crushed with a fine hemostat three times (10 s each time) with an interval of 10 s. For consistency of the procedure, all operations of sciatic nerve crush were done by the same well-experienced person. The surgical incisions were closed and animals were returned to their cages.

All experiments were conducted in accordance with guidelines established by the NIH, found in Guide for the Care and Use of Laboratory Animal (1985), and by the Society for Neuroscience, found in Guidelines for the Use of Animals in Neuroscience Research. The experiments were approved according to the Animal Care and Use Committee of Nantong University and the Jiangsu Province Animal Care Ethics Committee (Approval ID: SYXK(SU)2007-0021).

Determination of sciatic functional index (SFI)

In order to examine the extent of loss or recovery of locomotive function after sciatic nerve crush injury, we performed walking-track analysis with each rat before the blood collection. As described in a previous study (Liu et al., 2007; Dinh et al., 2009), the rats were allowed conditioning trials on an 8.2 x 50 cm walking track with a piece of white paper at the bottom of the track. The hind feet were dipped in red ink, leaving prints on the white paper. The print length (PL), toe spread (TS), and intermediary toe spread (IT) were thus obtained. In general, the maximal value was adopted for each measurement, and the data were recorded with the prefix E for the operated side and N for the normal non-operated side. The sciatic function index (SFI), an indicator of the degree of nerve dysfunction, varies from 0 to 100, with 0 corresponding to normal function and 100 to complete dysfunction. The sciatic nerve functional index (SFI) was then calculated by the following Bain formula:

SFI = 109.5 x (ETS-NTS)/NTS-38.3 x (EPL-NPL)/NPL+1 3.3 x (EIT-NIT)/NIT-8.8.

Wet weight ratio measure

On days 3, 7, 14, 21, 28 after surgery and including normal control (day 0), 4 rats in different operated groups were killed, and gastrocnemius muscles from both the operated and contralateral sham-operated limbs were removed and weighed for determination of muscle wet weight.

Serum sample collection

We used the method of rat cardiac puncture at the site below the xiphoid process for repeated blood sampling. In the normal group (day 0) and on day 3, 7, 14, 21, 28 after sciatic nerve crushed, under anesthesia, a 200-μL blood sample was collected from each rat. After blood collection, the needle was removed, and the puncture site was pressed for 1 min to stop any bleeding. After awakening, rats were returned to their cages with normal routine feeding. The blood samples were first incubated in a 37°C water bath for 30 min, then transferred to 4°C for 1 h, and finally centrifuged for 15 min at 10,000 rpm. The serum was transferred to a new 1.5-mL Eppendorf tube, and then aliquoted and stored at -80°C.

ELISA for rat myostatin and follistatin expression

According to the instruction of the ELISA kit, a serial dilution was used to set up a series of different standard samples. The final concentrations for the myostatin standard were 4.8, 3.2, 1.6, 0.8, 0.4, 0.2 and 0.1 ng/mL, respectively. For Follistatin standard sample, they were 0.6, 0.4, 0.2, 0.1 and 0.05 ng/mL. Meanwhile, blank wells did not contain sample or HRP-conjugate reagent. For the test sample well, 40 μL of sample diluting solution were first added and then 10 μL of serum sample, followed by gentle mixing. After covering with closure plate membrane, the plates were incubated at 37°C for 30 min. They were then washed 5 times, allowed to stand for 30 s, drained and patted dry. HRP-conjugate reagent (50 μL) was added to each well (except blank well), and the plates incubated at 37°C for 30 min. After washing 5 times, 50 μL of chromogen solution A and B mixture (A:B = 1:1) were added to each well and kept in the dark at 37°C for 15 min. Stop solution (50 μL) was added to complete the reaction. Within 15 min, the absorbance was read at 450 nm with a Synergy™ 2 Multi-Mode Microplate Reader (BioTek Instruments, USA).

Statistical analysis

All data analyses, statistical comparisons, and graphs were generated using Excel (Microsoft). Repeated measurements were performed in three or four separate experiments. All data represent means ± SE, and were analyzed by one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons and unpaired Student's t tests when necessary. Probability values less than 0.05 were considered significant. The final figure processing was performed with Photoshop (Adobe).

RESULTS

Sciatic nerve functional index (SFI)

SFI analysis showed that almost complete dysfunction of the sciatic nerve occurred on day 1 after nerve injury. Afterwards, a steady recovery of locomotive function of the crushed sciatic nerve in the rats was evidenced by continuous increases in the SFI value until the SFI reached around -20 at day 28 post-surgery (Figure 1). The model therefore was suitable for evaluating the degree of locomotive function recovery.

Muscle atrophy induced by nerve crush

In each repeated experiment, we randomly kept three rats in the sham-operated group and three rats in the injury group to monitor the degree of gastrocnemius muscle atrophy using the method of wet weight ratio. Figure 2A shows the change in the wet weight ratio of gastrovnemius muscle (the operated side/contralateral non-operated side) after crush injury to the sciatic nerve. Starting at day 1 after nerve injury, the muscle wet weight ratio gradually decreased, reaching a minimum level at about day 14, and then gradually recovered to close to the normal level at day 28. Significant differences were found on day 7 ($P < 0.05$), 14 ($P < 0.01$), and 21 ($P < 0.05$) after never injury as compared

to the control. The regression equation was $y = 0.0024x^2 - 0.0731x + 1.0092$, $R^2 = 0.9873$, $r = 0.9936$, $P < 0.001$.

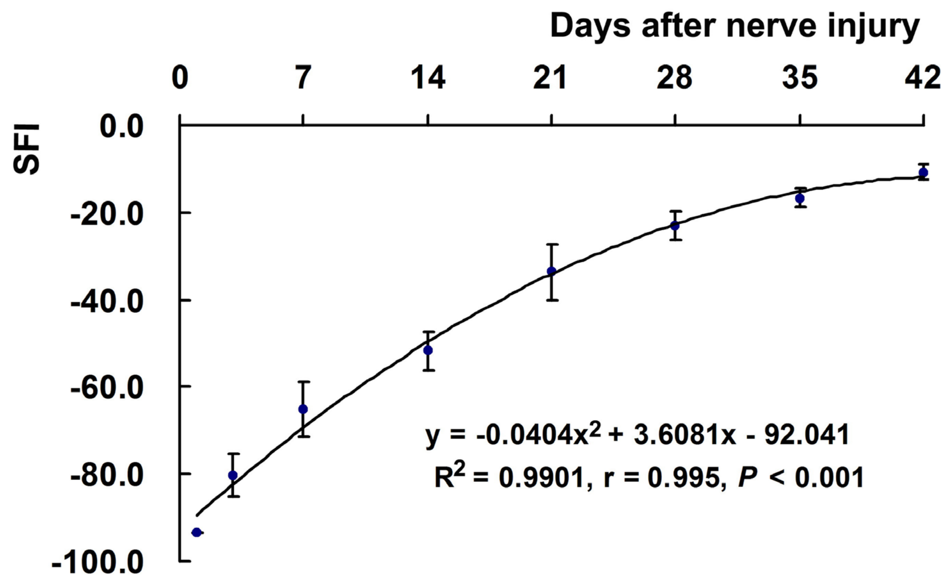

Figure 1. SFI values of rats at different time points after sciatic nerve crush injury.

ELISA analysis of rat myostatin

The ELISA method was so sensitive that we only needed 10 µL of serum sample to obtain the concentration of myostatin. In the normal rat, the concentration of myostatin was 2.233 ± 0.066 ng/mL in peripheral blood. As shown in Figure 2b, the ELISA results of rat myostatin (MSTN) indicated that the serum concentration of myostatin protein increased from day 0 to 14, followed by a decrease from day 14 to 28 after sciatic nerve crush. As found in our previous study, the ELISA analysis showed that expression of myostatin protein in whole blood was also dependent on time and degree of atrophy, and that this coincided with myostatin protein level in gastrocnemius muscle. At day 14 post-injury, we detected a significantly higher level of myostatin protein in blood compared to control. $[MSTN]_{14d} = 2.542 \pm 0.057$ ng/mL; $P = 0.024$. The regression equation was $y = -0.0013 x^2 + 0.0388 x + 2.2005$, $R^2 = 0.9223$, $r = 0.960$, $P < 0.01$. Moreover, the changes in MSTN concentration correlated linearly with change in wet weight ratio (Figure 2C, $y = -1.7602 x + 4.8809$, $R^2 = 0.952$, $r = 0.976$, $P < 0.001$).

ELISA analysis of rat follistatin

As shown in Figure 3A, the ELISA results of rat follistatin (FS) indicated that the serum concentration of follistatin protein increased from day 0 to 21, followed by a decrease after day 21 post-sciatic nerve crush. In the normal rat, the concentration of follistatin was 242.8 ± 10.49 ng/L in peripheral blood. At day 21 post-injury, we detected a significantly higher level of follistatin protein in serum compared to control. $[FS]_{21d} = 282.1 \pm 8.60$ ng/mL;

P = 0.044. The regression equation was $y = -0.0865x^2 + 3.7538x + 232.69$, $R^2 = 0.7894$, r = 0.888, P < 0.02. We knew that follistatin protein level in blood peaks around day 21 post-injury. ELISA analysis showed that expression of follistatin protein in blood was also dependent on time and degree of atrophy, findings very similar to those for myostatin, and therefore we examined the relationship between myostatin and follistatin.

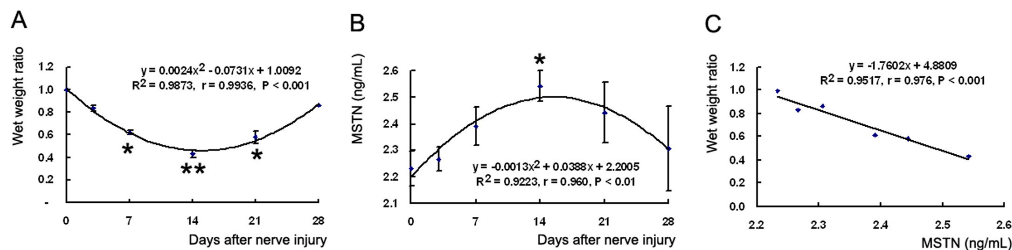

Figure 2. The wet weight ratio of the gastrocnemius muscle (operated side/contralateral non-operated side) (A) and the expression level of myostatin in circulation in rats at different times after sciatic nerve crush injury. *P < 0.05, **P < 0.01 vs. the control group. Non-linear correlation analysis: (A) $y = 0.0024x^2 - 0.0731x + 1.0092$, $R^2 = 0.9873$, r = 0.9936, P < 0.001; (B) $y = -0.0013 x^2 + 0.0388 x + 2.2005$, $R^2 = 0.9223$, r = 0.960, P < 0.01. Linear correlation analysis: (C) $y = -1.7602 x + 4.8809$, $R^2 = 0.952$, r = 0.976, P < 0.001.

Correlation between myostatin and follistatin in blood after nerve injury

Thus we analyzed the correlations between myostatin and follistatin protein levels over the entire time course from day 0 to day 28 after sciatic nerve injury. We converted the data of the samples to FS/MSTN ratios for each individual rat at different time points. As shown in Figure 3B, myostatin and follistatin showed a non-linear correlation of $y = -4E\text{-}05x^3 + 0.002x^2 - 0.0199x + 1$, R2 = 0.9907, r = 0.995, P < 0.001. If we assumed the expression level of myostatin or follistatin protein in the control group was 1, the ratio of FS/MSTN was below 1 during muscle atrophy from day 0 to day 14, and the ratio of FS/MSTN was above 1 during muscle recovery from day 14 to day 28.

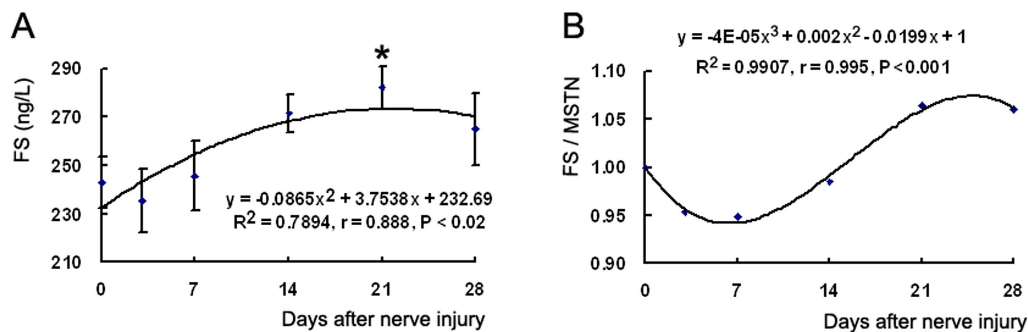

Figure 3. The expression level of follistatin in circulation in rats at different times after sciatic nerve crush injury and non-linear correlation analysis (A): *P < 0.05; $y = -0.0865x^2 + 3.7538x + 232.69$, $R^2 = 0.7894$, r = 0.888, P < 0.02; The follistatin/myostatin ratio in individual rats at different times after sciatic nerve crush injury and non-linear correlation analysis (B): $y = -4E\text{-}05x^3 + 0.002x^2 - 0.0199x + 1$, $R^2 = 0.9907$, r = 0.995, P < 0.001.

DISCUSSION

In the present study, we aimed to investigate the possibility of myostatin being an indicator of skeletal muscle atrophy caused by sciatic nerve injury. Based on our previous data (Liu et al., 2007), the expression level of myostatin protein in gastrocnemius muscle was time-dependent, consistently close to the degree of muscle atrophy. Since myostatin is a secreted protein, we considered to possibility of determining the expression level of myostatin protein in blood to estimate the degree of muscle atrophy. In 2009, Lakshman showed that myostatin ELISA has the characteristics of a valid, accurate, and sufficiently sensitive assay, to enable measurement of myostatin concentrations in the human body (Lakshman et al., 2009).

In this study, we chose the model of gastrocnemius muscle atrophy caused by sciatic nerve crush to examine the pattern of myostatin expression in whole blood of each animal. This kind of model could used to trace the change in blood myostatin levels in an individual over time post-injury. Our results showed that the expression level of myostatin in blood was similar to the expression pattern of myostatin in gastrocemius muscle after sciatic nerve crush injury. After sciatic nerve injury, the concentration of myostatin increased from day 0 to day 14, followed by a decrease from days 14-28. Correlation analysis showed that secreted myostatin protein was also time-dependent, and we found that peak value for myostatin protein in blood occurred around 18 days post-injury. We also analyzed the correlation of secreted myostatin with the wet weight ratio of gastrocemius muscle and found, as shown in Figure 2C, that MSTN concentration correlated with the wet weight ratio in a negatively linear fasion. This means that if we can determine the concentration of myostatin of the patient at different time points, and the change in MSTN expression in blood could assess the degree of gastrocemius muscle atrophy caused by denervation.

We also investigated the alteration of follistatin expression in the circulatory system of each individual rat, since myostatin propeptide, follistatin and the follistatin related gene (FLRG) can serve as inhibitory binding proteins of myostatin in normal serum (Hill et al., 2002; Rodino-Klapac et al., 2009). Our ELISA results demonstrated that after sciatic nerve injury, the concentration of follistatin graduately increased from day 0 to day 21, followed by a decrease after day 21. The correlations between follistatin and wet weight ratio, myostatin and follistatin were analyzed, and regression analysis showed that there was no significant correlation with follistatin and wet weight ratio.

We know that myostatin can be found in the blood in an inactive state when bound to follistatin, FLRG, or GASP-1. These peptides block the activation of the myostatin pathway. When the pathway is inhibited, the active myostatin dimer cannot bind to its receptor, ActRI-IB, and myostatin function is then disrupted. In our study, we found that follistatin expression increased during the recovery phase, lasting longer compared to myostatin. Here, we propose a hypothesis based on our results. If we assume that circulating myostatin and follistatin levels are in balance in normal individuals to maintain muscle mass, the ratio FS/MSTN is regarded as 1. Shown in Figure 3B, the ratio of FS/MSTN at different time points after nerve injury fit a curve of $Y = -4E\text{-}05x^3 + 0.002x^2 - 0.0199x + 1$, ($R^2 = 0.9907$, $r = 0.995$, $P < 0.001$). Taken together, the ratio of myostatin and follistatin was closely linked to the degree of denervated muscle atrophy. During the period of muscle atrophy and recovery, the amount of follistatin in blood represented a concomitant but delayed alteration with myostatin. At the turning point of muscle atrophy (day 14), the performance of follistatin was equal to that of myostatin, and

the ratio of follistatin/myostatin was almost 1. After 14 days post-nerve injury, the ratio was above 1. This means that the function of follistatin was stronger than that of myostatin, and that the target muscle was undergoing recovery. Conversely, from days 1 to 14, during the atrophy phase, the ratio was below 1.

In view of the above, the ratio of follistatin and myostatin can be calculated based on their concentration in blood at any time point after nerve injury. The state of muscle atrophy or recovery can be assessed according to the ratio. The present study could be used in a possible trial for clinical non-invasive diagnosis to monitor the process of muscle atrophy after nerve injury and nerve regeneration.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

ACKNOWLEDGMENTS

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