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Inhibition of ALDH2 expression aggravates renal injury in a rat sepsis syndrome model

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Abstract. Mitochondrial aldehyde dehydrogenase 2 (ALDH2) is closely associated with organ injury. The aim of the present study was to investigate the change of ALDH2 expression in a rat model of sepsis-induced acute renal injury, and to observe the effect of ALDH2 inhibition on the kidney. A model of sepsis syndrome was established in Sprague-Dawley (SD) rats by cecal ligation and puncture (CLP). The rats were divided into sham, CLP and CLP + cyanamide (CYA, an ALDH2 inhibitor) groups. The hemodynamic parameters heart rate (HR) and mean arterial blood pressure (MABP) were measured. Plasma creatinine (CRE) and urea nitrogen (BUN) levels were measured using an automatic biochemical analyzer. Malondialdehyde (MDA) content and superoxide dismutase (SOD) activity in the kidney tissue were measured. Histological changes of the kidney tissue were observed using hematoxylin and eosin staining and NF-κB p65 expression was observed by an immunohistochemical staining method. The expression of renal ALDH2 at the mRNA and protein levels was detected by reverse transcription-polymerase chain reaction and western blotting. In the CLP compared with the sham group after 24 h, the MABP was decreased, plasma CRE and BUN levels were elevated, the renal MDA level was increased and SOD activity was decreased. In addition, glomerular atrophy occurred, the renal protein expression of NF-κB p65 was increased, and the mRNA and protein expression levels of ALDH2 were decreased. In contrast with the CLP group, in the CLP + CYA group, the MABP and ALDH2 expression were further decreased while glomerular atrophy was aggravated. Furthermore, CRE, BUN, MDA levels and NF-κB p65 expression were further increased and SOD activity was further reduced. In this rat model of sepsis syndrome, the reduction of renal ALDH2 expression was accompanied by kidney injury. Inhibition of ALDH2 with CYA aggravated the renal injury, and was associated with the overproduction of reactive oxygen species and inflammatory reaction.

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Introduction

Sepsis is the leading cause of mortality in the intensive care unit (ICU), with a mortality rate ranging from 20% in sepsis to >60% in septic shock (1). In particular, sepsis-induced acute kidney injury (AKI) accounts for ~50% of all cases of AKI. Furthermore, ICU mortality is as high as 70% in patients with sepsis and concomitant AKI (2-4). The pathophysiology of septic AKI has not been completely established, but is likely to involve immunological, toxic and inflammatory factors that may affect the microvasculature and tubular cells, subsequently inducing renal necrosis and apoptosis (5).

Renal mitochondrial dysfunction also occurs during the course of sepsis-induced AKI (6). Mitochondrial aldehyde dehydrogenase 2 (ALDH2) is a member of the ALDH gene family, which is highly expressed in the heart, liver, kidney and muscle (7). ALDH2 catalyzes the oxidation of acetaldehyde to acetic acid in the metabolism of ethanol, and detoxifies reactive aldehydes (8). With regard to the role of ALDH2 in the pathogenesis of sepsis, Chen *et al* reported that hepatic ALDH2 activity was downregulated in septic rats following cecal ligation and puncture (CLP) (9). It has been reported that preconditioning with ethanol to stimulate ALDH2 activity prevented kidney ischemia and reperfusion injury in mice (10); however, the role of renal ALDH2 in sepsis-induced AKI is unclear. It remains to be determined whether ALDH2 is also a key factor associated with kidney injury.

In the present study, a rat model of septic injury was created by the CLP method, with the aim of observing whether the septic injury damaged kidney morphology and function, and evaluating the effects of the septic injury on ALDH2 in the kidney. Furthermore, the effects of treatment with an ALDH2 inhibitor were investigated.

Materials and methods

Animals. A total of 18 Male Sprague-Dawley rats (200-250 g; 7-8 weeks) were obtained from the Animal Center of Bengbu Medical College (Bengbu, China). The rats were housed in a controlled environment (a constant 12 h light/dark cycle with a temperature of 21+1˚C and 50-60% humidity), fed normal chow and had free access to tap water. All animal procedures were in conducted accordance with the 8th Edition of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, 2010, and were approved by the Animal Research Ethics Committee of Bengbu Medical College (Bengbu, China).

Chemicals and reagents. Cyanamide (CYA) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Mouse anti-ALDH2 monoclonal antibody (sc-100496) and anti-β-actin monoclonal antibody (sc-81178) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit anti-nuclear factor (NF)-κB p65 polyclonal antibody (PB0321) and horseradish peroxidase (HRP)-linked anti-mouse IgG secondary antibody (BA1050) were purchased from Wuhan Boster Biological Technology, Ltd, Wuhan, China. Malondialdehyde (MDA; cat. no. A003-1) and superoxide dismutase (SOD; cat. no. A001-1) detection kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China). The primers for ALDH2 were 5'-GTG TTC GGA GAC GTC AAA GA-3' and 5'-GCA GAG CTT GGG ACA GGT AA-3', with an amplified fragment length of 187 bp; the primers for β-actin were 5'-GAT GGT GGG TAT GGG TCA GAA GGA C-3' and 5'-GCT CAT TGC CGA TAG TGA TGA CT-3', with an amplified fragment length of 630 bp. All other chemicals were of the highest purity available.

Induction of sepsis and experimental protocol. Animals were randomly divided into sham, CLP and CLP + CYA groups (each n=6). Sepsis was induced in overnight-fasted rats by the CLP modeling method (11). All rats were injected with 0.4 g/kg chloral hydrate intraperitoneally to induce anesthesia. In the CLP group, under sterile surgical conditions, a 2-cm abdominal incision was made along the ventral surface of the abdomen to expose the cecum. The cecum was then punctured twice with a 22-gauge needle and fecal contents were allowed to leak into the peritoneum by gently squeezing cecum. The bowel was then returned to the abdomen and the abdominal cavity was closed. In the sham group, the animals were submitted to laparotomy and the cecum was manipulated but neither ligated nor punctured. In the CLP + CYA group, rats were subjected to CLP and then injected intravenously with CYA at a dose of 25 mg/kg body weight 30 min after the CLP procedure.

Hemodynamic parameter measurement. At 24 h after CLP, rats were injected with 0.4 g/kg chloral hydrate intraperitoneally to induce anesthesia. Hemodynamic parameters including the heart rate (HR) and mean arterial blood pressure (MABP) were measured continuously for 50 min by intubation of the left common carotid artery using a Medlab biological signal collecting and processing system (Nanjing, China).

Plasma creatinine (CRE) and blood urea nitrogen (BUN) measurement. After the hemodynamic parameters of the rats had been taken, 2 ml blood was obtained from the left common carotid artery, and the plasma CRE and BUN levels were measured using an automatic biochemical analyzer (AU5400; Olympus Corporation, Tokyo, Japan).

MDA content and SOD activity measurement. All rats were injected with 0.4 g/kg chloral hydrate intraperitoneally to induce anesthesia. Under sterile surgical conditions, an abdominal incision was made and kidneys were perfused with 0.01 mol/l PBS via the abdominal aorta, until the blood was washed out, and the parenchyma was pale in appearance. Then the kidneys were removed as quickly as possible, and the right kidney was stored at -80˚C for MDA content and SOD activity measurement. Kidney tissue (100 mg) was homogenized in ice-cold saline solution. The MDA content and SOD activity were measured using commercially available kits, according to the manufacturer's instructions (12).

Renal morphology observation and NF-κB p65 protein expression measurement. The renal tissues of left kidney were immersed in 10% buffered formalin. The tissues were gradually dehydrated, embedded in paraffin, cut into 5-μm sections and stained with hematoxylin and eosin (H&E) for histological evaluation. Renal NF-κB p65 protein expression was detected by an immunohistochemistry method. Immunohistochemical staining was performed using an Elivision Plus detection kit according to the manufacturer's protocol (KIT-9902, LabVision AB, Torsby, Sweden). Kidney sections (4 μm) were deparaffinized with xylene and rehydrated with decreasing percentages of ethanol, then washed with PBS (pH 7.2) for 10 min. The endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in methanol for 10 min at room temperature. Sections were subsequently placed in citrate buffer (pH 6.0) at 95˚C for antigen repair for 30 min, and the slides were rinsed with PBS 3 times. All sections were blocked with 10% goat serum (50012-8615; Zhejiang Tianhang Biotechnology Co., Ltd., Huzhou, China) for 30 min at room temperature. Kidney sections were incubated overnight at 4˚C with rabbit anti-NF-κB p65 polyclonal antibody (1:200). All sections were counterstained with hematoxylin for 30 sec at 60˚C, dehydrated, air-dried, and mounted. Histological sections were observed and images were captured using an Olympus CKX41 Inverted light microscope (Olympus Corporation).

Detection of ALDH2 mRNA by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was used to detect the renal expression of ALDH2 mRNA (12). Briefly, total RNA was extracted from rat renal tissue with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc. Waltham, MA, USA) according to the manufacturer's protocol. Total RNA was treated with DNase I (D8071; Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) to remove the genomic DNA. RNA was solubilized in ribonuclease-free water and quantified by measuring the absorbance at 260 nm by UV spectrophotometer (BioPhotometer plus; Eppendorf, Hamburg, Germany). The purity of RNA was confirmed by examining the optical density (OD) 260/280 as 1.8-2.0. Total RNA (2 μg) was reverse transcribed to cDNA using the

RevertAid First Strand cDNA Synthesis kit (K1622; Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. Briefly, the mix (2 μg total RNA, 1 μl OligodT_{18} primer, 4 μl 5X Reaction Buffer, 1 μl 20 U/μl RiboLock RNase Inhibitor, 2 μl 10 mmol/l dNTPs Mix, 1 μl 200 U/μl RevertAid M-MuLV RT and ribonuclease-free water to give a final colume of 20 μl) was incubated for 60 min at 42˚C, and the reaction was terminated by heating the mixture to 70˚C for 5 min. PCR was performed using PCR Master Mix (2X; K0171; Thermo Fisher Scientific, Inc.) using the aforementioned primers according to the manufacturer's protocol. The reaction mixture contained 25 μl 2X PCR Master Mix (0.05 U/μl *Taq* DNA polymerase, reaction buffer, 4 mmol/l $MgCl_2$, and 0.4 mmol/l of each dNTP), 2 μl template DNA (50 ng), 0.5 μl of each 20 pmol/μl primer, and 22 μl of double distilled H_2O to give a total volume of 50 μl. The PCR conditions were as follows: 95˚C for 3 min, then 30 cycles of 95˚C for 50 sec, 60˚C for 45 sec, and 72˚C for 60 sec, followed by a final extension step at 72˚C for 10 min. PCR products were separated on 1% agarose gel. Densitometry results for ALDH2 were analyzed with a Tanon Gel Image System 1D (version 4.1.2; Tanon, Shanghai, China) and compared with the corresponding β-actin levels to account for loading differences. All experiments were repeated three times.

Detection of ALDH2 protein by western blotting. Rat renal tissue (0.1 g) was homogenized in a lysis buffer (P0013; Beyotime Institute of Biotechnology, Haimen, China) supplemented with 1% protease inhibitor cocktail. Following centrifugation at 12,000 x g for 30 min at 4˚C, the supernatant was collected, and the protein concentration was determined using an enhanced bicinchoninic acid protein assay kit according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Protein (80 μg) was separated on sodium dodecyl sulfate-polyacrylamide gels in a minigel apparatus and then transferred electrophoretically to a polyvinylidene difluoride membrane. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline prior to overnight incubation at 4˚C with anti-ALDH2 (1:500) and anti-β-actin (1:500) antibody. Membranes were then incubated for 1 h at 37˚C with HRP-linked anti-mouse IgG (1:5,000). Autoradiographs were scanned using the ChemiDoc XRS Gel Image System and analyzed with Image Lab software (version 3.0; Bio-Rad Laboratories, Inc., Hercules, CA, USA) (12).

Statistical analysis. All values are expressed as the mean ± standard error of the mean. Statistical comparisons were performed by one-way analysis of variance and the Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference.

Results

Changes of hemodynamic parameters. The MABP in the CLP group was decreased significantly compared with that in the sham group, and the MABP was decreased further in the CLP + CYA group at 24 h after the CLP procedure. No significant difference in HR was observed among the different groups (Table I).

Table I. Hemodynamic data in the three groups (n=6).

Group	HR (beats/min)	MABP (mmHg)
Sham	398.56±14.30	106.93±3.84
CLP	385.84±17.01	92.35±4.79[a]
CLP + CYA	398.93±25.59	79.85±6.33[a,b]

[a]P<0.01 vs. the sham group; [b]P<0.01 vs. the CLP group. HR, heart rate; MABP, mean arterial blood pressure; CLP, cecal ligation and puncture; CYA, cyanamide.

Table II. Renal functionin the three groups (n=6).

Group	BUN (mmol/l)	CRE (μmol/l)
Sham	9.17±0.21	34.80±2.58
CLP	19.25±7.23[a]	65.25±7.50[b]
CLP + CYA	32.24±8.43[b,c]	82.66±10.07[b,c]

[a]P<0.05 and [b]P<0.01 vs. the sham group; [c]P<0.01 vs. the CLP group. BUN, blood urea nitrogen; CRE, creatinine; CLP, cecal ligation and puncture; CYA, cyanamide.

Table III. Renal SOD activity and MDA content in the three groups (n=6).

Group	SOD (U/mg.protein)	MDA (nmol/mg.protein)
Sham	144.80±8.66	3.27±0.42
CLP	120.38±7.98[a]	8.19±1.33[a]
CLP + CYA	102.33±10.81[a,b]	11.42±2.56[a,b]

[a]P<0.01 vs. the sham group; [b]P<0.01 vs. the CLP group. SOD, superoxide dismutase; MDA, malondialdehyde; CLP, cecal ligation and puncture; CYA, cyanamide.

Changes of plasma CRE and BUN levels. In comparison with the plasma CRE and BUN levels in the sham group, those in the CLP group were increased, and those in the CLP + CYA group were further increased (Table II).

Changes of renal MDA content and SOD activity. The renal MDA content was increased and SOD activity was decreased in the CLP group compared with the sham group. Furthermore, the MDA content was higher and SOD activity was lower in the CLP + CYA group compared with the CLP group (Table III).

Histological analysis of the renal tissue. In the sham group, the renal corpuscles appeared normal with dense, rounded structures, and the Bowman's spaces surrounding the glomeruli were narrow. In the CLP group, the glomeruli appeared wrinkled and the Bowman's spaces were wider than those in the sham group. In the CLP + CYA group, the wrinkling of the glomeruli was aggravated compared with that in the CLP group (Fig. 1).

Table IV. Renal ALDH2 expression at the mRNA and protein levels in the three groups (n=6).

Group	ALDH2/β-actin mRNA	ALDH2/β-actin protein
Sham	0.86±0.02	0.71±0.17
CLP	0.52±0.05[a]	0.53±0.08[b]
CLP + CYA	0.45±0.08[a,c]	0.38±0.09[a,c]

[a]P<0.01 and [b]P<0.05 vs. the sham group; [c]P<0.05 vs. the CLP group. ALDH2, aldehyde dehydrogenase 2; CLP, cecal ligation and puncture; CYA, cyanamide.

Change of NF-κB p65 in renal tissue. In the sham group, the renal expression of NF-κB p65 protein was low. Compared with that in the sham group, renal NF-κB p65 protein was highly expressed in the CLP and CLP + CYA groups, and was particularly high in the CLP + CYA group (Fig. 2).

Changes of renal ALDH2 at the mRNA and protein levels. Renal ALDH2 expression was decreased at the mRNA and protein levels in the rats of the CLP and CLP + CYA groups compared with the sham group, and was particularly low in the CLP + CYA group (Table IV and Fig. 3).

Discussion

In the current study, it was identified that in the CLP-induced rat sepsis model, renal expression of ALDH2 at the mRNA and protein levels was decreased, which was accompanied by a reduction of renal function, the overproduction of reactive oxygen species and increased NF-κB protein expression when compared with rats subjected to a sham procedure. When the septic rats were treated with CYA, an ALDH2 inhibitor, renal injury, oxidative stress and inflammation were aggravated as the renal ALDH2 activity was reduced, which suggests that ALDH2 could be a key factor regulating the pathological changes of sepsis-induced renal injury.

Despite advances in modern medicine, sepsis and its sequelae remain a major cause of patient mortality. CLP in rats creates a realistic sepsis model. Sepsis is a systemic inflammatory response that induces tissue damage and multiple organ dysfunction syndrome, including AKI, septic shock, septic lethality and even death (13).

The activation of NF-κB plays a central role in the pathophysiology of septic shock. During sepsis, NF-κB is activated in many organs and has a potent effect on downstream signaling pathways and tissue injury. Inhibiting NF-κB expression restores systemic hypotension, ameliorates septic myocardial dysfunction and vascular derangement, inhibits the expression of multiple proinflammatory genes, diminishes intravascular coagulation, reduces tissue neutrophil influx and prevents microvascular endothelial leakage (14). In a study of patients with sepsis, NF-κB activity was found to be markedly increased in various organs, and higher NF-κB activity exhibited an association with higher mortality rates and worse clinical outcomes (15), which indicates that inflammatory pathways play an important role in sepsis-induced organ dysfunction. In the present study, it was observed that in the rat model of sepsis, renal injury was

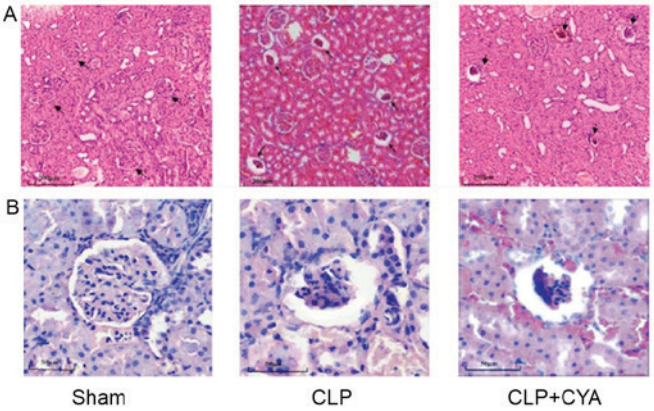

Figure 1. Histological observation of the kidney tissue in the three groups with hematoxylin and eosin staining. (A) Magnification, x100 and (B) magnification, x400. In the sham group, renal corpuscles appeared normal with dense, rounded structures, and the glomeruli (indicated by arrows) were surrounded by narrow Bowman's spaces. In the CLP group, the glomerulus was wrinkled with wider Bowman's spaces. In the CLP + CYA group, the wrinkling of the glomerulus was aggravated. CLP, cecal ligation and puncture; CYA, cyanamide.

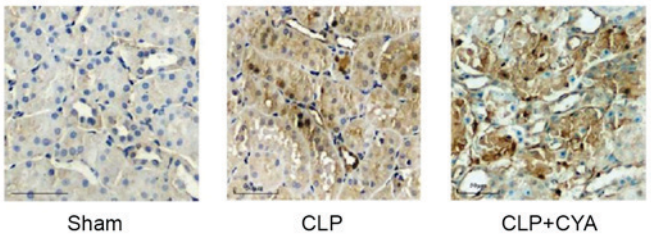

Figure 2. Protein expression of nuclear factor-κB p65 in the kidney tissue of rats from the three groups, stained using the Elivision plus two-step System and counterstained with hematoxylin (magnification, x400). CLP, cecal ligation and puncture; CYA, cyanamide.

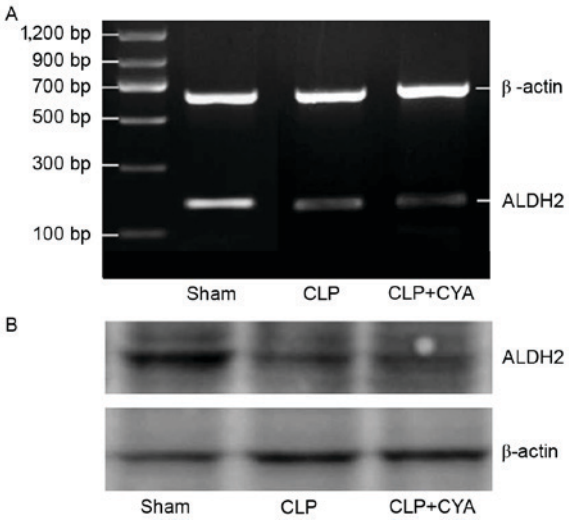

Figure 3. Expressions of renal ALDH2 at the mRNA and protein levels in the three groups. Representative (A) reverse transcription-polymerase chain reaction and (B) western blotting results are shown. ALDH2, aldehyde dehydrogenase 2; CLP, cecal ligation and puncture; CYA, cyanamide.

associated increased renal NF-κB expression, suggesting that sepsis-induced renal injury is associated with an inflammatory response.

In the present study, it was observed that the inhibition of renal ALDH2 using CYA was accompanied by oxidative stress in the septic rats. Furthermore, renal function injury, oxidative stress and the inflammatory response were further aggravated when ALDH2 activity was reduced. Sepsis is associated with mitochondrial dysfunction and impaired oxygen consumption (16,17), ALDH2 is localized in the mitochondrial fraction, and mitochondrial dysfunction changes ALDH2 function. A number of studies have reported that ALDH2 protects against organ injury, such as that affecting the kidneys, heart and neurons (10,18-20). Our previous studies showed that cardiac ALDH2 expression was decreased in rats with diabetes, or myocardial ischemia and reperfusion injury, and that upregulation of ALDH2 protected against myocardial injury and protected the lungs against diabetes-induced lung injury, all of which indicate that ALDH2 could be an intrinsic factor regulating the occurrence of disease (12,21,22). Whether ALDH2 is also an intrinsic factor for the regulation of sepsis-induced organ injury was thus considered. It has previously been reported that ALDH2 activity is reduced in the livers of rats with CLP-induced sepsis (9). ALDH2 has also been demonstrated to be protective against LPS-induced cardiac dysfunction (23), and increased ALDH2 expression was observed to reduce renal cell apoptosis during ischemia and reperfusion injury (24). However, the role of ALDH2 in sepsis-induced renal injury remains poorly elucidated. ALDH2 has been reported to attenuate oxidative stress. Sepsis induces mitochondrial dysfunction, and inflammation associated with the overproduction of reactive oxygen species (17). The scavenging of mitochondrial reactive oxygen species protects mitochondrial function, attenuates tissue inflammation, and improves whole organ activities in the heart during sepsis (25). Since ALDH2 can rapidly detoxify toxic reactive aldehydes and attenuate the oxidative stress response, when ALDH2 activation in sepsis rat was inhibited, the kidney injury was aggravated, suggesting that ALDH2 plays a key protective role against sepsis-induced organ injury.

The present study has certain limitations. The study simply measured the changes of ALDH2 in rats with sepsis-induced renal injury, and observed the changes of renal function when the rats were treated with CYA, a non-specific ALDH2 inhibitor. However, this is a preliminary study conducted to verify whether ALDH2 participates in sepsis-induced kidney injury. In future studies, we will firstly select a specific ALDH2 inhibitor such as daidzin to observe its effects on the kidney functions of the septic rats, then use the ALDH2 activator alda-1 to observe whether the activation of ALDH2 attenuates kidney injury. The likely mechanism for the protective effect of ALDH2 in sepsis-induced renal injury will be investigated further in the future.

From the present study, it was concluded that in sepsis-induced renal injury, renal ALDH2 expression was decreased, renal function was attenuated, and oxidative stress and inflammation occurred. Inhibition of ALDH2 expression in the kidney aggravated the renal injury and inflammation, which suggests that ALDH2 might be an intrinsic regulator of sepsis-induced kidney dysfunction. Further studies are necessary to confirm the role of ALDH2.

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