

## Original Article

# The regulation effect of ulinastatin on the expression of SSAT2 and AQP4 in myocardial tissue of rats after cardiopulmonary resuscitation

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Received June 30, 2015; Accepted August 20, 2015; Epub September 1, 2015; Published September 15, 2015

**Abstract:** Objective: This study aims to investigate the regulation effects of ulinastatin (UT1) on the expression of spermidine/spermine-N1-acetyltransferase 2 (SSAT2) and aquaporin 4 (AQP4) in myocardial tissue of rats after cardiopulmonary resuscitation (CPR) and their correlations. Methods: A total of 90 adult SD rats were divided into sham operation group (A, n=30), model group (B, n=30) and UT1 group (C, n=30). The cardiac arrest (CA) and CPR model was established by asphyxia method. Left ventricular fractional shortening (LVFS), left ventricular ejection fraction (LVEF) and E/A peak ratio of mitral valve in three groups were collected by ultrasonic echocardiography. Apoptosis of myocardial cells was detected by DAPI staining. The expression levels of SSAT2 and AQP4 were detected by RT-PCR, Western blotting and immunohistochemical methods. Results: UT1 could significantly improve the levels of LVFS, LVEF and E/A ratio and decrease myocardial cell apoptosis. As compared with group B, the expression level of SSAT2 increased and the expression level of AQP4 decreased in group C ( $P<0.01$ ). SSAT2 was the most in group A and the least in group B while AQP4 was the least in group A and the most in group B ( $P<0.01$ ). There was positive correlation between SSAT2 and cardiac function in CRP model while there was negative correlation between AQP4 and cardiac function ( $P<0.01$ ). The expression of SSAT2 and AQP4 protein in myocardial tissue was negatively correlated in CRP model ( $r=-0.920$ ,  $P<0.01$ ). Conclusions: UT1 can effectively reduce the cardiac function damage caused by CRP, which could be related with the increased SSAT2 and decreased AQP4.

**Keywords:** Ulinastatin (UT1), cardiopulmonary resuscitation (CPR), spermidine/spermine-N1-acetyltransferase 2 (SSAT2), aquaporin 4 (AQP4)

## Introduction

Cardiopulmonary resuscitation (CPR) after cardiac arrest (CA) often causes multiple organ dysfunction or failure. Cardiac dysfunction is one of the main causes of death in patients after resuscitation [1]. The successful treatment of myocardial tissue after CPR plays a decisive role in the patient's prognosis [2]. Ulinastatin (UT1) is a glycoprotein that has the roles of scavenging oxygen free radicals, inhibiting inflammatory reaction, reducing ischemia reperfusion injury and so on. Previous studies found that UT1 could improve the heart function of patients after CPR and reduce myocardial injury, and had a certain protective effect on the heart [3, 4]. However, the underlying mechanism of protecting heart remains to be elucidated.

Rat CPR model induced by asphyxia undergoes the process of anoxia and re-oxygenation, ischemia and reperfusion. Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) plays an on-off action in this process. Spermidine/spermine-N1-acetyltransferase2 (SSAT2) is a key protein in the degradation of HIF-1 $\alpha$  which is closely related to the CPR process [5]. Aquaporin 4 (AQP4) is four polymers and homology with water channel protein family with extensive distribution in the brain, and its main function is to transport water. AQP4 is highly expressed by heart and brain tissues after cerebral hemorrhage, cerebral trauma and CRP [6, 7]. These observations suggest that SSAT2 and AQP4 may be one of the important factors for systemic syndrome after CPR. In this study, we observed the effects of CPR and UT1 on SSAT2 and AQP4 in a rat CPR model induced by asphyxia and investigate the mech-

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**Table 1.** PCR primers used in this study

Gene	Primer (5'-3')	bp
SSAT2	For: ATCTTCCAGCGCCCGGAAGCTACTG	369
	Rev: CTCAAGACAGAGATCCTCCTAGGGATG	
AQP4	For: TTGGACCATCATAGGCGC	250
	Rev: GCATGTGATCGACATTGACC	
$\beta$ -actin	For: GTCGTACCACTGGCATTGTG	291
	Rev: CTCTCAGCTGTGGTGGTAA	

**Table 2.** Effects of UT1 on LVEF, LVFS and E/A of rats after CRP ( $\bar{x} \pm s$ ,  $n=30$ )

Group	EF (%)	FS (%)	E/A
A	74.12 $\pm$ 13.78	44.91 $\pm$ 9.86	1.91 $\pm$ 0.42
B	36.31 $\pm$ 9.76*	31.51 $\pm$ 7.94*	1.44 $\pm$ 0.31*
C	57.94 $\pm$ 10.62#	37.34 $\pm$ 8.69#	1.68 $\pm$ 0.39#

\* $P<0.01$  compared with group A; # $P<0.01$  compared with group B. A: sham operation group; B: model group; C: UT1 group.

anism of the protective effect of UT1 on myocardial injury after CPR.

## Materials and methods

### Experimental animals

A total of 90 adult SD rats (mean weight was 335 $\pm$ 70 g) were purchased from Qinghai experimental animal center. They were bred and maintained in a specific pathogen-free environment. SD rats were divided into sham operation group (A,  $n=30$ ), model group (B,  $n=30$ ) and UT1 group (C,  $n=30$ ). The cardiac arrest (CA) and CPR model was established by asphyxia method. Experimental parameters and records are based on the Utstein model [8]. The rats were anaesthetized with intraperitoneal injection of chloral hydrate. Tracheal intubation was closed at the end of expiration in group B and group C for CA for 5 min and then CPR was performed. The presence of spontaneous rhythm and pulse wave was the criterion for restoration of spontaneous circulation (ROSC). Injection of 100 kU/kg UT1 was performed through carotid artery in group C in 2 min after ROSC and the intervention time was 12 h. Sterile saline was injected in group B.

### Cardiac function monitor

Left ventricular fractional shortening (LVFS), left ventricular ejection fraction (LVEF) and E/A

peak ratio of mitral valve in three groups were collected by ultrasonic echocardiography.

### Apoptosis detection of myocardial cells

Myocardial tissues of rats in each group were collected and nuclear staining was performed with DAPI. DAPI (1:1000) was added and incubated for 5 min. Slices were mounted and observed under fluorescence microscope. Image-proplus 6.0 software was used for image analysis.

### Histopathological analysis

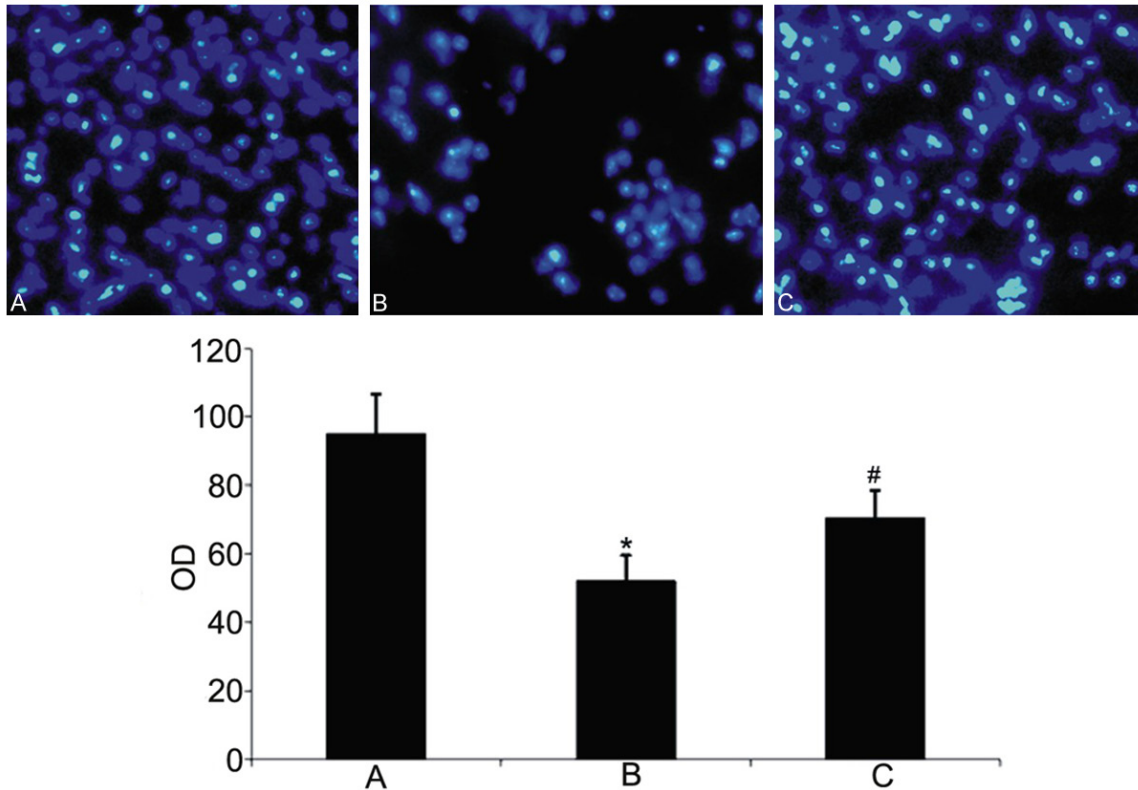
Myocardial tissues was fixed in 4% paraformaldehyde for 48 h, embedded in paraffin, and cut into 10- $\mu$ m-thick sections. The sections were washed with PBS and then blocked with goat serum and Triton-X-100. The primary antibodies (1:1000 SSAT2 and 1:1,500 AQP4) were added respectively and incubated at 4°C overnight. Biotin labeled second antibodies were added after washing with PBS and incubated for 2 h. They were developed with DAB solution and observed under microscope. Image-proplus 6.0 software was used for image analysis.

### Real-time PCR

Total RNA was isolated from myocardial tissues using TRIzol reagent kit (Invitrogen Life Technologies, USA) according to the manufacturer's protocol. The concentration and purity of RNA were evaluated by spectrometry at 260 and 280 nm. The primers used in this study were shown in **Table 1**. Reverse transcription and RT-PCR were performed using RT-PCR kit according to the manual. The amplification conditions were as follows: 45 s at 94°C, 40 cycles of 10 s at 95°C, 40 s at 60°C, and 60 s at 72°C. For relative quantification, we calculated the  $n$ -fold differential expression by the  $2^{-\Delta\Delta Ct}$  method (Ct denoting the threshold cycle of PCR amplification at which product is first detected by fluorescence) to compare the amount of target gene amplification after normalization to the mice  $\beta$ -actin (endogenous reference).

### Western blotting analysis

Myocardial tissues were homogenized by RIPA buffer (10  $\mu$ g/ml) in the presence of protease inhibitors. Total proteins were isolated and their concentration was measured by the bicincho-



**Figure 1.** Inhibitory apoptosis of UT1 on heart tissue sections from CRP rats. A: Sham operation group; B: Model group; C: UT1 group. DAPI staining in heart tissue sections from CRP rats (scale bar =200  $\mu$ m). \* $P$ <0.01 compared with group A; # $P$ <0.01 compared with group B.

nicinic acid (BCA) method. Samples were separated by 12% sodiumdodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk for 1 h and incubated at 4°C overnight with primary antibodies, followed by incubation with secondary antibodies for 1 h at room temperature.  $\beta$ -actin was applied as an internal control. The photos were analyzed with NIH Image J software.

#### Statistical analysis

Data were expressed as the mean  $\pm$  SD. SPSS 19.0 software was used for data analysis. Pearson correlation analysis and t-test were used to analyze the differences between groups.  $P$  value <0.05 was considered statistically significant.

#### Results

##### Effect of UT1 on cardiac function of CRP rats

As compared with group A, the LVEF, LVFS and E/A ratio significantly decreased in group B

( $P$ <0.01), while as compared with group B, the LVEF, LVFS and E/A ratio significantly increased in group C ( $P$ <0.01), which suggested that UT1 could improve the cardiac function of rats after CRP (Table 2).

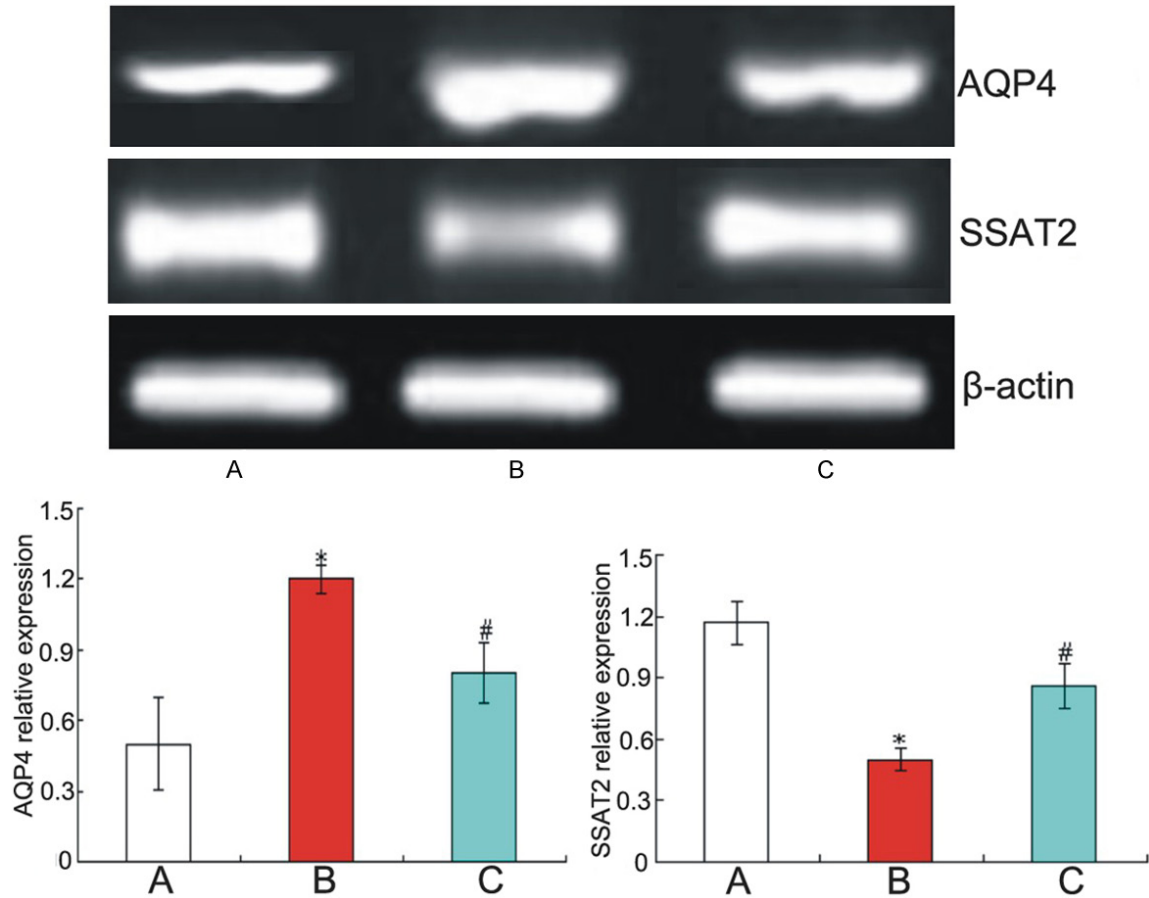
##### Effect of UT1 on the apoptosis of myocardial cells in CRP rats

DAPI staining results showed that cellular apoptosis significantly decreased after treatment by UT1. As compared with group A, optical density (OD) values significantly decreased in group B ( $P$ <0.01), while as compared with group B, OD values significantly increased in group C ( $P$ <0.01), which suggested that UT1 could decrease cellular apoptosis after CRP (Figure 1).

##### Measurement of gene expressions of SSAT2 and AQP4 by RT-PCR

RT-PCR results were shown in Figure 2. It showed that as compared with group A, the expression level of SSAT2 significantly decreased in group B ( $P$ <0.01). As compared with

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**Figure 2.** RT-PCR results of expression of SSAT2 and AQP4 mRNA in myocardium tissues \* $P < 0.01$  compared with group A; # $P < 0.01$  compared with group B. A: Sham operation group; B: Model group; C: UT1 group.

group B, the expression level of SSAT2 significantly increased in group C ( $P < 0.01$ ). Furthermore, as compared with group A, the expression level of AQP4 significantly increased in group B ( $P < 0.01$ ), while the expression level of AQP4 significantly decreased in group C as compared with group B ( $P < 0.01$ ). These results suggest that UT1 increases the expression of SSAT2 but decreases the expression of AQP4 in myocardial tissues after CRP.

### Histopathological analysis

Positive expression of SSAT2 and AQP4 was brown yellow and distributed in myocardial cell membrane. They were shown in **Figure 3** and **Table 3**. There were more positive expressions of SSAT2 in group A and C and less in group B, while there were more positive expressions of AQP4 in group B and less in group A and C. The results were consistent with RT-PCR results of which UT1 could increase the expression of

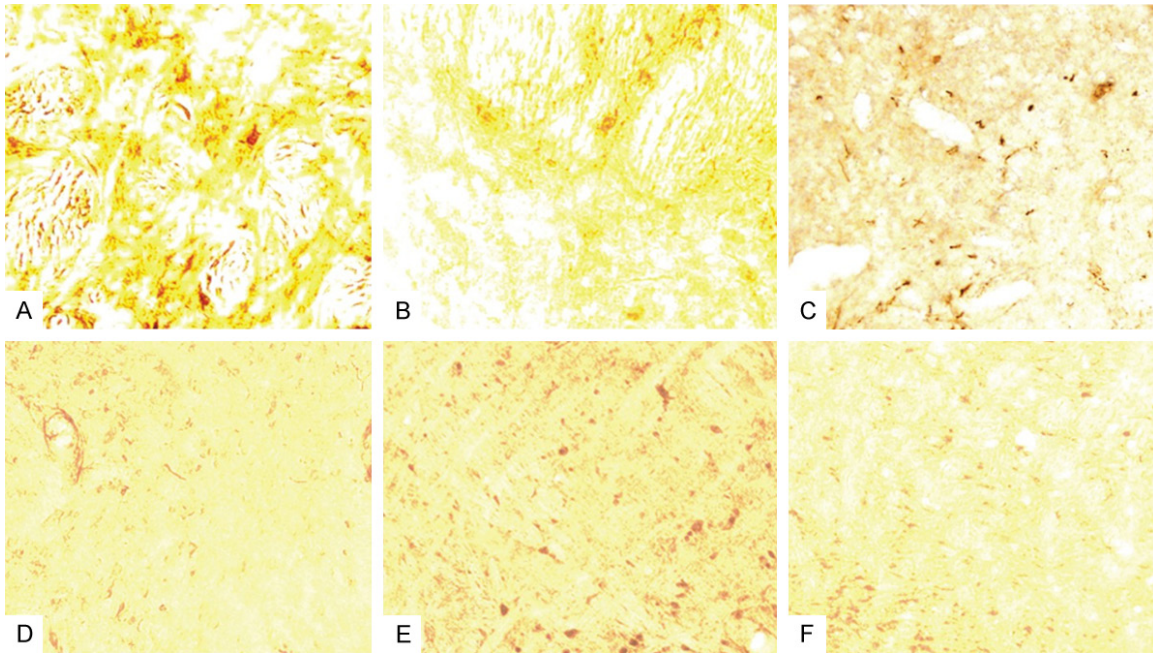
SSAT2 while decrease the expression of AQP4 in myocardial tissues after CRP.

### Western blotting results

Western blotting results were shown in **Figure 4**. They were similar with both RT-PCR and histopathological results. SSAT2 was the most in group A and least in group B, while AQP4 was the least in group A and most in group B.

### Correlation between cardiac function and SSAT2 and AQP4

The correlation analysis was shown in **Table 4** of which there was a positive correlation between SSAT2 and cardiac function in CRP model while there was a negative correlation between AQP4 and cardiac function ( $P < 0.01$ ). The levels of SSAT2 and AQP4 protein in myocardial tissue were negatively correlated in CRP model ( $r = -0.920$ ,  $P < 0.01$ ).



**Figure 3.** The immunohistochemical results of SSAT2 and AQP4 protein in myocardium tissues (scale bar =200 μm). A: SSAT2 positive expression in group A; B: SSAT2 positive expression in group B; C: SSAT2 positive expression in group C; D: AQP4 positive expression in group A; E: AQP4 positive expression in group B; F: AQP4 positive expression in group C.

**Table 3.** Comparison of SSAT2 and AQP4 expression in myocardium tissues ( $\bar{x} \pm s$ ,  $n=15$ )

Group	Slice	SSAT2		AQP4	
		OD	AD	OD	AD
A	75	6.90±2.25	12.57±3.79	2.26±0.44	6.12±1.90
B	75	1.82±0.39*	3.51±0.94*	7.52±2.03*	11.85±3.72*
C	75	4.53±1.49*,#	8.94±2.78*,#	4.62±1.79*,#	8.23±2.89*,#

\* $P<0.01$  compared with group A; # $P<0.01$  compared with group B. A: Sham operation group; B: Model group; C: UT1 group.

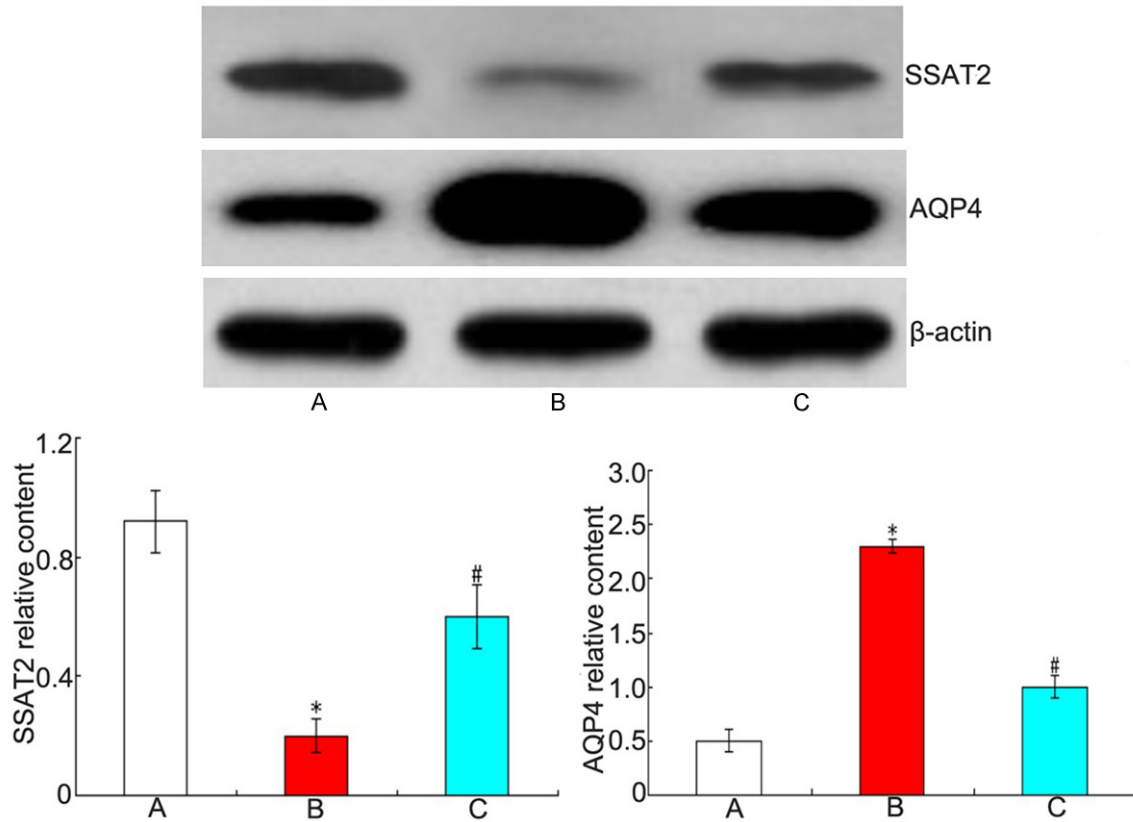
### Discussion

CPR rescue measures were often taken for the treatment of CA patients. However, the survival rate of patients was still low after CPR. The long-term survivors were only about 10% [9]. The main reason is the function damage of multiple organs caused by reperfusion after CPR, especially cardiac dysfunction. In physiological conditions, HIF-1 $\alpha$  produced by the body is rapidly degraded by SSAT2 [10]. SSAT2 is interacted with the 531-826 amino acid of HIF-1 $\alpha$ , and interacted with the 81-200 amino acid of HIF-1 $\alpha$  in 293 cells [11]. The expression of SSAT2 increased in myocardial tissues of ischemia reperfusion injury rat model [12] and myo-

cardial hypertrophy rat model [13]. SSAT2 was overexpressed in ischemic injury myocardium [14]. These suggest that the level of myocardial SSAT2 has important role for evaluating the prognosis of CPR patients. In this study we found that the levels of SSAT2 decreased in myocardial tissue of CPR rats, which was

inconsistent with that of myocardial tissue damage caused by other external factors. The possible reasons may be because the test time was not the same, or the model establishment method was not the same. AQP4 was widely used in the diagnosis of brain damage after CPR recently [4]. The detection of the water channel protein has an important role in the diagnosis of myocardial damage after CPR. It has been confirmed that AQP4 has good sensitivity in the evaluation of myocardial injury [15]. Feng et al. found in the study of cardiac arrest model rats that AQP4 in cerebral cortex was up-regulated early after heart and lung resuscitation [16]. Xiao et al. found that cerebral edema occurred in cardiac arrest rats after cardiopul-

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**Figure 4.** Western blotting results of SSAT2 and AQP4 protein in myocardium tissues. A: Sham operation group; B: Model group; C: UT1 group. \* $P < 0.01$  compared with group A; # $P < 0.01$  compared with group B.

**Table 4.** Correlation analysis of SSAT2 and AQP protein and heart function ( $\bar{x} \pm s$ ,  $n=30$ )

SSAT2 and AQP4 and heart function	$r$
SSAT2 and EF	0.712 <sup>a</sup>
SSAT2 and FS	0.850 <sup>a</sup>
AQP4 and EF	-0.843 <sup>a</sup>
AQP4 and FS	-0.791 <sup>a</sup>

<sup>a</sup> $P < 0.01$ .

monary resuscitation for 1 h with up-regulated AQP4 expression in cerebral cortex [17]. Taniguchi et al. found in cerebral ischemia model rat that AQP4 increased in astrocyte of cerebral cortex necrosis area [18]. High expression of AQP4 was closely related to myocardial edema and cardiac dysfunction [19-21]. In this study we found that AQP4 increased in myocardial tissue of CPR rats, suggesting that there was myocardial damage in rats after CPR.

UT1 is a broad-spectrum protease inhibitor and can inhibit the activity of a variety of enzymes,

block the release of cytokines and inflammatory factors, scavenge oxygen free radicals and reduce reperfusion injury [3, 4]. Sun et al. found that UT1 could decrease the expression of TLR4 and the activity of NF- $\kappa$ B and reduce the apoptosis of myocardial cells [22]. Hu et al. also found that UT1 could reduce the apoptosis of myocardial cells through inhibiting the expression of NF- $\kappa$ B, TNF- $\alpha$  and IL-6 [23]. In this study we found that UT1 could significantly improve the levels of LVFS, LVEF and E/A ratio and decrease myocardial cell apoptosis, which suggested that UT1 could improve the cardiac function of rats after CRP. As compared with group B, the expression level of SSAT2 increased and the expression level of AQP4 decreased in group C, suggesting that UT1 could effectively reduce the cardiac function damage caused by CRP and may be related with the increased SSAT2 and decreased AQP4.

In this study we found that there was a positive correlation between SSAT2 and cardiac function in CRP model while there was a negative

correlation between AQP4 and cardiac function. The levels of SSAT2 and AQP4 protein in myocardial tissue were negatively correlated in CRP model. Hypoxic ischemic injury and the related inflammatory reaction are one of the main pathways of myocardial injury after CPR [24] and HIF-1 $\alpha$  plays an important role in the pathophysiological process [25]. There was a negative correlation between SSAT2 and HIF-1 $\alpha$  in the process of myocardial resuscitation [5], and inhibition of HIF-1 $\alpha$  could reduce the expression of AQP4 [26]. Therefore, the underlying mechanism of SSAT2 and AQP4 action may be related with HIF-1 $\alpha$ .

In summary, we found that UT1 could effectively reduce the cardiac function damage caused by CRP, which could be related with the increased SSAT2 and decreased AQP4.

## Acknowledgements

This study was supported by Science and Technology Department of Qinghai Province Fund (2014-Z-741).

## Disclosure of conflict of interest

None.

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