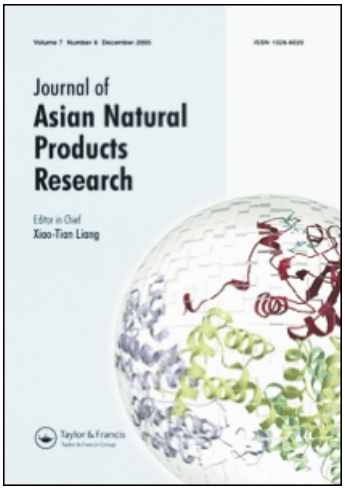

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Therapeutic effects of hydroxysafflor yellow A on focal cerebral ischemic injury in rats and its primary mechanisms

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To cite this Article Zhu, Hai-Bo , Zhang, Ling , Wang, Zheng-Hua , Tian, Jing-Wei , Fu, Feng-Hua , Liu, Ke and Li, Chang-Ling(2005) 'Therapeutic effects of hydroxysafflor yellow A on focal cerebral ischemic injury in rats and its primary mechanisms', Journal of Asian Natural Products Research, 7: 4, 607 — 613
To link to this Article: DOI: 10.1080/10286020310001625120
URL: http://dx.doi.org/10.1080/10286020310001625120

Journal of Asian Natural Products Research, Vol. 7, No. 4, August 2005, 607–613

Therapeutic effects of hydroxysafflor yellow A on focal cerebral ischemic injury in rats and its primary mechanisms

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(Received 2 June 2003; revised 9 July 2003; in final form 16 July 2003)

The therapeutic effects of hydroxysafflor yellow A (HSYA), extracted from *Carthamus tinctorius.* L, on focal cerebral ischemic injury in rats and its related mechanisms have been investigated. Focal cerebral ischemia in rats were made by inserting a monofilament suture into internal carotid artery to block the origin of the middle cerebral artery and administrated by HSYA *via* sublingular vein injection in doses of 1.5, 3.0, 6.0 mg kg^{-1} at 30 min after the onset of ischemia, in comparison with the potency of nimodipine at a dose of 0.2 mg kg^{-1}. Then, 24 h later, the evaluation for neurological deficit scores of the rats were recorded and postmortem infarct areas determined by quantitative image analysis. At the end of the experiment, blood samples were taken to determine plasma 6-Keto-$PGF_{1\alpha}$/TXB_2 by radioimmunoassays and blood rheological parameters. The effects exerted by HSYA on thrombosis formation by artery vein by-pass method and ADP-induced platelet aggregation *in vivo* and *in vitro* were investigated, respectively. The results indicated that more than 30% of the area of ischemic cerebrum was observed in the ischemic model group. HSYA dose-dependently improved the neurological deficit scores and reduced the cerebral infarct area, and HSYA bore a similarity in potency of the therapeutic effects on focal cerebral ischemia to nimodipine. The inhibition rates of thrombosis formation by HSYA at the designated doses were 20.3%, 43.6% and 54.2%, respectively, compared with saline-treated group. Inhibitory activities of HSYA were observed on ADP-induced platelets aggregation in a dose-dependent manner, and the maximum inhibitory aggregation rate of HSYA was 41.8%. HSYA provided a suppressive effect on production of TXA_2 without significant effect on plasma PGI_2 concentrations. Blood rheological parameters were markedly improved by HSYA, such as whole blood viscosity (from 21.71 ± 4.77 to 11.61 ± 0.90 mPa.s), plasma viscosity (from 2.73 ± 0.53 to 1.42 ± 0.07 mPa.s), deformability (from 0.66 ± 0.26 to 0.77 ± 0.33) and aggregation of erythrocyte (from 3.24 ± 0.41 to 2.57 ± 0.30), but no significant effect of HSYA on homatocrit was found (from 51.38 ± 4.68% to 49.91 ± 2.32%). HSYA appears to be a good potential agent to treat focal cerebral ischemia, and the underlying mechanisms exerted by HSYA might be involved in its inhibitory effects on thrombosis formation and platelet aggregation as well as its beneficial action on regulation of PGI_2/TXA_2 and blood rheological changes in rats.

Keywords: *Carthamus tinctorius* L; Hydroxysafflor yellow A; Focal cerebral ischemia; Thrombosis formation; PGI_2/TXA_2; Blood rheology

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Journal of Asian Natural Products Research
ISSN 1028-6020 print/ISSN 1477-2213 online © 2005 Taylor & Francis Group Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/10286020310001625120

1. Introduction

The brain is susceptible to ischemia-induced damage followed by thrombotic block. Once occluded, dysfunction in neurons will happen within 5 min. Generally, cerebral ischemia is characterized by the state of hypercoagulability and hyperviscosity in circulation, which is prone to form thrombosis. Hydroxysafflor yellow A, (abbreviated HSYA; figure 1) is a pure compound extracted from *Carthamus tinctorius.* L. Our previous work demonstrated that HSYA markedly extended coagulation time in mice, which raises the possibility that HSYA might exert therapeutic actives on cerebral ischemia induced by thrombosis. Currently, experimental studies on cerebral ischemia had been based upon the models of cerebral ischemia in animals [1]. Of the methods of establishment of focal cerebral ischemic models, the way of inserting a monofilament suture into internal carotid artery to block the origin of the middle cerebral artery has been widely used. The pathophysiological process in this model is confirmed to correspond to human stroke, and especially stimulates the patients with initial focal cerebral ischemia, since most of these patients have thrombosis blocked in the middle cerebral artery, according to epidemiological and clinical studies. Therefore, in this study, the evaluation of therapeutic effect afforded by HSYA on focal cerebral ischemia was investigated in the middle cerebral artery occlusion model.

2. Results and discussion

2.1 Therapeutic effects of HSYA on focal cerebral ischemia in rats

In a therapeutic experiment, 24 h after the onset of MCAO, neurological deficit scores and ratios of infarct area and cerebrum in MCAO rats were significantly higher than those of a sham group, indicating that the MCAO model was established successfully. Treatment with HSYA at doses of 3.0 and 6.0 mg kg^{-1} significantly reduced the infarct area by 60% and 85% respectively *vs*. MCAO group, and improved the score in the neurological deficit tests. However, the 1.5 mg kg^{-1} dose of HSYA was not found to be ineffective. HSYA at dose of 6.0 mg kg^{-1} showed a similar potency as compared with that of nimodipine at a dose of 0.2 mg kg^{-1} (table 1).

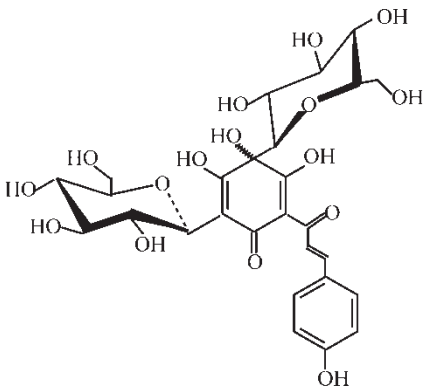

Figure 1. Structure of HSYA.

Table 1. Effects of hydroxysafflor yellow A on focal cerebral ischemic injury in MCAO rats (mean ± SD).

Group	*Dose (mg kg^{-1})*	*Neurological deficit scores*	*Infarction area of brain (%)*
Sham	–	0	1.2 ± 0.55
NS	–	10.20 ± 0.92[a]	31.9 ± 9.2[a]
HSYA	1.5	9.30 ± 1.06[b,c]	30.1 ± 12.9[c]
HSYA	3.0	7.20 ± 0.63[c,d]	13.4 ± 6.3[c,d]
HSYA	6.0	5.80 ± 1.14[d]	4.8 ± 2.9[d]
Nimodipine	0.2	4.90 ± 1.45[d]	4.9 ± 2.6[d]

Ten rats in each group. [a]$P < 0.01$ *vs.* sham; [b]$P < 0.05$ *vs.* saline; [c]$P < 0.01$ *vs.* nimodipine; [d]$P < 0.01$ *vs.* saline.

2.2 Effects of HSYA on PlaHSYA 6-Keto-PGF$_{1\alpha}$ and TXB$_2$ concentrations in MCAO rats

Plasma 6-Keto-PGF1α and TXB2 concentrations, which are steady metabolites of PGI2 and TXA2, were determined, respectively. As shown in table 2, there was a slight increase in 6-Keto-PGF1α in MCAO rats, but the changes were unlikely to be of experimental significance. The results showed that HSYA exerted a inhibitory effect on the production of TXB2 in a dose-dependent manner, but no obvious effect of HSYA was observed on the formation of 6-Keto-PGF1α, indicating that HSYA increased the ratio of 6-Keto-PGF1α and TXB2 (table 2).

2.3 Effects of HSYA on blood rheological parameters in MCAO rats

The results of the main blood rheological parameters are summarized descriptively in tables 3 and 4. 24 h post MCAO, the blood rheological parameters of the rats such as blood viscosity, plasma viscosity, deformability and aggregation of erythrocyte were significantly higher than those in the sham group. HSYA provided an inhibitory effect on the parameters mentioned above, but no dose-dependent relationship existed. There was no apparent difference in homatocrit changes between saline-treated rats and HSYA-treated rats.

2.4 Antithrombotic effects of HSYA in rats

In the artery vein bypass-induced thrombosis experiment, HSYA at doses of 1.5, 3.0, 6.0 mg kg^{-1} markedly reduced thrombotic weight in a dose-dependent fashion, and the inhibitory activity of HSYA at dose of 6.0 mg kg^{-1} was equivalent to the 87% potency bore by heparin sodium at dose of 0.2 mg kg^{-1} (figure 2).

Table 2. Effects of hydroxysafflor yellow A on production of plasma 6-keto-PGF$_{1\alpha}$ and TXB$_2$ in MCAO rats (mean ± SD).

Group	*Dose (mg kg^{-1})*	*6-Keto-PGF$_{1\alpha}$ (ng L^{-1})*	*TXB$_2$ (ng L^{-1})*
Sham	–	613.15 ± 143.54	456.08 ± 251.98
NS	–	642.08 ± 191.74[a]	976.57 ± 306.47[a]
HSYA	1.5	623.65 ± 223.87	737.42 ± 256.55[b,c]
HSYA	3.0	627.43 ± 236.09	581.19 ± 232.32[b,c]
HSYA	6.0	631.87 ± 209.41	537.12 ± 245.11[c,d]
nimodipine	0.2	637.07 ± 237.13	521.43 ± 297.45[c]

Ten rats in each group. [a]$P < 0.01$ *vs.* sham; [b]$P < 0.01$ *vs.* nimodipine; [c]$P < 0.01$ vs saline; [d]$P < 0.05$ *vs.* nimodipine.

Table 3. Effects of hydroxysafflor yellow A on blood viscosity in MCAO rats (mean ± SD).

Group	Dose ($mg\,kg^{-1}$)	Whole blood viscosity (mPa.s)		Plasma viscosity (mPa.s)
		Low shear rate	High shear rate	
Sham	–	7.23 ± 1.75	3.32 ± 1.01	1.12 ± 0.08
NS	–	21.71 ± 4.77[a]	9.23 ± 2.13[a]	2.73 ± 0.53[a]
HSYA	1.5	13.57 ± 2.50[b,c]	4.73 ± 0.43[b,c]	1.60 ± 0.08[b,c]
HSYA	3.0	13.34 ± 2.19[b,c]	4.72 ± 0.65[b,c]	1.58 ± 0.07[b,c]
HSYA	6.0	11.61 ± 0.90[b,c]	4.51 ± 0.40[b,c]	1.42 ± 0.07[b]
Nimodipine	0.2	9.85 ± 1.78[b]	4.25 ± 0.49[b]	1.37 ± 0.05[b]

Ten rats in each group. [a]$P < 0.01$ *vs.* sham; [b]$P < 0.01$ *vs.* saline; [c]$P < 0.01$ *vs.* nimodipine.

2.5 *Effects of HSYA on platelet aggregation induced by ADP* in vitro

Figure 3 summarizes the inhibitory effects of HSYA at different final concentrations on ADP-induced platelet aggregation was observed *in vitro*, but there was no concentration-dependence. The suppression afforded by HSYA at dose of $100\,\mu g\,ml^{-1}$ (final concentrations) on platelet aggregation was equal to the 77.5% potency exerted by acetosalic acid at final concentrations of $30\,\mu g\,ml^{-1}$.

In this study, the therapeutic effects of hydroxysafflor yellow A, extracted from *Carthamus tinctorius. L* on focal cerebral ischemic injury in rats were systemically investigated. The results indicated that HSYA exerted therapeutic activities in a dose-dependent fashion in MCAO rats, characterised by a significant reduction in both neurological deficit scores and the cerebral infarct area.

As long as 30 years ago it was noticed that platelets were activated in the process of cerebral ischemia [2]. Recently, ever more studies have confirmed that platelets usually are in the active state in both general cerebral ischemia and focal cerebral ischemia in rats, and platelets also were found in infarct tissue [3]. The aggregation of platelets and their adhesion to leukocyte contributes greatly to the formation of thrombosis in an evolving brain infarct, and the major factors which induce the aggregation of platelets are ADP, TXA_2 and thrombin, etc. Additionally, a line of evidence suggests that the production of thrombosis followed by misbalance of PGI_2 and TXA_2 plays an important role in the occurrence of focal cerebral ischemia [4]. In our study, the results indicating the inhibitory effects exerted by HSYA on ADP-mediated platelets aggregation, taken together with the beneficial effects exerted by HSYA on the regulations of PGI_2/TXA_2, might give us a better understanding of its inhibition on the formation of thrombosis *in vivo*. Nevertheless, it is necessary to know how PGI_2/TXA_2 and platelets or other unknown factors participate in the suppression of

Table 4. Effects of hydroxysafflor yellow A on blood rheological parameters of erythrocyte in MCAO rats (mean ± SD).

Group	Dose ($mg\,kg^{-1}$)	Erythrocyte aggregation index	Erythrocyte deformability index	Homatocrit (%)
Sham	–	1.95 ± 0.41	0.66 ± 0.18	48.44 ± 2.12
NS	–	3.24 ± 0.41[a]	0.98 ± 0.26[a]	51.38 ± 4.68
HSYA	1.5	2.82 ± 0.34[b,c]	0.88 ± 0.30[b,c]	51.43 ± 3.46
HSYA	3.0	2.63 ± 0.21[b,c]	0.77 ± 0.21[b,c]	49.52 ± 4.29
HSYA	6.0	2.57 ± 0.30[b,c]	0.75 ± 0.33[b,d]	49.91 ± 2.32
Nimodipine	0.2	2.41 ± 0.37[b]	0.71 ± 0.25[b]	50.92 ± 3.64

Ten rats in each group. [a]$P < 0.01$ *vs.* sham; [b]$P < 0.01$ *vs.* saline; [c]$P < 0.01$ *vs.* nimodipine; [d]$P < 0.05$ *vs.* nimodipine.

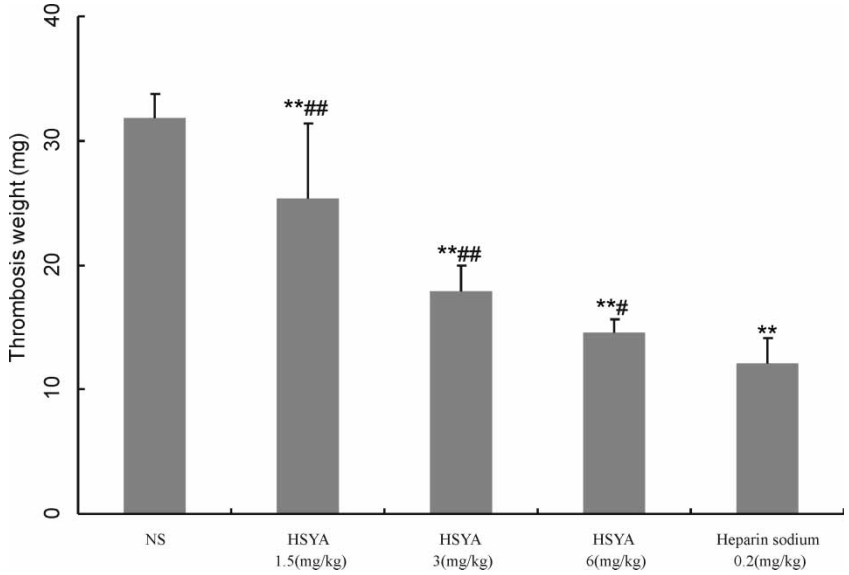

Figure 2. Effects of hydroxysafflor yellow A on ADP induced platelets aggregation *in vitro*.

HSYA on thrombosis formation is for further investigation. As for nimodipine, its inhibitory effect on TXA_2 production could be supported by recent data showing that nimodipine reversed the imbalance of 6-keto-$PGF1_\alpha$/TXB_2 in serum after thrombosis [5].

Both aggregation and deformability of erythrocyte are major factors causing an increase on blood viscosity that triggers blood stagnation. Our observations suggested that focal cerebral ischemia for 24 h induced a significant decrease in erythrocyte deformability and an increase in erythrocyte aggregation as well as blood viscosity, which worsen the dysfunction of cerebral microcirculation after an ischemic event. HSYA provided a significant beneficial

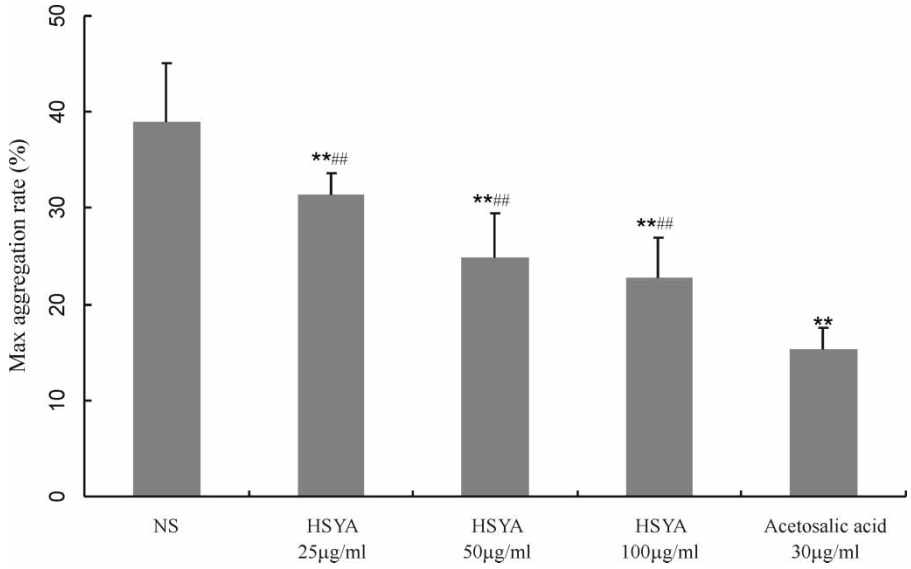

Figure 3. Effects of hydroxysafflor yellow A on artery vein by pass-induced thrombosis formation.

effect on blood rheological parameters. It appears likely that HSYA exerted therapeutic activity in MCAO rats benefited from its action of anticoagulation. In support of this suggestion, it has been observed that HSYA dramatically extended the bleeding time in mice (author's unpublished data). In this study, therefore, these data, taken together with suppression of HSYA on thrombosis formation followed by its inhibition on platelets aggregations and its adjustment of PGI_2/TXA_2, at least in part, could be used to explain the mechanisms underlying the therapeutic effects afforded by HSYA in MCAO rats. However, whether direct neuroprotective activity of HSYA on ischemic neurons exists remains to be proven on cultured cerebral cortical cells.

In conclusion, we discovered that HSYA produced a dose-dependent reduction in infarct area, beginning with a dose of $3\,mg\,kg^{-1}$, implicating that the use of HSYA may be considered as a promising candidate to treat ischemic tissue damage following focal cerebral ischemia. In addition, its underlying mechanisms might be associated with its inhibitory effects on blood rheological changes and thrombosis formation followed by platelet aggregation as well as its beneficial action on the PGI_2/TXA_2 ratio.

3. Experimental

3.1 Materials and methods

3.1.1 Drugs and reagents. HSYA (purity $>99\%$, molecular weight: 611.16 Da) was supplied by the Shandong Engineering Research Center for Natural Drugs. Nimodipine was produced by the Shandong Xinhua Pharmaceutical Co. 2,3,5-Triphenyltetrazolium chloride (TTC) was purchased from Sigma Chemical Co (St. Louis, MO). 6-Keto-$PGF_1\alpha$ and TXB_2 kits were products of the Academy of Military Medical Sciences, Beijing. Heparin sodium was product of Light Biochemical Institute of Jiangsu Province. Acetosalic acid powder was purchased from Medica Institute of Guanzhou Hejigong Pharmaceutical Co. All other chemicals used were the best grade available commercially.

3.1.2 Effects of HSYA on focal cerebral ischemic injury, plasma HSYA 6-Keto-$PGF_{1\alpha}/TXB_2$ concentrations and blood rheological parameters in rats. Male Wistar-Kyoto (WKY) rats weighing 382 ± 18 g were obtained from the Experimental Animal Center of Shangdong (Grade II, Certificate No. 980201). Rats were housed for one week to become accustomed to our environmental conditions before experiments. In therapeutic experiments, the effects of a single injection of HSYA were studied in rats with middle cerebral artery occlusion (MCAO). Rats were randomly divided into six groups: sham-operation; MCAO model; 1.5; 3; $6\,mg\,kg^{-1}$ HSYA-treated groups; Nimodipine $0.2\,mg\,kg^{-1}$, ten in each group. Rats were anesthetized with $350\,mg\,kg^{-1}$ chloral hydrate intraperitoneally and placed on dorsal position. Under sterile conditions, the right cervical artery was isolated and a monofilament suture was inserted into internal carotid artery to block the origin of the middle cerebral artery as described by Zea Longa *et al.* [6]. The MCAO rats were treated with HSYA *via* sublingular vein injection in doses of 1.5, 3.0, $6.0\,mg\,kg^{-1}$ or administered nimodipine at a dose of $0.2\,mg\,kg^{-1}$ at 30 min point after the onset of ischemia. The HSYA or nimodipine-untreated group was injected with the same volume of saline as that for HSYA. 24 h post MCAO, the neurological deficit scores of the rats were evaluated by a single

experimenter, who was blind to the experimental treatment groups. Then each rat was decapitated and the brain removed. Brain slices were stained by TTC and photographed. These photographic images were digitized and used to determine the area of infarct and the area of each hemisphere for each slice on a Compix system computer (C imaging 1280 system; Compix Inc Image Systems). At the end of the experiment, blood samples were taken to determine the 6-Keto-$PGF_{1\alpha}$/TXB_2 by radioimmunoassays and blood rheological parameters (blood viscosity, plasma viscosity, deformability and aggregation of erythrocyte, etc.) were determined with a blood rheology automatic analysis apparatus (LBY-N6A, PULESENG Co, Beijing).

3.1.3 Effects of HSYA on thrombosis formed by artery vein by-pass in rats. Male rats weighing 365 ± 17 g were randomly divided into six groups (ten in each group): sham-operation; artery vein by-pass model; 1.5; 3; 6 mg kg^{-1} HSYA-treated groups; heparin sodium 0.2 mg kg^{-1}. The operation procedure was performed as previously described by Liao [7]. The rats were pretreated with HSYA or heparin sodium *via* sublingual vein 5 before thrombosis formation. At 15 min post thrombosis formation, the wet weight of the thrombosis was measured.

3.1.4 Effects of HSYA on platelets aggregation induced by ADP in vitro. Male rats (362 ± 23 g) were randomly divided into five groups: saline (NS); HSYA at final concentrations of 25; 50; 100 μg ml^{-1} groups; acetosalic acid at final concentrations of 30 μg ml^{-1} group. Ten rats were in each group. Blood (4.5 ml) was taken from each rat and poured into the tube containing 3.8% sodium citrate (0.5 ml). The blood samples were then centrifuged at 1000 rpm for 10 min, and the supernatant was removed as platelet-rich plaHSYA (PRP) The remains were then centrifuged at 3000 rpm for 10 min; its supernatant was platelet-poor plaHSYA. The following procedure was performed as described by Wang *et al.* [8]. The platelet aggregation induced by ADP was measured on a platelet agglutometer (LBY-NJ2, PULESENG Co, Beijing).

3.1.5 Statistical analysis. Data are expressed as mean $\pm$ SD and were analyzed with the *t* test.

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