Int J Clin Exp Med 2016;9(6):11058-11064
www.ijcem.com /ISSN:1940-5901/IJCEM0022285

*Original Article*

# Astragalus polysaccharide protects cardiomyocytes from 5-Fluorouracil-induced injury via decreasing ROS production

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Received December 19, 2015; Accepted April 9, 2016; Epub June 15, 2016; Published June 30, 2016

**Abstract:** Chemotherapy-induced cardiotoxicity has reportedly restricted the clinical application of drugs. However, the potential for astragalus polysaccharides (APS) to ameliorate 5-FU-induced cardiotoxicity remains largely unknown. In the present study, an MTT assay was applied to determine whether 5-FU affected cardiomyocyte viability. For in vivo study, the SD rats were randomly divided into three groups by direct gastric gavage for 7 days: Group I: saline group; Group II: 5-FU (1 mg/Kg body weight); and Group III: 5-FU+APS (1.5 g/kg body weight). The in vivo effects of 5-FU on cardiac function were explored through echocardiography. The SOD and MDA contents were also determined. We found that 5-FU significantly enhanced ROS production in primary cardiomyocytes in a dose-dependent manner. Primary cardiomyocytes viability was decreased by 5-FU in a dose- and time-dependent manner. 5-FU significantly enhanced the activation of caspase3, thereby prompting cardiomyocyte apoptosis. In addition, treatment with 5-FU obviously reduced the SOD content and enhanced the MDA level. Preincubation with APS could partially reverse 5-FU-induced SOD reduction and MDA upregulation. Western blot analysis demonstrated that treatment with APS decreased 5-FU-induced activation of caspase3 and reduced the expression of Bax. In conclusion, treatment with APS was shown to suppress 5-FU-induced cardiomyocyte apoptosis primarily by suppression of ROS production.

**Keywords:** APS, 5-Fluorouracil, ROS production, cardiotoxicity, apoptosis

## Introduction

As an analogue of uracil, 5-Fluorouracil (5-FU) pro-drugs are widely applied for the treatment of breast, gynecological and gastrointestinal cancers [1]. Through inhibiting the availability of thymidylate, 5-FU can suppress DNA synthesis during the S phase of the cell cycle [1, 2]. In addition, 5-FU can also repress RNA synthesis and processing [3]. Although 5-FU is widely applied for tumor treatment, its side effects cannot be disregarded [4]. Reported side effects of 5-FU include leukopenia, diarrhea, stomatitis, cardiac toxicity and nausea [5, 6]. As the second major cause of chemotherapy-induced cardiotoxicity, the cardiac toxicity of 5-FU can be a severe threat for patients [7].

Chemotherapy-induced cardiotoxicity significantly restricts the clinical application of drugs [8]. It is widely accepted that reactive oxidative stress (ROS) is the major origin of chemotherapy-induced cardiotoxicity [9, 10]. When the balance of the ROS-generation system and antioxidant defense system is disturbed, enhanced ROS production leads to obvious cellular damages and abnormal responses [11]. Aberrant ROS production can function as a secondary signaling pathway involved in cell proliferation and cell death [12]. Thus, maintenance of the normal level of ROS production is of great importance for cellular homeostasis [13]. In the heart, oxidative stress can result in cellular hypertrophy, cell death, and ventricular remodeling that may further develop into cardiomyopathy and heart failure [14].

Astragalus polysaccharide (APS) is a major component of astragalus that has been proven effective in the treatment of cardiac ischemia

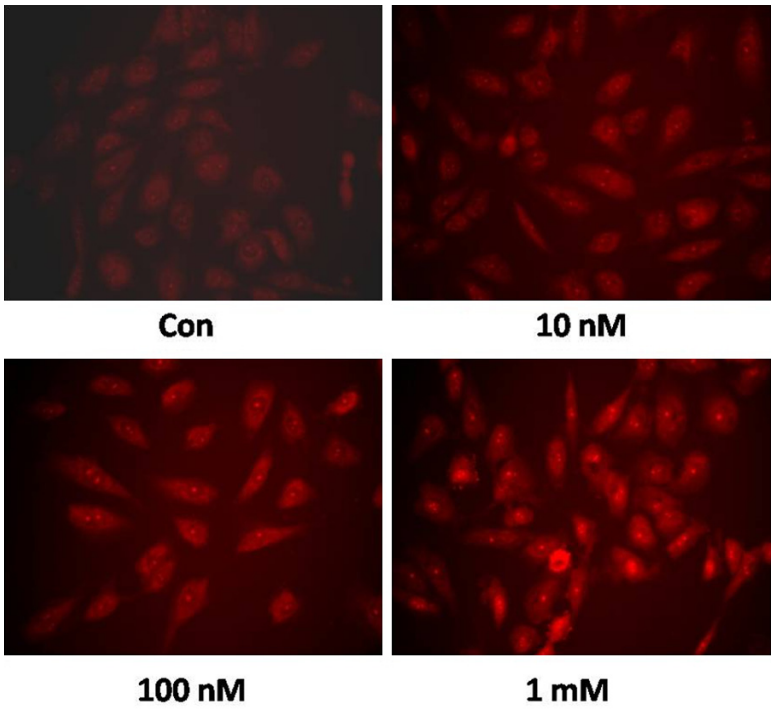


**Figure 1.** 5-FU significantly enhanced ROS production in primary cardiomyocytes in a dose-dependent manner at 10 nM, 100 nM and 1 mM.

[15]. Specifically, this compound can protect the heart through improving coronary blood flow, LPO content and superoxide dismutase activity [16-18]. However, whether APS could improve 5-FU-induced cardiotoxicity remains largely unknown. In this study, we first identified that 5-FU could significantly enhance ROS production in primary cardiomyocytes; then, we tested treatment with APS to see if it could obviously protect cardiomyocytes from 5-FU-induced injury by decreasing ROS production.

## Materials and methods

*Primary cardiomyocyte culture*

Primary cardiomyocytes from rat neonatal hearts were isolated as previously described [19]. The animal protocol was approved by the affiliated Hospital of Qingdao University. In brief, hearts were isolated and digested with collagenase type II (Worthington) solution. After digestion, the cells were cultured for 2 hr to collect cardiomyocytes. The attached cells were then discarded, as the unattached cells were primarily cardiomyocytes.

*Study protocols: in vivo*

Eight-week-old male Sprague-Dawley (SD) rats were purchased from the affiliated Hospital of Qingdao University. Then, the SD rats were randomly divided into three groups by direct gastric gavage for 7 days: Group I: saline group; Group II: 5-FU (1 mg/Kg body weight); and Group III: 5-FU+APS (1.5 g/kg body weight). The hearts were subsequently excised, and the proteins were extracted.

*Cell proliferation assay*

Primary cardiomyocytes were cultured in 1% gelatin coated 96-well tissue culture plates at 5,000 cells/well. After 24 h, 5-FU dissolved in DMSO was added in the medium at a final concentration of 1 nM, 10 nM, 100 nM or 1 mM. DMSO alone was added as a control. The MTT reagent was added to each well at a final concentration of 0.5 mg/ml and incubated at 37°C for 5 h. The medium was then removed and formazan crystals were dissolved with 100 μl of DMSO. The absorbance was determined at 570 nm with a microplate reader. The cells were subsequently preincubated with 100 nM 5-FU for 12, 24, 48 or 72 h, and cell viability was determined with the same method as previously described. Each experiment was independently performed at least 3 times.

*Reactive oxygen species (ROS) detection*

Cells were cultured on slides in six-well chambers at a 60% confluence. After 24 h, the cells were treated with 5-FU at a final concentration of 1 nM, 10 nM, 100 nM or 1 mM. After 48 h, the cardiomyocytes were collected, centrifuged and washed in PBS three times (5 min/time). The slides were then treated with 5 μM DHE (Vigorous Biotechnology Beijing Co., Ltd) in serum-free DMEM F-12 medium for 30 min at 37°C in darkness. The cells were fixed in 4% paraformaldehyde for 30 min at RT. The slides were washed with cold PBS three times and mounted. Immunofluorescent images were captured by fluorescence microscopy. To quantify the intracellular ROS, relative fluorescence intensities were analyzed with flow cytometry (Becton-Dickinson) of the primary cardiomyocytes.

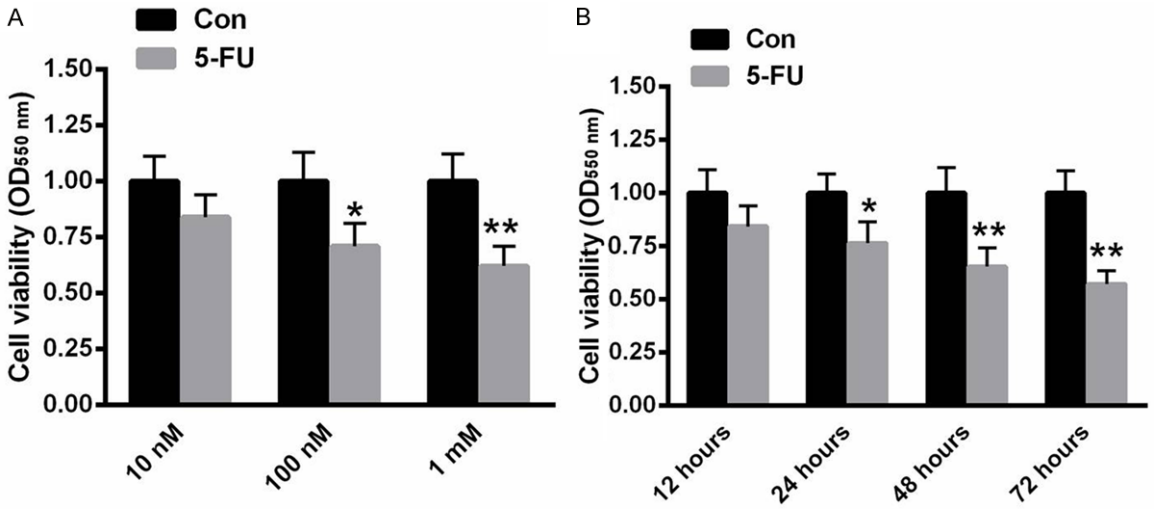


**Figure 2.** 5-FU decreased primary cardiomyocytes viability in a dose- and time-dependent manner. A. Incubation of primary caridiomyocytes with 5-FU significantly decreased cell viability at 100 nM and 1 mM. B. Treatment with 100 nM 5-FU reduced cardiomyocyte viability by 34.5% and 42.3% at 48 h and 72 h, respectively. Data represent the means ± SEM, n=3 independent experiments. $**P<0.01$, versus control.

*Western blot analysis*

Total lysates were collected with cell lysis buffer (Cell Signaling Technology), and the protein concentrations were determined using the BCA Protein Assay (Millipore, Billerica, MA, USA). Equal amounts of proteins were separated on a 12% SDS-PAGE gel and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with 5% non-fat milk powder (w/v) for 2 h at room temperature and then incubated with primary antibody rabbit anti-caspase3, Bcl-2, Bax and GAPDH (Cell Signaling). The antibody was diluted in 5% bovine serum albumin according to the manufacturer's instructions. Horseradish peroxidase-conjugated secondary antibodies were then added, and the resulting signal was detected through autoradiography using chemiluminescence (ECL, Amersham Biosciences). GAPDH served as the internal control.

*Enzyme activity assay*

The tissue homogenate was centrifuged at 2000 rpm for 10 min. The contents of total protein, SOD, CAT and MDA were determined using assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions.

*Echocardiography*

Echocardiography was performed using Vevo 770 and Vevo 2100 (VisualSonics) instruments. Fraction shortening (FS), ejection fraction (EF), left ventricular internal diameter (LVID) during systole, LVID during diastole, end-systolic volume, and end-diastolic volume were calculated with Vevo Analysis software (version 2.2.3) as previously described [20].

*Statistical analyses*

The data were expressed as the means ± SEM. The statistical evaluation was performed using SPSS10.0 software. The statistical comparisons were performed using a one-way analysis of variance (ANOVA), and Dunn's method was used to discriminate the differences between groups. $P<0.05$ was considered statistically significant.

## Results

*5-FU significantly enhances ROS production in primary cardiomyocytes*

As shown in **Figure 1**, pretreatment with 5-FU significantly enhanced the production of ROS in primary cardiomyocytes in a dose-dependent manner.

*5-FU decreases primary cardiomyocytes viability in a dose and time dependent manner*

To explore the effect of 5-FU on cell viability, an MTT assay was applied. As shown in **Figure 2A**, incubation of primary cardiomyocytes with 5-FU significantly decreased cell viability at

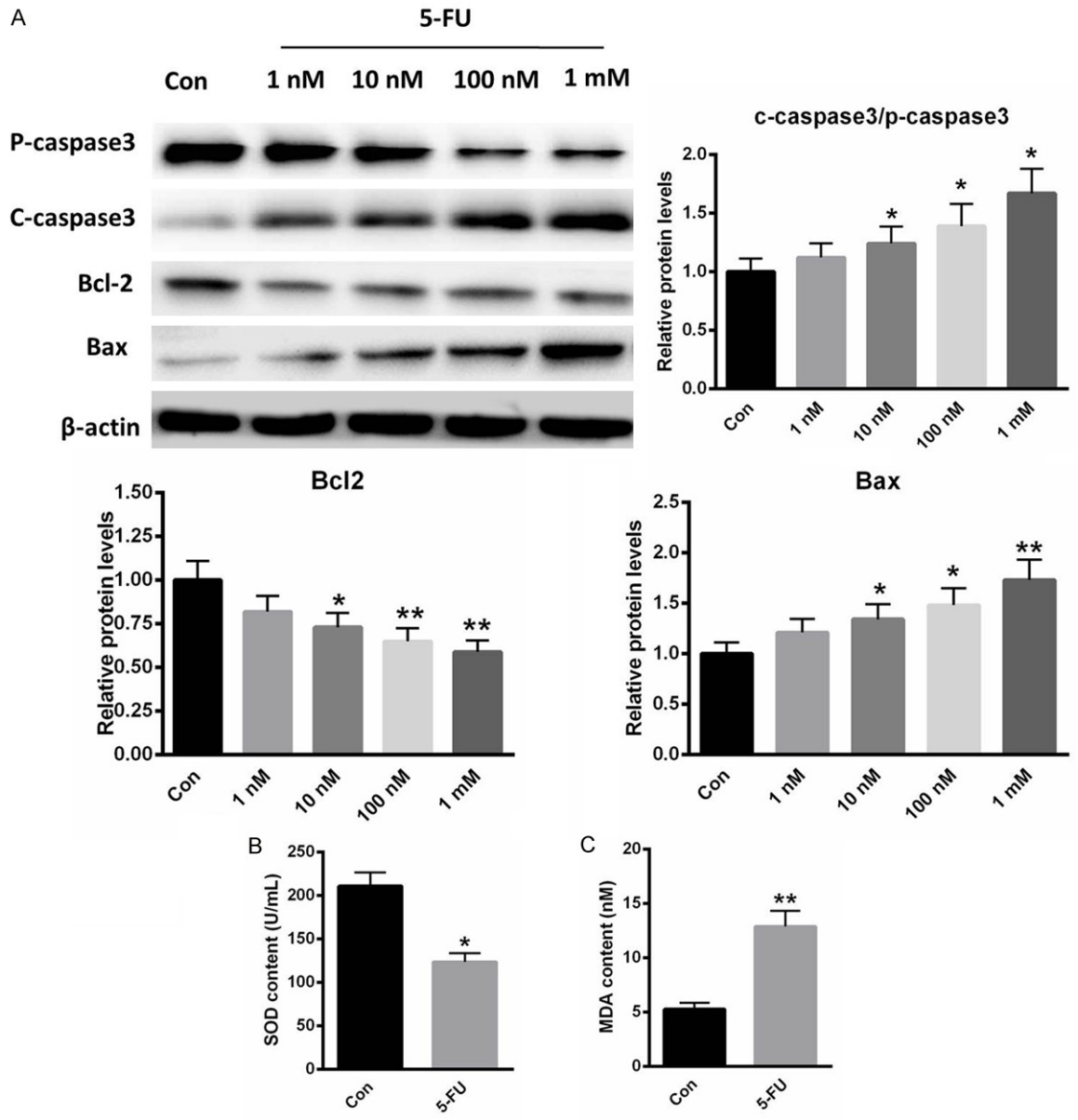


**Figure 3.** 5-FU induced cardiotoxicity and apoptosis in vivo. (A) 5-FU significantly enhanced the activation of caspase3. Treatment with 5-FU significantly reduced the SOD contents (B) and enhanced the MDA level (C). Data represent the means ± SEM, n=3 independent experiments. $^{**}P<0.01$, versus control.

100 nM and 1 mM. Meanwhile, treatment with 100 nM 5-FU reduced cardiomyocyte viability by 34.5% and 42.3% at 48 h and 72 h, respectively (**Figure 2B**).

*5-FU induces cardiotoxicity and apoptosis in vivo*

The in vitro study found that treatment with 5-FU significantly enhanced the activation of caspase3 (**Figure 3A**). Meanwhile, the Bcl-2 protein level was obviously decreased, while the Bax protein level was significantly enhanced (**Figure 3A**). We also detected the SOD and MDA levels when primary cardiomyocytes were treated with 5-FU at 100 nM. The data showed that treatment with 5-FU significantly reduced the SOD content and enhanced the MDA level, suggesting the cardiotoxic effect of 5-FU (**Figure 3B** and **3C**).

*APS ameliorates 5-FU-induced cardiac injury by regulating ROS production*

To explore the protective role of APS treatment on 5-FU-induced cardiac injury, echocardio-

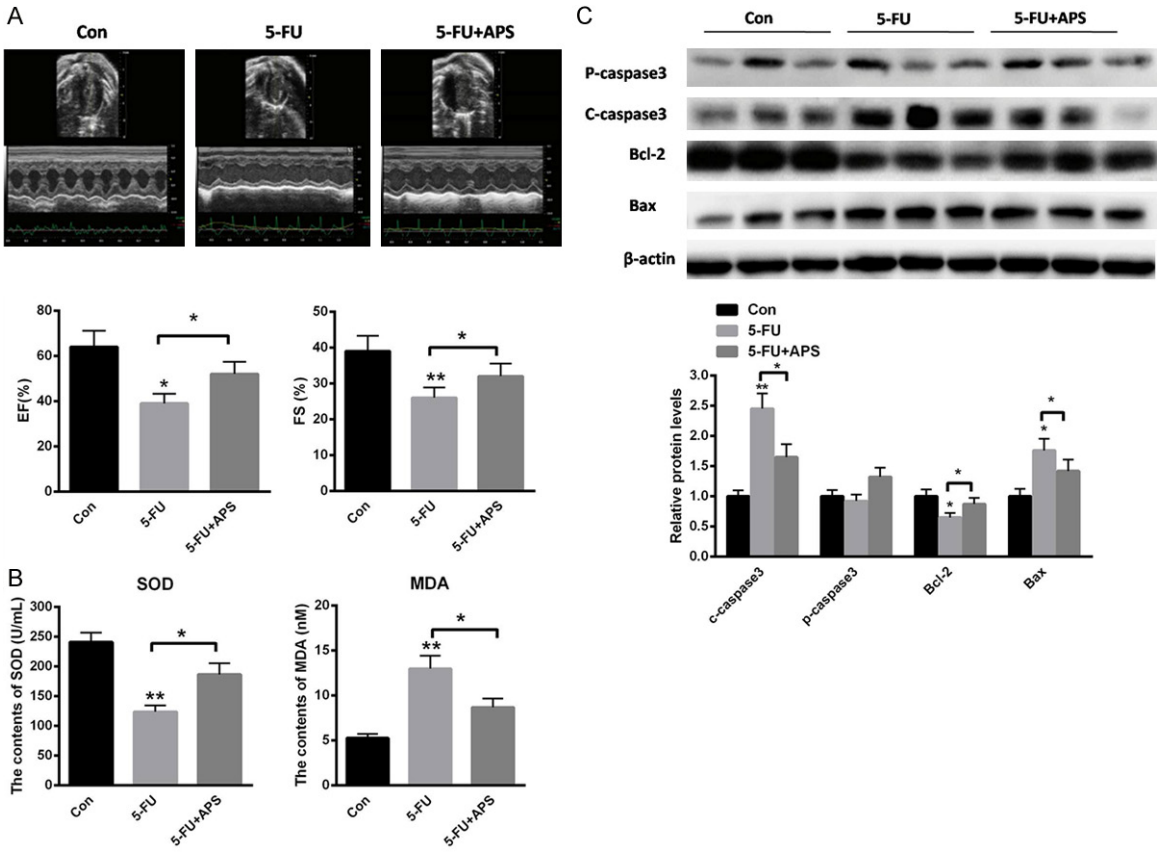


**Figure 4.** APS ameliorated 5-FU-induced cardiac injury by regulating ROS production. A. APS could improve the 5-FU treatment-induced decreased heart function as measured by the ejection fraction (EF)% and fraction shortening index (FS)% compared with the control. B. Preincubation with APS significantly increased the SOD level and decreased the MDA content. C. APS decreased 5-FU-induced activation of caspase3 and the expression of Bax. Data represent the means ± SEM, n=3 independent experiments. $^{**}P<0.01$, versus control.

graphic analysis was conducted. Compared with the control rats, heart function was decreased by 5-FU treatment as measured by the ejection fraction (EF)% and fraction shortening index (FS)% (**Figure 4A**). Compared with 5-FU treatment, APS significantly enhanced the ejection fraction (EF)% and fraction shortening index (FS)% (**Figure 4A**). In addition, preincubation with APS significantly reversed the SOD level and the MDA content decrease caused by 5-FU treatment (**Figure 4B**). Western blot analysis demonstrated that treatment with APS decreased 5-FU-induced activation of caspase3 and the expression of Bax in vivo. In comparison, APS treatment could upregulate the level of Bcl2 compared with 5-FU treatment alone (**Figure 4C**).

## Discussion

Chemotherapy-induced cardiotoxicity is a severe complication that significantly limits the clinical application of drugs [21]. Understanding the mechanism of cardiotoxicity induction is key in reducing undesirable effects on normal tissues and ameliorating tumor treatment.

5-FU has been widely applied for cancer treatment [22, 23]. A high rate of occurrence of cardiac toxicity is reported, ranging from 20% to 100% [24, 25]. To develop effective strategies for cardiotoxicity prevention, the specific mechanism of 5-FU was explored in this study. We treated primary cardiomyocytes with 5-FU at different concentrations and found it can significantly enhance the production of ROS. Furthermore, the MTT assay showed that 5-FU treatment decreased cardiomyocyte viability in a dose- and time-dependent manner and that treatment with 5-FU obviously enhanced the activation of caspase3. More importantly, 5-FU decreased the SOD content and enhanced the MDA level. These in vitro experiments indicated

that 5-FU could significantly induce neonatal rat ventricular myocyte injury.

APS has long been applied as an effective traditional medicine that can enhance immunity, reduce cancer cell growth and reduce inflammation [26]. Previous studies have indicated that APS may act as a potent protective medicine that can reduce heart injury, such as myocardial hypertrophy and heart failure [27]. However, few studies have explored whether APS could protect from cardiac injury induced by 5-FU. In this study, we verified that APS treatment could markedly reduce 5-FU-induced cardiac injury.

In both human and animal heart failure models, myocardial oxidative stress clearly led to ventricular dilatation [28, 29]. ROS are often released by cardiomyocytes in response to chemotherapeutic drugs [30]. We identified that ROS are induced by 5-FU in a dose-dependent manner. In comparison, treatment with APS could significantly lower the ROS production that is crucial for the activation of cell apoptosis.

In this study, we showed that treatment with APS reduces 5-FU-induced apoptosis of cardiomyocytes, primarily by suppressing ROS production. APS treatment could clearly protect the heart from the side effects induced by 5-FU treatment. Thus, our findings may shed light on novel therapeutic strategies for preventing 5-FU-induced heart injury.

## Disclosure of conflict of interest

None.

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