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## Original Article

# Gene expression profiles of sodium-dependent vitamin C transporters in mice after alcohol consumption

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**Alcoholic liver disease (ALD) is a serious liver problem in western countries. Our previous study has demonstrated that vitamin C plays a protective role in ALD. The vitamin C homeostasis is tightly regulated by sodium-dependent vitamin C transporters (SVCTs) 1 and 2. But the role of two SVCTs in ALD is less understood. In this study, we examined the expression patterns of two SVCTs in mice after alcohol consumption. Our results suggested that alcohol consumption obviously increased the expression of two SVCTs in liver and SVCT1 in kidney and intestine, which is important for vitamin C absorption. Vitamin C supplement increased the sera vitamin C content and ameliorated the symptom of ALD. Intestinal absorption and renal re-absorption mediated by SVCT1 are key factors to increase the sera vitamin C content after alcohol consumption. We proposed that both reactive oxygen species and low vitamin C concentration regulate the expression of SVCTs, and the protective role of vitamin C is mediated by suppressing the stability of hypoxia-inducible factor-1α. Thus, our study is significant for the understanding of vitamin C homeostasis in ALD and for better use of other antioxidants in ALD therapy.**

*Keywords*    alcoholic liver disease; vitamin C; iron overload; sodium-dependent vitamin C transporter

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## Introduction

Alcoholic liver disease (ALD) is one of the most common diseases in western countries and common reasons for liver transplantation [1]. The most prevalent types of ALD include fatty liver, alcoholic hepatitis, and chronic hepatitis with hepatic fibrosis or cirrhosis [2]. The exact mechanism of ALD is not completely understood, but it is known that alcohol metabolism can lead to many liver injuries, including oxidative stress, lipid peroxidation, and acetaldehyde toxicity [3]. Many factors can increase the risk of ALD development after alcohol consumption, and iron overload is an important factor [4]. Iron is essential for cell growth and division, but excess iron causes tissue damage and organ failure through the production of reactive oxygen species (ROS) [5]. ROS are highly reactive, oxygen-containing molecules that can damage complex cellular molecules such as lipids, proteins, or DNA [6]. Acute and chronic alcohol treatments increase the production of ROS and decrease antioxidant activity in the liver [7]. These studies implied that antioxidants can prevent or delay the primary and secondary injuries associated with ALD [8].

Antioxidant therapy has been considered to have the possibility of beneficial effects on the management of many liver diseases including ALD [9]. Many antioxidants such as glutathione, vitamin E, and 2-hydroxy-4-methoxy benzoic acid afford protection against alcohol-induced liver injury in rats [10]. Vitamin C, also known as L-ascorbic acid, is an essential nutrient and an antioxidant, which can protect the body against oxidative stress [11]. Many studies have indicated that vitamin C can protect the liver from the deleterious effects of chronic high-dose alcohol and has hepatoprotective effect [12]. Our previous study also provided evidence that vitamin C supplement is important for the amelioration of ALD [13].

Although vitamin C is required for a range of essential metabolic reactions in all animals, it cannot be synthesized in several animals such as guinea pig, monkey, and human. It is essential for these species to absorb vitamin C from the diet. There are two biological forms of vitamin C: the reduced form, ascorbic acid (AA), and the oxidized form, dehydroascorbic acid (DHA) [14]. Vitamin C can enter the cell both in AA and DHA forms using sodium-dependent vitamin C transporters (SVCTs) and glucose transporters [15], respectively. There are two SVCTs, SVCT1 and SVCT2, which are encoded by solute carrier family 23 member 1 (*Slc23a1*) and 2 (*Slc23a2*), respectively [16]. The

vitamin C homeostasis is tightly regulated by intestinal absorption, tissue accumulation, and renal re-absorption and excretion [17]. SVCT1 is largely confined to epithelial surfaces such as intestine and kidney, and is involved in the maintenance of whole-body homeostasis through dietary absorption and renal re-absorption. SVCT2 exists in metabolically active tissues including brain, liver, and muscle in order to protect them from oxidative damage [18]. A previous study has indicated that antioxidants, ursodeoxycholic acid and bilirubin, exert protective effect on cholestasis-induced oxidative stress in liver cells through up-regulation of SVCT1 and/or SVCT2 [19]. It means that up-regulation of two SVCTs may be the essential mechanism for the antioxidant protective role.

To date, little information is available on vitamin C homeostasis after alcohol consumption. In this study, we examined the expression patterns of two SVCTs and several iron metabolism-related proteins including hepcidin, Tfr1 (transferrin receptor 1), Fpn1 (ferroportin1), IRP2 (iron regulatory protein 2), and L-ferritin (ferritin-light chain) in mice after alcohol consumption. Our results suggested that abnormal expression of iron metabolism-related proteins cause liver iron overload after alcohol drinking, and up-regulation of SVCT1 and SVCT2 expressions is essential to increase vitamin C absorption and to protect liver from oxidative injury.

## Materials and Methods

### Animal feed and treatment
Twenty male Kunming mice (KM), aged 4 weeks, were purchased from Laboratorial Animals Center of Hebei Medical University (Shijiazhuang, China), and divided into four groups (five mice per group): control group, alcohol group, alcohol + 1.5 mM vitamin C group, and alcohol + 3.0 mM vitamin C group. The mice in the control group received normal water supplement, and mice in other three groups received 25% alcohol (final concentration), which is dissolved in water. For vitamin C experiments, mice received no vitamin C, 1.5 mM vitamin C (Sangon Biotech Co., Ltd, Shanghai, China), and 3.0 mM vitamin C, which was dissolved in water, for 2 weeks, respectively. Then, all the mice were sacrificed. Liver, spleen, intestine, and blood were collected.

This study was carried out in strict accordance with animal ethical standard. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Hebei Medical University. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

### Cell culture and treatment
Human hepatocellular carcinoma cell line HepG2, human embryonic cell line HEK293, and human epithelial colorectal adenocarcinoma cell line Caco-2 were purchased from cell resource center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). These cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, USA), 100 IU/ml penicillin, 100 $\mu$g/ml streptomycin at $37^\circ$C in 5% $CO_2$. HepG2 cells were seeded into culture dishes (Corning, Corning, USA) and treated with 50 mM alcohol (no vitamin C supplement, 75 $\mu$M vitamin C supplement, and 150 $\mu$M vitamin C supplement, respectively). Untreated cell were used as controls. After being treated for 24 h, cells were collected for total RNA and protein extractions.

### Hematoxylin and eosin staining of liver tissue
Liver samples were fixed in 4% buffered formalin for 24 h and then immersed in phosphate-buffered saline (PBS; pH 7.4) for 4 h; treated with gradient concentrations of ethanol and xylene. Finally, liver samples were embedded with paraffin and sectioned to a thickness of 10 $\mu$m. After being dewaxed and rehydrated, tissue sections were stained with hematoxylin and eosin for 10 and 5 min, respectively. After being washed with water, sections were observed and analyzed under a microscope.

### Serum iron and alanine aminotransferase measurement
The measurements of iron and alanine aminotransferase (ALT) in mice serum were preformed by colorimetric method and Reitman method as previously described [20].

### High-performance liquid chromatography measurement of vitamin C
Vitamin C contents in serum samples of mice were determined by high-performance liquid chromatography (HPLC) as described previously [21], and the experiment was carried out on the LC-16A HPLC instrument (Shimadzu, Tokyo, Japan). First, sera or standard vitamin C samples were diluted in the mobile phase (60 mM phosphoric acid, pH 3.1). Secondly, a 20 $\mu$l mixed sample was injected onto a Nucleosil C18 column (Sigma, St Louis, USA) and eluted with an acetonitrile gradient (0%–60%) at a flow rate of 0.8 ml/min. Thirdly, vitamin C elution was monitored at 254 nm. Finally, relative peak area was measured and standard curve of pure vitamin C ranging from 25 to 500 $\mu$M was used to calculate the content of vitamin C in serum samples. Livers were homogenized in 5.4% metaphosphoric acid and the supernatants were prepared with centrifugation. Total vitamin C levels in supernatant were determined with HPLC.

### Total RNA extraction and cDNA synthesis
Total RNA of mouse liver, kidney, duodenum, and cells was extracted using the Trizol reagent (Invitrogen, Carlsbad,

USA) according to the manufacturer's protocol. The total RNA concentration was determined with Nanodrop 2000 (Thermo Scientific, Wilmington, USA). RNA (2 μg) was reversely transcribed into first-strand cDNA using a reverse transcription system according to the manufacturer's instructions (Invitrogen). The conditions for reverse transcription are 42°C for 60 min and 70°C for 15 min.

### Semi-quantitative polymerase chain reaction

The mRNA expression levels of iron metabolism-related proteins were analyzed by semi-quantitative polymerase chain reaction (semi-qPCR). The primer of mouse *hepcidin*, *TfR1*, *Fpn1*, *L-ferritin*, and *β-actin* were synthesized by Sangon Biotech Co., Ltd and the sequences are listed in **Table 1**.

PCR amplification was performed by adding 2 μl aliquot of cDNA sample to 20 μl of reaction mixture (Fermentas, Glen Burnie, USA) containing both the forward and reverse primers. Amplification was carried out in DNA Thermal Cycler (Applied Biosystems, Foster City, USA) under the following conditions: denaturation at 94°C for 10 min, annealing at 58°C for 45 s, and extension at 72°C for 30 s, with final extension at 72°C for 10 min. Based on the results of preliminary studies, 25 cycles were used for *β-actin*, 30 cycles for *hepcidin*, 33 cycles for *TfR1*, and 27 cycles for *Fpn1*. Each PCR product (5 μl) was subjected to electrophoresis on 1.5% agarose gel in Tris-acetic acid-EDTA buffer and stained with ethidium bromide. Gels were viewed, and the images were stored and analyzed digitally with Fujifilm LAS-4000 (Tokyo, Japan). For each sample, the densitometric units of the amplified cDNA fragments were counted for semi-quantitative evaluation by normalization with the *β-actin*.

### Quantitative real-time PCR

The mRNA expression levels of vitamin C transporters were determined by quantitative real-time PCR

**Table 1 Primer sequences for semi-qPCR**

| Gene | Primer sequence | Size (bp) |
|---|---|---|
| *Hepcidin* | 5′-CCTATCTCCATCAACAGATG-3′ | 171 |
| | 5′-AACAGATACCACACTGGGAA-3′ | |
| *TfR1* | 5′-GCAGCTATTGCACTAGTC-3′ | 420 |
| | 5′-TGACTGCACTATGGTCAC-3′ | |
| *Fpn1* | 5′-CCAGCATCAGAACAAACACG-3′ | 175 |
| | 5′-ACTGCAAAGTGCCACATCC-3′ | |
| *L-ferritin* | 5′-AATCAGGCCCTCTTGGATC-3′ | 82 |
| | 5′-GATAGTGGCTTTCCAGGAAGTC-3′ | |
| *β-actin* | 5′-AGCCATGTACGTAGCCATCC-3′ | 250 |
| | 5′-TTTGATGTCACGCACGATTT-3′ | |

**Table 2 Primer sequences for qRT-PCR**

| Gene | Primer sequence | Size (bp) |
|---|---|---|
| *SVCT1* (mouse) | 5′-CCCACGCCAACAGTGAGACG-3′ | 171 |
| | 5′-CTTGGTACACACAGACCCAGTTTC-3′ | |
| *SVCT2* (mouse) | 5′-GCAAGAACACGGCATCCAAGTC-3′ | 147 |
| | 5′-TGTCCTCGTTGTCCTGCTCTCC-3′ | |
| *18S* (mouse) | 5′-GTAACCCGTTGAACCCCATT-3′ | 151 |
| | 5′-CCATCCAATCGGTAGTAGCG-3′ | |
| *SVCT1* (human) | 5′-CCGGCGCGTGGTGCAGTATG-3′ | 89 |
| | 5′-GGTCAGGGAGCGAGGCGAAGAG-3′ | |
| *SVCT2* (human) | 5′-TCCAGCGGTGAGCAGGACAATGAG-3′ | 119 |
| | 5′-GGGTCCAGACTGCCAGTGCTATCC-3′ | |
| *18S* (human) | 5′-CGGCGGCTTTGGTGACTCTAG-3′ | 175 |
| | 5′-CCGTTTCTCAGGCTCCCTCTCC-3′ | |

(qRT-PCR). The primer sequences to mouse/human *SVCT1*, *SVCT2*, and *18S* were shown in **Table 2**. qRT-PCR was carried out by adding 0.4 μl aliquot of cDNA sample to 20 μl of SYBR green PCR Mix (Tiangen, Beijing, China) including 0.4 μl forward and inverse primers. Amplification was performed using 7500 Real-Time PCR System (Applied Biosystems) under the following conditions: 95°C for 2 min, 95°C for 20 s, 55°C for 30 s, and 68°C for 45 s, 40 cycles. Relative expression levels of *SVCT1* and *SVCT2* were normalized to the internal reference *18S* RNA. The data were analyzed using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method.

### Western blot analysis

Cells and mice tissues including liver, spleen, and intestine were homogenized using supersonic method. The lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, pH 8, and 1% NP-40) included inhibitors of proteases (1 mM phenylmethanesulfonyl fluoride, 10 mg/ml leupeptin, and 10 mg/ml pepstatin A). Proteins were quantified using Coomassie brilliant blue staining method. A total of 50 μg protein was dissolved in reducing loading buffer, separated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and transferred onto nitrocellulose (NC) membranes. Then the membranes were blocked with 5% non-fat milk in TBST (50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween-20) for 2 h, incubated with 1 : 5000 primary antibodies overnight, and then incubated with 1 : 5000 diluted peroxidase-conjugated goat anti-rabbit IgG (secondary antibody; Sigma) for 1 h. After being washed with TBST four times (5 min/time), the NC membrane was exposed to chemical luminescence substrate (Pierce, Rockford, USA) for 5 min and then detection was performed with Fujifilm Las-4000.

The primary antibodies used in this study include anti-TfR1 (Alpha Diagnostic, San Antonio, USA), anti-Fpn1 (Alpha Diagnostic), anti-L-ferritin (Sigma), anti-IRP2 (Alpha Diagnostic), anti-hypoxia-inducible factor-1α (HIF-1α; Alpha Diagnostic), anti-SVCT1 (Santa Cruz, Canta Cruz, USA), anti-SVCT2 (Santa Cruz), and anti-β-actin (Sigma).

### Immunohistochemical analysis

Mice liver specimens embedded with paraffin were serially sectioned to a thickness of 5 μm, dewaxed, and rehydrated. The endogenous peroxidase activity of section was blocked with 3% $H_2O_2$/methanol at room temperature for 10 min. The sections were heated to 95°C in 0.01 M citrate buffer (pH 6.0) for 10 min to restore antigen activity. Non-specific binding was blocked with 5% bovine serum albumin in a humidified chamber at room temperature for 30 min. Sections were then incubated overnight at 4°C with 1 : 200 dilution of rabbit polyclonal antibodies against SVCT1 and SVCT2. After being washed twice using PBS, the sections were incubated with peroxidase-conjugated goat anti-rabbit IgG (ZSGB Bio, Beijing, China) at room temperature for 30 min and then reacted with DAB kit (ZSGB Bio) according to the manufacturer's protocol. Sections were observed and analyzed under the Axioscope A1 microscope (Carl Zeiss, Jena, Germany).

### Statistical analysis

All results were expressed as the mean ± standard deviation (SD). Comparisons between groups were analyzed by a two-tailed paired-sample Student's t-test. Probability value of $P < 0.05$ was considered statistically significant. All data were managed by SPSS 17.0.

## Results

### Alcohol consumption leads to obvious liver injuries and vitamin C supplement ameliorates the condition

Similar to our previous study [13], alcohol consumption (30 ml water and 3 g alcohol per day per mouse) causes abnormal physiological indices in mice including weight gain, rate of weight gain, liver iron, and serum ALT (Table 3). Alcohol consumption also leads to disorder of iron metabolism and liver injuries (**Supplementary Figs. S1** and **S2**). Vitamin C supplement ameliorates the damage effect of alcohol consumption.

### Up-regulated expression of two SVCTs in liver after alcohol consumption

To understand the role of vitamin C transporters in alcohol metabolism, we further examined the change of SVCT1 and SVCT2 expression levels in liver. The results indicated that both vitamin C transporters are up-regulated after 2 weeks' alcohol consumption (**Fig. 1**). The increased level of SVCT1 was more obvious than that of SVCT2 in liver [**Fig. 1(A,B)**], which indicated that SVCT1 may be more important in hepatic vitamin C absorption after alcohol consumption. On the other hand, SVCT2 protein was decreased after vitamin C supplement [**Fig. 1(B,D)**], which was inconsistent with the change of mRNA level [**Fig. 1(A)**]. The possible explanation is that post-translational modification is involved in SVCT2 protein stability. Our results indicated that alcohol consumption also up-regulated the contents of SVCT1 and SVCT2 after 1.5 mM vitamin C supplement (**Supplementary Fig. S3**).

### Vitamin C supplement increases the content of vitamin C in serum and liver

In order to understand the effect of vitamin C supplement, the vitamin C concentration in sera was determined with HPLC method. Chromatograms showed that HPLC method is specific for vitamin C measurement [**Fig. 2(A)**]. Standard curve indicated that linear relationship is very good between vitamin C concentrations and peak areas ($R^2 = 0.9987$) [**Fig. 2(B)**]. Vitamin C supplement after alcohol consumption obviously increased vitamin C concentration in serum ($P < 0.01$) [**Fig. 2(C)**], which is effective for protecting liver from oxidative damage. Consistent with this, vitamin C

**Table 3 The change of related indices in mice after 2 weeks' alcohol consumption ($n = 5$)**

|  | Control group | Alcohol group | Alcohol + 1.5 mM vitamin C group | Alcohol + 3.0 mM vitamin C group |
| --- | --- | --- | --- | --- |
| Weight gain (g) | 5.8 ± 1.1 | 1.6 ± 2.3* | 6.3 ± 2.4# | 6.2 ± 2.1# |
| Rate of weight gain (%) | 15.5 ± 3.9 | 6.2 ± 6.2* | 20.4 ± 1.6# | 19.2 ± 3.2# |
| ALT (U/L) | 58.94 ± 39.10 | 148.64 ± 53.46** | 67.73 ± 34.07# | 115.81 ± 30.55 |
| Liver iron (μg/g wet) | 221.34 ± 33.51 | 830.49 ± 225.28** | 632.16 ± 129.94 | 724.90 ± 212.02 |
| Liver vitmain C (μg/g wet) | 120.31 ± 9.18 | 102.72 ± 4.36 | 170.15 ± 7.71# | 201.24 ± 8.53# |

*$P < 0.05$ or **$P < 0.01$ vs. control group.
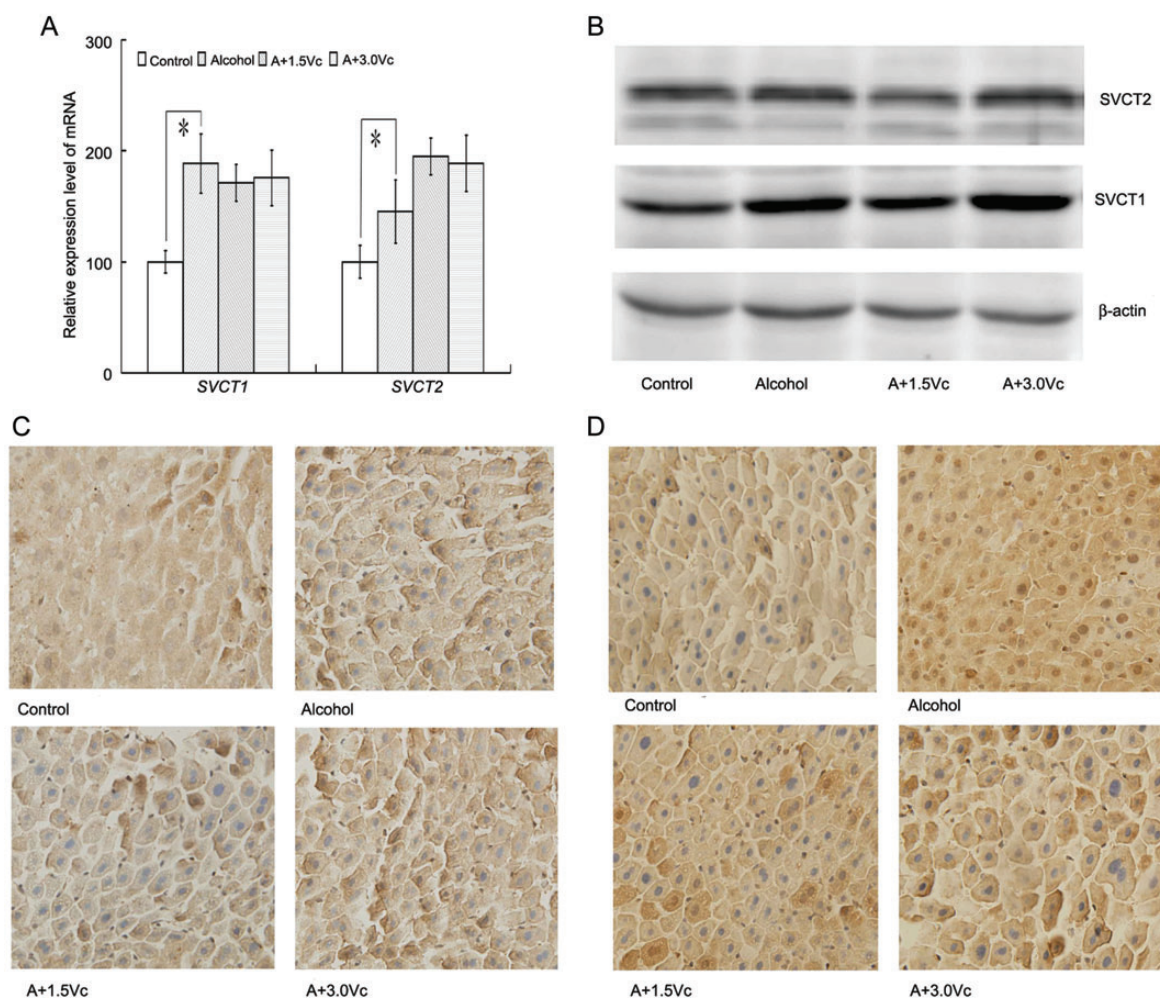#$P < 0.05$ vs. alcohol group.

**Figure 1 Expression levels of SVCT1 and SVCT2 in liver** (A) qRT-PCR of *SVCT1* and *SVCT12*. (B) Western blot analysis of SVCT1 and SVCT12. (C) Immunohistochemistry of SVCT1 in liver. (D) Immunohistochemistry of SVCT2 in liver. Alcohol consumption increases the expression of two SVCTs and absorption of vitamin C. For each experiment, the mRNA levels of various groups were normalized to the control, which has been set to 100%. The data were shown as the mean $\pm$ SD from five mice. The experiment was repeated three times. *$P < 0.05$ vs. control.

supplement also increased vitamin C levels in liver ($P <$ 0.05) (**Table 3**).

**The content of sera vitamin C is mainly determined by intestinal absorption and renal re-absorption after alcohol consumption**

A previous study has indicated that intestinal absorption and renal re-absorption were major determinants of vitamin C concentration in sera [17]. In the present study, the expression of SVCT1 in three cell lines and three tissues was investigated. SVCT1 expression was significantly increased in HEK293 and Caco-2 compared with that in HepG2 [**Fig. 3(A)**]. The expression levels of SVCT1 in kidney and intestine were also higher than that in liver [**Fig. 3(A)**]. Further experiments showed that the relative levels of *SVCT1* mRNA and SVCT1 protein were obviously increased after alcohol consumption in kidney and intestine (both $P < 0.05$) [**Fig. 3(B−D)**].

**The stability of HIF-1α protein is partly affected by vitamin C content**

The experimental and clinical data have confirmed that high alcohol levels in blood can lead to liver hypoxia, which increased the liver damage of ALD [20]. HIF-1α can modulate expression of liporegulatory genes and cause hepatic lipid accumulation [22]. In this study, the protein expression of HIF-1α was obviously increased accompanied with lipid accumulation in ALD mice compared with control mice [**Supplementary Fig. S2(D)**]. Vitamin C supplements reduced the expression level of HIF-1α protein and attenuated lipid accumulation [**Supplementary Figs. S1(C)** and **S2(D)**].

**Treatment with alcohol also increases the levels of SVCTs in cells**

The experiments further provided evidence that alcohol treatment increased the expression of SVCT1 at both mRNA
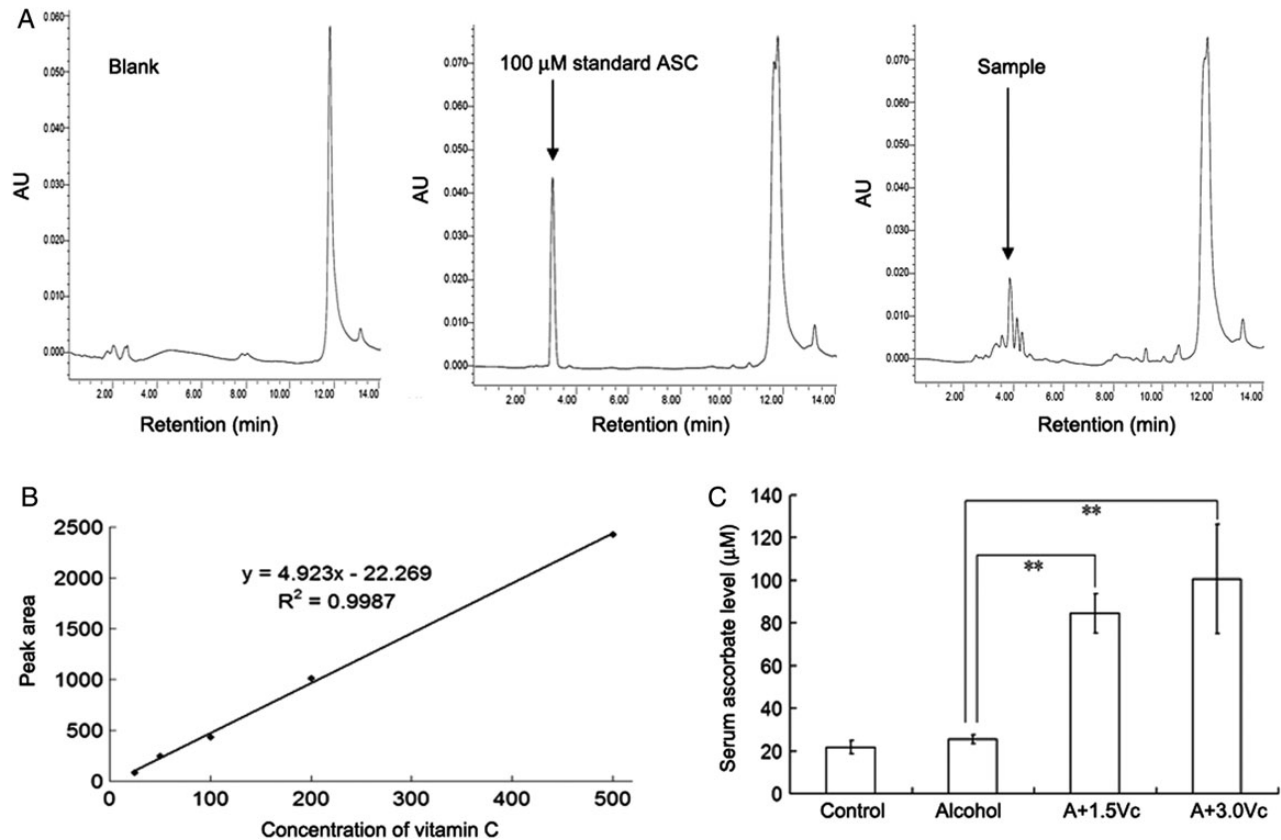
**Figure 2 HPLC determination of ascorbate concentrations** (A) Exemplary chromatograms derived from samples of blank (60 mM phosphoric acid), standard vitamin C solution (100 μM), and sample, respectively. (B) Standard curve was obtained after plotting the peak areas of vitamin C elution (given in arbitrary units; AU) against known vitamin C concentrations. (C) Serum vitamin C concentration in different groups. Vitamin C supplement in alcohol consumption water can obviously increase serum vitamin C content. The data were shown as the mean $\pm$ SD from five mice. The experiment was repeated twice. **$P < 0.01$ vs. alcohol group.

and protein levels ($P < 0.05$) (**Fig. 4**). But it is not evident that SVCT2 expression was up-regulated. Because SVCT2 is a major determinant of ascorbate accumulation in tissues lacking SVCT1 [23], it is possible that SVCT1 is more important than SVCT2 after cell treatment with alcohol.

## Discussion

Because iron constitutes a major source of toxicity due to its ability to generate ROS that can damage cellular macromolecules, excessive iron in the body of ALD patients is extremely dangerous [24]. Many antioxidants play protective roles in the alcohol-induced hepatotoxicity. Our previous study has demonstrated that an antioxidant, vitamin C, can ameliorate the liver damage [13]. Alcohol consumption led to abnormal expressions of iron metabolism-related proteins, which was an important cause for hepatic iron overload. Our results indicated that vitamin C can partly ameliorate disorder of iron metabolism.

Compared with other natural antioxidants, vitamins such as vitamin C and vitamin E have been widely used in the clinic, which also play protective roles in ALD [13,25].

A better understanding of the mechanism by which oxidative stress leads to liver damage during alcohol exposure is essential for vitamin C and vitamin E therapy for ALD in humans [26]. Iron overload in liver was associated with oxidative stress and up-regulation of antioxidant defense [27]. In this study, we showed that increased levels of two SVCTs were an important feature after hepatic iron overload in mice after alcohol consumption, which is beneficial to vitamin C intake.

But there is a question required to be solved. How these antioxidants are absorbed into blood from the intestine and then entered the body? Considering that vitamin C homeostatic mechanism has been extensively studied in the past years, we determine to further study the change of vitamin C transports in the alcohol-induced hepatotoxicity. It has been shown that vitamin C is more effective than any other antioxidants such as quercetin and thiamine to treat hepatotoxicity induced by alcohol administration [12]. We proposed that the existence of two SVCTs is important for high efficiency of vitamin C absorption, but the mechanism of other antioxidants absorption remains to be investigated. Perez *et al.* [19] has proved that antioxidants can affect the expression of two SVCTs in
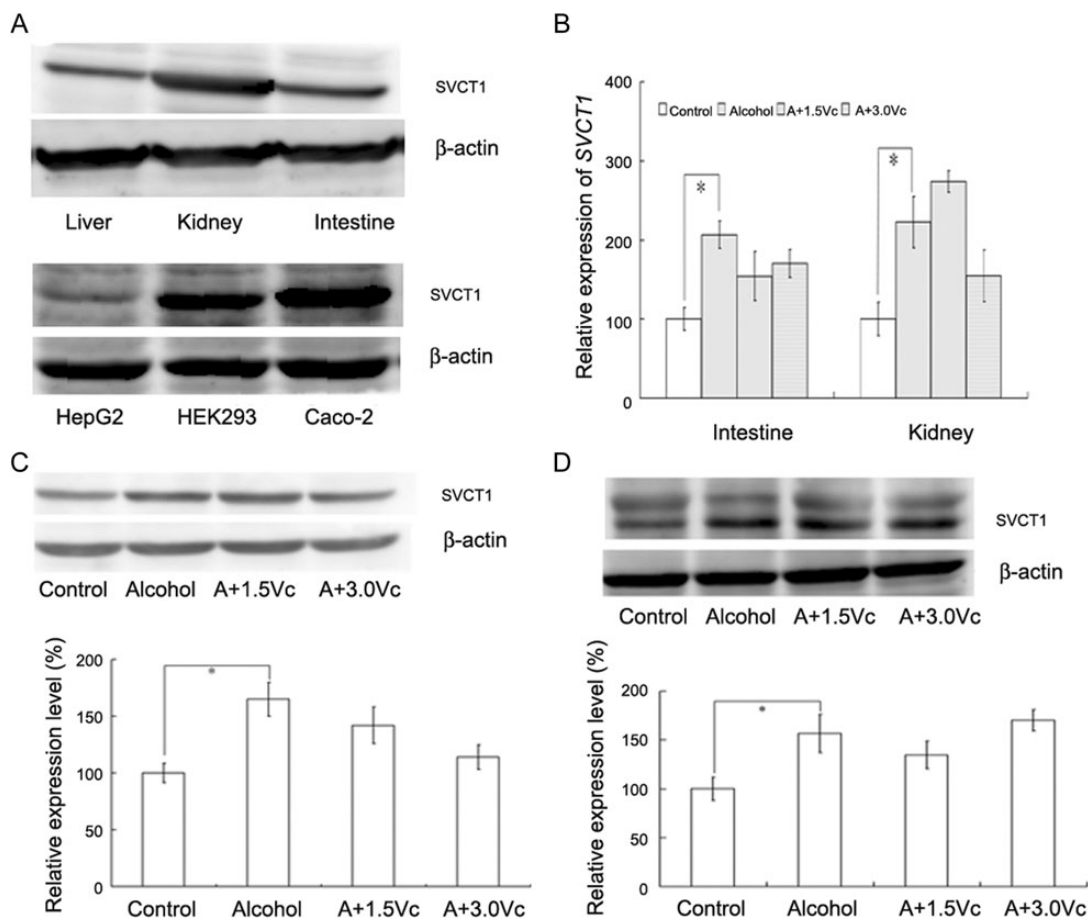
**Figure 3 Expression patterns of SVCT1 in different tissues** (A) Protein expression of SVCT1 in three tissues and cell lines. (B) qRT-PCR of *SVCT1* mRNA in intestine and kidney. (C) Protein expression of SVCT1 in intestine. (D) Protein expression of SVCT1 in kidney. Alcohol consumption obviously up-regulates the expression of SVCT1 in intestine and kidney. For each experiment, the mRNA/protein levels of various groups were normalized to the control, which has been set to 100%. The data were shown as the mean ± SD from five mice. The experiment was repeated three times. *$P < 0.05$ vs. control.

liver, so we speculated that liver protective role of some anti-oxidants after alcohol consumption may be mediated by up-regulation of SVCT1 and/or SVCT2.

SVCT1 and SVCT2 are crucial for maintaining vitamin C homeostasis and intracellular vitamin C concentrations in most cell types [28]. It has been shown that intracellular vitamin C is an important regulator of SVCT1 and SVCT2 expression in the liver, and vitamin C depletion can enhance expression levels of two SVCTs [29]. The expression levels of two SVCTs were increased in the liver of rats that were not administered by vitamin C and decreased in those that were administered by vitamin C, which further demonstrated that vitamin C level can regulate the expression of two SVCTs [30]. Our result indicated that two SVCTs are obviously up-regulated in ALD mice and moderately down-regulated after vitamin C supplement. Because the need of vitamin C during chronic consumption of moderate alcohol doses is enhanced [31], the increased levels of two SVCTs are important for vitamin C supplement. A previous study demonstrated that SVCT2 expression was increased through vitamin C

depletion in the liver [32]. Considering that SVCT2 is mainly expressed in metabolically active tissues, the up-regulation of SVCT2 is essential to reduce oxidative damage [33,34].

On the regulatory and functional mechanisms of SVCTs in ALD, we hypothesize that ROS plays an important role. Because iron participates in the formation of ROS, iron overload in liver can lead to oxidative stress and elicit toxic effects [35]. Vitamin C can antagonize the ROS-induced cytotoxic effects and tissue damage [36,37]. ROS stabilizes HIF-1α protein and then increases the expression of hypoxia-response genes [38,39]. Our previous study indicated vitamin C could scavenge the ROS and diminish hypoxia effect [40]. In this study, the expression of HIF-1α protein was obviously increased in liver after alcohol consumption, and vitamin C supplement antagonized this effect. It was confirmed that vitamin C is a major regulator of the hypoxic response [41].

In conclusion, the present study provides the evidence that the levels of SVCT1 and SVCT2 are regulated by alcohol metabolism through iron overload (**Fig. 5**). It was known
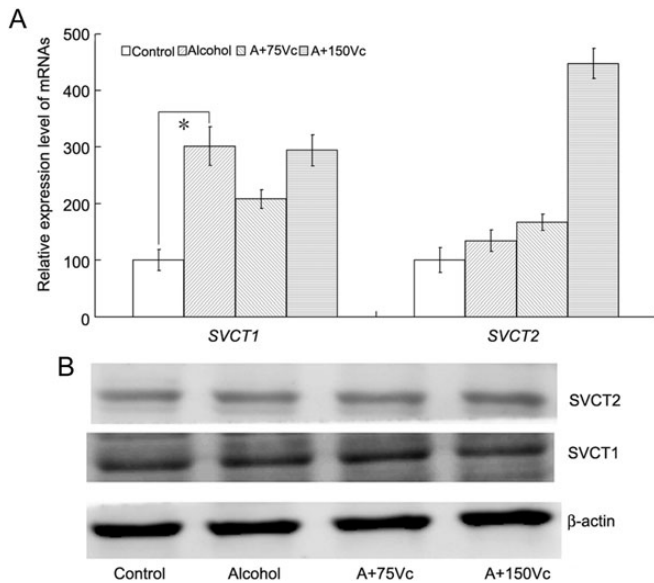
**Figure 4 Expression of SVCT1 and SVCT2 in cell line HepG2** (A) qRT-PCR of *SVCT1* and *SVCT2*. (B) Western blot analysis of SVCT1 and SVCT2. Alcohol treatment (50 mM) up-regulates the expression of SVCT1, but it is not significant. For each experiment, the mRNA levels of various treatments (alcohol, alcohol + 75 μM vitamin C, and alcohol + 150 μM vitamin C) were normalized to the control, which has been set to 100%. The data were shown as the mean ± SD from triplicates. The experiment was repeated three times. *$P < 0.05$ vs. control.
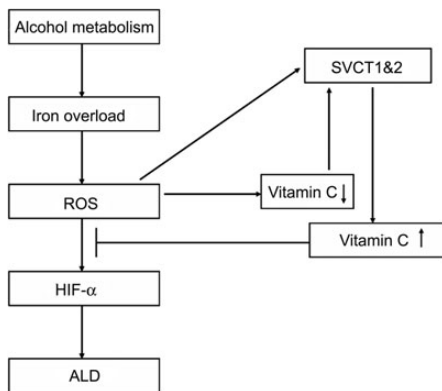


**Figure 5 Proposed model of two SVCTs** Alcohol metabolism can lead to iron overload in liver, which further induces the production of ROS. ROS can directly contribute to the up-regulation of SVCT1 and SVCT2 expression, but also consume the vitamin C. While low vitamin C is also an inducer of SVCT1 and SVCT2 expression. Both SVCT1 and SVCT2 can increase the absorption of vitamin C, which lowers the ROS content and further reduces the stability of HIF-α.

that a variety of factors may regulate the expression of SVCTs such as SVCT2 [42,43], but the detailed mechanism was poorly understood. Both SVCT2 and IRP2 were regulated by redox status [43,44], and the expression of these two proteins were up-regulated after alcohol consumption. This result demonstrates that there is an association between them. Furthermore, alcoholic liver toxicity is complex and involved in various signal transduction pathways [45].

SVCT1 and SVCT2 have different expression patterns in liver and may play different roles under physiological and pathological conditions [46]. So, the role and regulation of SVCTs in ALD still remains to be investigated.

## Supplementary Data

Supplementary data are available at *ABBS* online.

## Acknowledgments

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