

## Original Article

# Pyrrolizidine alkaloid clivorine-induced oxidative stress injury in human normal liver L-02 cells

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**ABSTRACT:** Clivorine is an otonecine-type pyrrolizidine alkaloid isolated from the traditional Chinese medicine *Ligularia hodgsonii* Hook. Pyrrolizidine alkaloids (PAs) are well-known hepatotoxins widely distributed around the world. The present study sought to evaluate clivorine-induced oxidative injury in human normal liver L-02 cells. After cells were treated with various concentrations of clivorine for 48 h, cellular total antioxidant capacity, glutathione-S-transferase (GST) and glutathione reductase (GR) were determined to evaluate oxidative injury. Results showed that cellular total antioxidant capacity and GST activity both increased in clivorine-treated L-02 cells, while clivorine decreased GR activity in cells. Further, the protective effects of some antioxidants such as ascorbic acid (vitamin C, Vc), Trolox, dithiothreitol (DTT) and mannitol against clivorine-induced cytotoxicity were observed. Results showed that Trolox, which is an analogue of tocopherol (vitamin E, Ve), prevented clivorine-induced cytotoxicity in L-02 cells. Taken together, these results revealed clivorine-induced oxidative injury in human liver L-02 cells. These results also indicated the possible use of Trolox in the reduction of clivorine-induced hepatotoxicity.

**Keywords:** Pyrrolizidine alkaloid, clivorine, oxidative injury, antioxidant, Trolox

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## 1. Introduction

Mammalian cells are generally kept in a reducing environment that is maintained by various antioxidants. The overproduction of reactive oxygen species (ROS) will occur after the disruption of the cellular oxidant/antioxidant balance by exogenous oxidative injury and thus leading to cell death by the damage of cellular macromolecules such as lipids, proteins, and nucleic acids (1). The cellular redox balance is maintained by both enzymatic and nonenzymatic antioxidant systems to maintain the normal reducing environment in cells. Cellular nonenzymatic antioxidants primarily include  $\alpha$ -tocopherol, ascorbic acid, and dithiothreitol (DTT), while the enzymatic antioxidant system includes catalase, superoxide dismutase, and reduced glutathione (GSH)-related enzymes such as glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx) (2). Many studies have found that oxidative stress injury was involved in hepatotoxin-induced toxicity in hepatocytes involving alcohol, acetaminophen, carbon tetrachloride, etc. (3-5).

Pyrrolizidine alkaloids (PAs) are natural hepatotoxins widely distributed in various species of plants around the world. Due to their serious liver injury, the U.S. Food and Drug Administration (FDA) proposed a series of research programs and instructions to alert people to the toxicity of PAs-containing herbs, while the British Medicines Healthcare Products Regulatory Agency (MHRA) and bodies in other European countries all formulated standards to limit the dosage of PAs per day. The hepatotoxicity of PAs has been widely investigated around the world. Clivorine is an otonecine-type PA abundant in *Ligularia hodgsonii* Hook and *Ligularia dentata* Hara, which are generally used to treat coughs, hepatitis, and inflammation in traditional Chinese medicine (6,7). Previous studies by the current authors have found that clivorine inhibited cell growth and induced cell apoptosis in normal human liver L-02 cells via cellular mitogen-activated protein kinases and mitochondrial-mediated apoptotic signal

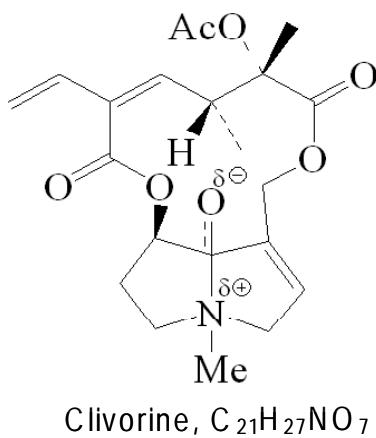
pathways (6,8,9).

The present study was designed to observe clivorine-induced oxidative injury in human liver L-02 cells and protection provided by various antioxidants.

## 2. Materials and Methods

### 2.1. Cells and reagents

A L-02 cell line was derived from adult normal human livers (10,11) (Cell Bank, Type Culture Collection of Chinese Academy of Sciences), and cells were cultured in RPMI1640 supplemented with 10% (v/v) fetal bovine serum. Clivorine (Figure 1) was isolated from *Ligularia hodgsonii* Hook with a purity  $\geq 99.5\%$ . GSH, oxidized glutathione (GSSG), and NADPH were purchased from Roche Diagnostics GmbH (Mannheim, German). Unless indicated, other reagents were from Sigma Chemical Co. (St. Louis, MO, USA).



**Figure 1.** The chemical structure of clivorine.

### 2.2. Cell viability assay

After various treatments, cells were incubated with 500  $\mu\text{g}/\text{mL}$  3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) for 4 h. The functional mitochondrial dehydrogenases in surviving cells converted MTT to blue formazan, which was dissolved in 10% SDS-5% iso-butanol-0.01 M HCl (12). The optical density was measured at 570 nm, with 630 nm as a reference, and cell viability was normalized as a percentage of the control.

### 2.3. Measurement of cellular total antioxidant capacity

The ferric reducing antioxidant power (FRAP) assay is a simple and reliable colorimetric method commonly used to measure total antioxidant capacity (13). After treatment, cells were harvested in cold phosphate buffer (pH 7.0) and sonicated (2  $\times$  5 sec) on ice and then

centrifuged at 5,000  $\times$  g for 10 min. The supernatant was transferred to new tubes for determination. The FRAP testing buffer consisted of 300 mM acetate buffer (pH 3.6), 20 mM ferric chloride and 10 mM TPTZ in 40 mM hydrochloric acid. The three above solutions were mixed together at a ratio of 25:2.5:2.5 (v/v/v), and the absorbance of the reaction mixture at 593 nm was measured after incubation with the supernatant at 25°C for 10 min. The FRAP values, expressed in mmol FeSO<sub>4</sub> per mg protein, were derived from a prepared standard curve.

### 2.4. Measurement of cellular glutathione S transferase

After treatment, cells were harvested in cold phosphate buffer (pH 7.0) and sonicated (2  $\times$  5 sec) on ice and then centrifuged at 5,000  $\times$  g for 10 min. The supernatant was used for enzymatic assays. GST activity was measured in accordance with a previously reported method (14) using 1-chloro-2,4-dinitrophenol (CDNB) as the substrate. A unit of GST activity is defined as the amount of enzyme catalyzing the formation of 1  $\mu\text{M}$  of product per min under the conditions of the specific assay. Specific GST activity is defined as the units of enzyme activity per mg of protein. Protein concentrations were determined by the Bradford method (15) with bovine serum albumin as a standard.

### 2.5. Measurement of cellular glutathione reductase

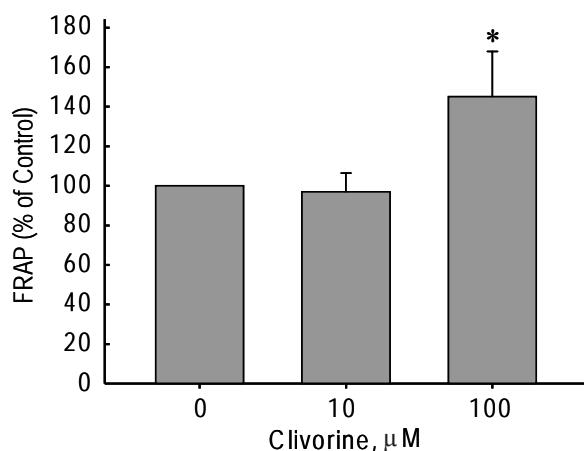
After treatment, cells were harvested in cold phosphate buffer (pH 7.0) and sonicated (2  $\times$  5 sec) on ice and then centrifuged at 5,000  $\times$  g for 10 min. The supernatant was used for enzymatic assays. The glutathione reductase (GR) activity was assayed according to a reported method (16). The enzymatic activity of GR was expressed as mU/mg protein, where 1 unit of GR activity is defined as 1 mmol GSSG catalyzed per minute.

### 2.6. Statistical analysis

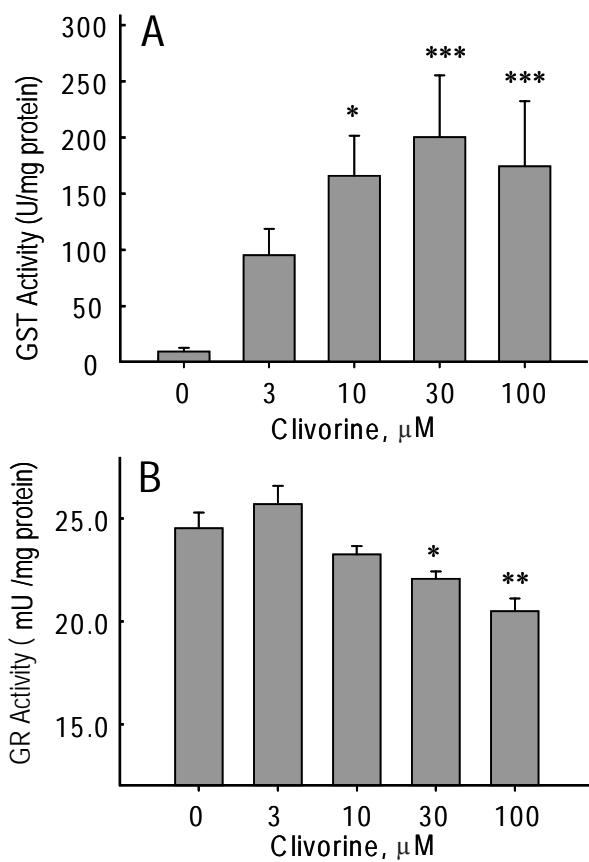
For all experiments, data were expressed as means  $\pm$  SEM. Statistical comparisons were subjected to an analysis of variance (ANOVA) and LSD-test using SPSS version 11.5, and  $p < 0.05$  was considered as statistically significant difference. All statistical analysis were performed using SigmaPlot version 10.0 software.

## 3. Results

Previous studies have revealed the cytotoxicity of clivorine on human liver L-02 cells (6,8). As shown in Figure 2, clivorine at a concentration of 100  $\mu\text{M}$  increased cellular total antioxidant capacity, which may be due to the cellular defense against the oxidative stress injury induced by clivorine.



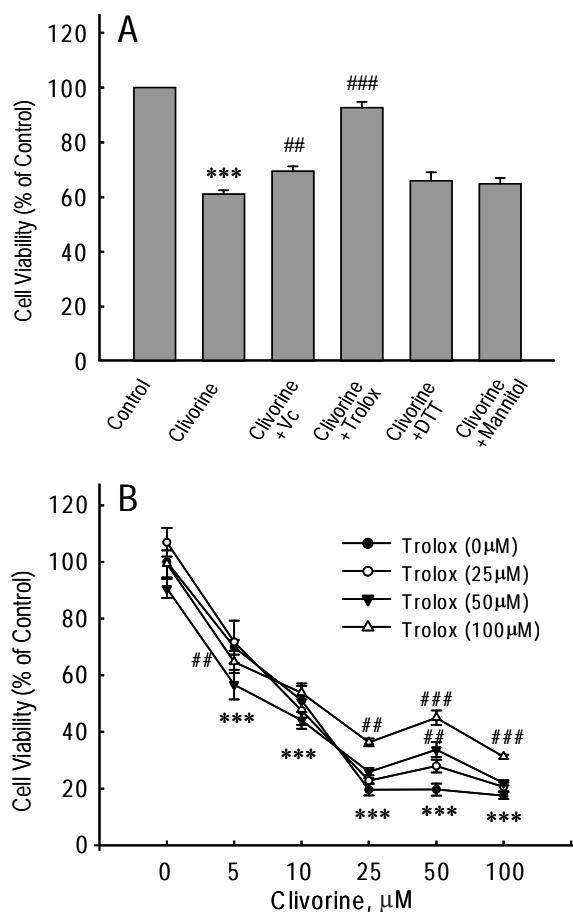
**Figure 2. Effects of clivorine on cellular total antioxidant capacity in L-02 cells.** Cells were treated with 10  $\mu\text{M}$  and 100  $\mu\text{M}$  clivorine for 48 h, and then cellular total antioxidant capacity was analyzed as described in "Materials and Methods". The results (means  $\pm$  SEM,  $n = 6$ ) are expressed as a percent of the control in the absence of clivorine.\* $p < 0.05$  in comparison to the absence of clivorine.



**Figure 3. Effects of clivorine on GST (A) and GR (B) activity in L-02 cells.** (A) Cells were treated with various concentrations of clivorine for 48 h, and then cellular GST activity was analyzed as described in "Materials and Methods". (B) Cells were treated with various concentrations of clivorine for 48 h, and then cellular GR activity was analyzed as described in "Materials and Methods". Data are means  $\pm$  SEM ( $n = 6$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  in comparison to the absence of clivorine.

L-02 cells were treated with various concentrations of clivorine (3, 10, 30, and 100  $\mu\text{M}$ ) for 48 h. As shown in Figure 3A, clivorine significantly increased GST activity in a concentration-dependent manner, while clivorine decreased GR activity (Figure 3B).

The effect of several well-known antioxidants, such as ascorbate (Vc), Trolox, mannitol, and DTT, on clivorine-induced cytotoxicity was also observed in L-02 cells. As shown in Figure 4A, cell viability decreased to 60% of the control after cells were incubated with 50  $\mu\text{M}$  clivorine for 48 h. When cells were pretreated with Trolox (50  $\mu\text{M}$ ) for 15 min, clivorine-induced cytotoxicity significantly reversed, and cell viability increased from 60% to 90% of the control. The effect of various concentrations of Trolox on clivorine-induced cytotoxicity was also observed. As shown in Figure 4B, the protection provided by



**Figure 4. Effects of various antioxidants on clivorine-induced cytotoxicity in L-02 cells.** (A) Cells were pretreated with Vc (500  $\mu\text{M}$ ), Trolox (50  $\mu\text{M}$ ), DTT (500  $\mu\text{M}$ ), or mannitol (500  $\mu\text{M}$ ) for 15 min and then incubated with clivorine (50  $\mu\text{M}$ ) for another 48 h. The number of viable cells was determined by MTT assay. (B) Cells were pretreated with various concentrations of Trolox (0, 25, 50, and 100  $\mu\text{M}$ ) for 15 min and then incubated with clivorine (50  $\mu\text{M}$ ) for another 48 h. The number of viable cells was determined by MTT assay. All the results (means  $\pm$  SEM,  $n = 6$ ) are expressed as a percent of the control in the absence of clivorine. \*\*\* $p < 0.001$  versus control in the absence of clivorine, ## $p < 0.01$ , ### $p < 0.001$  in comparison to clivorine.

100 μM Trolox was better than that provided by 50 μM and 25 μM Trolox.

#### 4. Discussion

Clivorine is an otonecine-type PA that is abundant in the traditional Chinese medicines *Ligularia hodgsonii* Hook and *Ligularia dentata* Hara. Previous reports by the current authors have shown their direct toxicity on human normal liver L-02 cells (6,8). The ferric reducing antioxidant power (FRAP) assay is a simple and reliable colorimetric method commonly used to measure total antioxidant capacity (13). The FRAP assay has been used to test for free radical scavenging and antioxidant activity in tissues (17). The present study showed that clivorine increased FRAP values in cells, which indicated that clivorine changed the redox balance in hepatocytes.

GSTs are enzymes of a multi-gene family that is involved in eliminating xenobiotic hepatotoxins. GSTs have the capacity to catalyze the conjugation of cellular GSH with electrophilic compounds, thereby decreasing their reactivity with important cellular macromolecules (18). The current results showed that clivorine increased GST activity in a concentration-dependent manner, which suggests that cellular GST may play a critical role in regulating clivorine-induced hepatotoxicity. Mammalian soluble GSTs are primarily divided into 4 main classes, Alpha (A), Mu (M), Pi (P), and theta (T) (19), and further research is needed to determine which types of GSTs are crucial to clivorine-induced toxicity.

GR is an important enzyme that helps replenish the key cellular antioxidant GSH via reduction of GSSG into GSH (16). A previous study by the current authors showed that clivorine decreased cellular GSH. S-adenosyl-L-methionine (SAM), which is a precursor of cellular GSH, significantly prevented clivorine-induced hepatotoxicity (20). As shown in Figure 3B, clivorine decreased GR activity in a concentration-dependent manner, which may explain the reduced amounts of GSH.

The cellular antioxidant system consists of enzymes and numerous nonenzymatic antioxidants including vitamins A, E, and C, ubiquinone, and flavonoids. Next, we observed the protection from clivorine-induced cytotoxicity provided by various antioxidants. As shown in Figure 4A, both Trolox and Vc prevented the cytotoxicity of clivorine, and Trolox provided better protection than Vc, while other antioxidants such as DTT and mannitol had no protective effect. As shown in Figure 4B, various concentrations of Trolox all protected cells from clivorine-induced cytotoxicity, and 100 μM Trolox provided the best protection.

Ve is a naturally occurring antioxidant in biological systems, and Trolox is a chemical analogue of Ve. Many studies have examined the protection from oxidative stress injury provided by Ve (21,22). The

current results are the first to demonstrate the potential use of Trolox or Ve in the reduction of clivorine-induced hepatotoxicity. The present study helps to direct the search for alexipharmac substances for use in the clinical removal of clivorine or other PAs.

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