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# The "double-faced" effect of VC-12 on free-radical-induced haemolysis of human erythrocytes: antioxidant and prooxidant

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## Abstract

Vitamin C is a popular antioxidant; however, its water solubility limits its function in the lipid environment. As a result, the antioxidative properties of its lipophilic derivatives have aroused research attention, especially L-ascorbyl-6-laurate (VC-12). We have investigated the effect of a high concentration of VC-12 on 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH)-induced haemolysis of human erythrocytes. The findings indicated that VC-12 was capable of protecting erythrocytes against AAPH-induced haemolysis when its concentration was below 60 μM. With an increase in the concentration of VC-12 and a decrease in the concentration of AAPH, VC-12 promoted haemolysis remarkably, the mechanism of which has been proposed as VC-12-mediated peroxidation. When the concentration of VC-12 was increased to above 150 μM, VC-12 promoted haemolysis by its function as a surfactant, to unbalance the osmotic pressure within and outside erythrocytes. High concentrations of VC-12 may have generated radicals via autooxidation, resulting in eventual haemolysis. Therefore, the double-faced effect of VC-12 on haemolysis of human erythrocytes was due to its concentration. This information may be useful for the safe use of lipophilic vitamin C.

## Introduction

Vitamin C is a popular antioxidant, which efficiently scavenges toxic radicals and other reactive oxygen species (ROS) formed in cell metabolism (Arrigoni & De Tullio 2002). However, its water solubility limits the antioxidative function in the lipid environment. Cousins et al (1977) proposed that esterification of the hydroxyl group at 6-position in vitamin C by a fatty acid, such as lauric acid, could enhance its lipophilicity and may function as a lipophilic antioxidant, as shown in Figure 1.

Thus, many reports have focused on the influence of the chain length of the fatty acid in lipophilic vitamin C on the antioxidative activity. For example, Liu et al (1992) measured the reaction rate of lipophilic vitamin C in repairing α-tocopherol radical by means of electron spin resonance (ESR), and found that the antioxidant activity of lipophilic vitamin C was much better than vitamin C itself in protecting erythrocytes and low density lipoprotein (LDL) against peroxidation induced by 2,2′-azobis(2-amidinopropane hydrochloride) (R-N=N-R, R=$-C(CH_3)_2C(=NH)NH_2$, AAPH) (Kuang et al 1994; Liu et al 1998). Liu et al (1990) explored the influence of micelle charges on the reaction between lipophilic vitamin C and α-tocopherol radical. A "flip-flop diffusion" model was applied to explain the reason why the antioxidant activity of L-ascorbyl-6-laurate (VC-12) was better than other forms of lipophilic vitamin C (Liu et al 1995). Those results showed VC-12 to be an attractive prospect for further investigation. After Bowry et al (1992) reported that the endogenous α-tocopherol in LDL was able to initiate lipid peroxidation of LDL called tocopherol-mediated peroxidation (TMP) (Bowry & Stocker 1993), the prooxidative properties of antioxidants have attracted much research attention. Podmore et al (1998) found that excess supplementation of vitamin C to healthy volunteers increased the level of 8-oxoguanine and 8-oxoadenine in their DNA, and the autooxidation of vitamin C accelerated the peroxidation of glycerol trioleate in micelles (Liu 2006a). Vitamin C can also react with $Fe^{3+}$-EDTA and $H_2O_2$ to generate high reactive hydroxyl radical (·OH) (Aeschbach et al 1994). We have observed (Liu 2006b) that a high concentration of α-tocopherol

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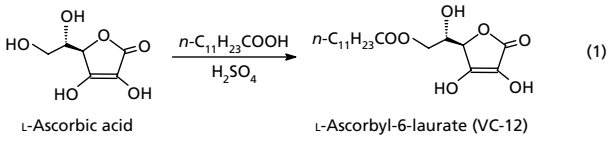


**Figure 1** Esterification of the hydroxyl group at 6-position in vitamin C.

promoted haemolysis of human erythrocytes in the presence of low concentrations of AAPH, demonstrating that exogenous $\alpha$-tocopherol could act as prooxidant in AAPH-induced haemolysis. This has motivated us to investigate whether vitamin C and its lipophilic derivative, VC-12, could play a prooxidative role in the same experimental system. We have studied the haemolysis of human erythrocytes induced by different concentrations of AAPH, and its inhibition or promotion by various concentrations of VC-12.

## Materials and Methods

### Materials

Fresh human erythrocytes were provided by the Red Cross Center for Blood (Changchun, China), which has a legal responsibility in Changchun to provide human erythrocytes for clinical and scientific usage. Before the experiment, erythrocytes were washed three times with phosphate-buffered saline (PBS; 150 mM NaCl, 8.1 mM $Na_2HPO_4$, 1.9 mM $NaH_2PO_4$, and 10 $\mu$M EDTA, pH 7.4) to remove the residual plasma. During the final washing the erythrocytes were centrifuged at 1700 *g* for exactly 10 min to obtain a packed cell volume for the further experimentation (Zavodnik et al 1999). AAPH was purchased from Fluka, and vitamin C from Sinowestern Pharmacy Co., Shenyang, China. VC-12 was synthesized as detailed by Cousins et al (1977) and dissolved in dimethyl sulfoxide (DMSO) as a stock solution. AAPH and vitamin C were dissolved in PBS.

### Process for experimental haemolysis

The procedure to obtain haemolysis was followed as described in the literature (Niki et al 1988; Liu 2006b). In brief, VC-12/DMSO solution (the added volume less than 1% the total volume of the mixture to avoid the influence of DMSO on haemolysis (Liu et al 2003)) and AAPH/PBS solution were added in turn to 3.0% erythrocytes suspended in PBS. The mixture was incubated at 37°C to initiate haemolysis. Samples were taken at appropriate intervals and centrifuged at 1700 *g* to obtain a supernatant. The absorbance of the supernatant was measured at 535 nm (May et al 1998).

### Statistical analysis

AAPH-induced haemolysis of erythrocytes was an in-vitro experimental system, which meant that the results avoided completely the influence of the metabolism, unlike an in-vivo biological system. A controlled experiment was carried out in every determination. Thus, the data points of absorbance in Figures 2, 3 and 5 were the average values from three individual measurements within 10% error, and plotted vs incubation time to illustrate the haemolysis process. The concentrations of VC-12 vs lag times of haemolysis, together with the linear relationship between the concentration of vitamin C and the corresponding lag time of haemolysis (see Figure 4), were analysed statistically by one-way analysis of variance using Origin 6.0 professional Software. $P < 0.05$ indicated a significant correlation.

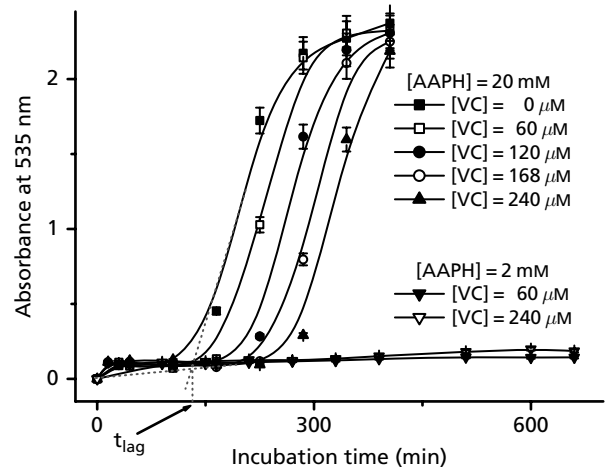


**Figure 2** Haemolysis procedure of erythrocytes (3.0% suspension in PBS) initiated by 20 and 2 mM AAPH at 37°C in the presence of vitamin C at various concentrations indicated in the figure.

## Results and Discussion

### The antioxidant role of vitamin C

A high concentration of $\alpha$-tocopherol (larger than 200 $\mu$M) in the presence of a low concentration of AAPH (less than 2 mM) promoted haemolysis of erythrocytes. Liu (2006b) explained that this was because the radical derived from $\alpha$-tocopherol was oxidized by AAPH which initiated haemolysis eventually. Herein, the aforementioned experiment was performed in the presence of vitamin C instead of $\alpha$-tocopherol. Figure 2 outlines the haemolysis initiated by 20 and 2 mM AAPH in the presence of various concentrations of vitamin C.

The lag time ($t_{lag}$) of haemolysis increased proportionally with the concentration of vitamin C ranging from 0 to 240 $\mu$M when the concentration of AAPH was 20 mM, which can be expressed quantitatively as:

$$t_{lag}(\text{min}) = 0.555(\pm 0.036)\ [\text{vitamin C}(\mu\text{M})] + 136.8\,(\pm 5.22)\ (\text{correlation coefficient} = 0.9937,\ P < 0.001)$$

and the image of the equation is illustrated in Figure 4. It is worthy to note that, when the concentration for AAPH decreased to 2 mM, vitamin C did not promote haemolysis, even when the concentration was as high as 240 $\mu$M. This was

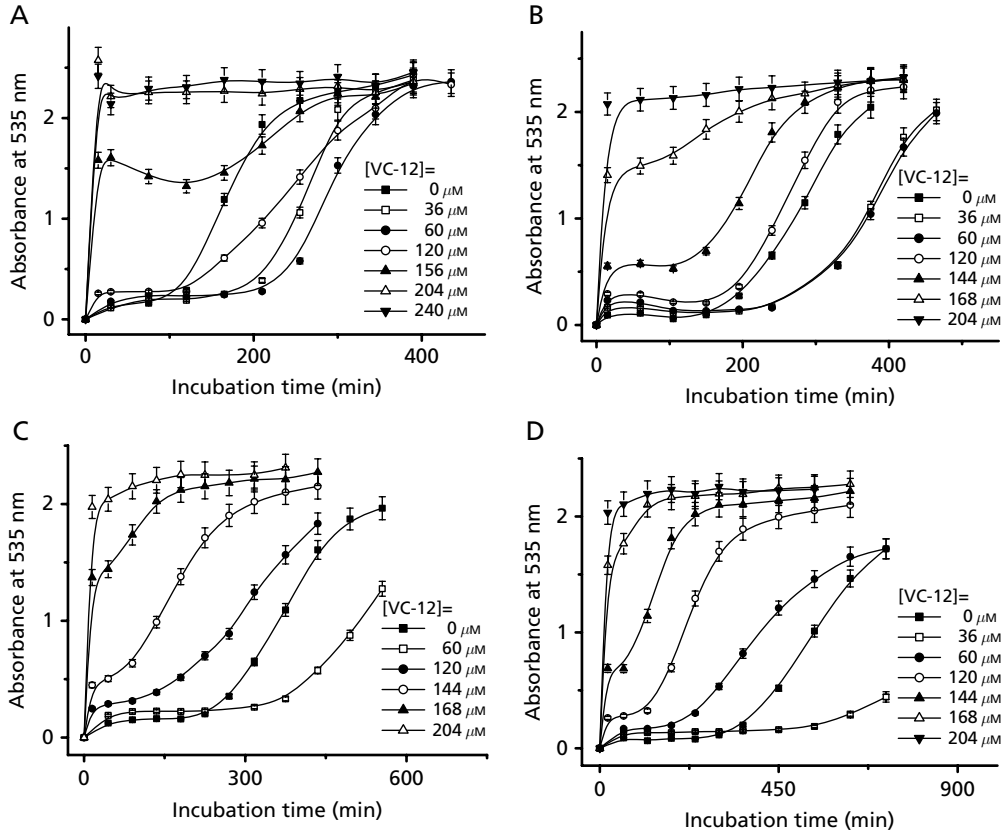


**Figure 3** Haemolysis procedure of erythrocytes (3.0% suspension in PBS) initiated by 20 mM AAPH (A), 10 mM AAPH (B), 4 mM AAPH (C) and 2 mM AAPH (D) at 37°C in the presence of various concentrations of VC-12, which are indicated on the figure.

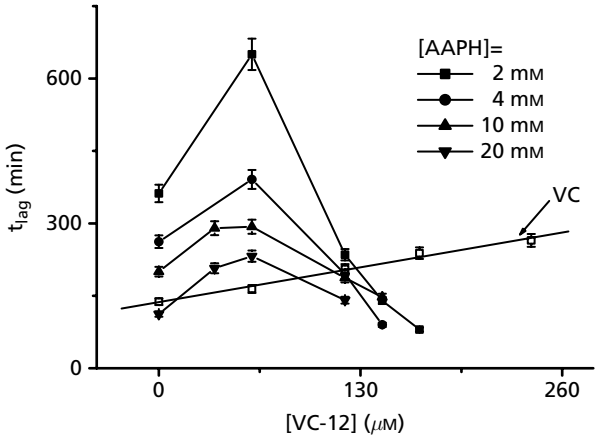


**Figure 4** The relationship between the concentrations of VC-12 and lag times of haemolysis ($t_{lag}$) initiated by various concentrations of AAPH, which are indicated on the figure. VC, vitamin C.

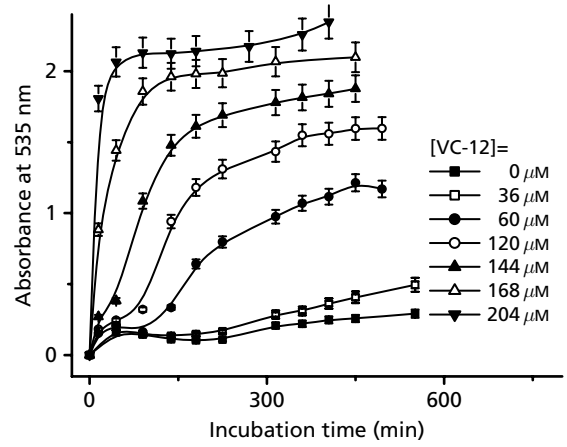


**Figure 5** Haemolysis procedure of erythrocytes (3.0% suspension in PBS) at 37°C in the presence various concentrations of VC-12, which are indicated on the figure.

because vitamin C, as a water-soluble antioxidant, could trap the initiating radical generated from AAPH in aqueous solution directly and avoid the contact between the initiating radical and the membrane of erythrocytes. Therefore, it could be concluded that vitamin C acted as a perfect antioxidant to protect erythrocytes against AAPH-induced haemolysis whatever the concentrations of vitamin C and AAPH used.

### Acting as a prooxidant, VC-12 promoted haemolysis in the presence of AAPH

The above experiment was performed in the presence of VC-12 instead of vitamin C. Figure 3 illustrates the haemolysis procedure when the concentration of VC-12 ranged from 60 to 240 μM, and the concentration of AAPH decreased from 20 to 2 mM.

As can be seen in Figure 3A, when the concentration of VC-12 exceeded 120 $\mu$M, the $t_{lag}$ could not be obtained because haemolysis occurred at the beginning of the reaction and reached a platform immediately. This phenomenon was still observed along with the decrease of the concentration of AAPH from 10 to 4 to 2 mM (see Figure 3B, C and D, respectively). Furthermore, the concentration of VC-12 for obtaining $t_{lag}$ increased remarkably; that is, when the concentrations of AAPH were 10 and 4 mM, the concentration of VC-12 for obtaining $t_{lag}$ increased to 144 $\mu$M, and even to 168 $\mu$M when the concentration of AAPH was as low as 2 mM. All the obtained values of $t_{lag}$ were plotted vs the concentration of VC-12 (Figure 4).

We have found that $t_{lag}$ increased proportionally with the concentration of VC-12 below 50 $\mu$M (Tang & Liu 2007). Figure 4 illustrates the relationship between $t_{lag}$ and the concentration of VC-12 (higher than 60 $\mu$M), and indicated that, no matter how high a concentration of AAPH was used, $t_{lag}$ increased to a maximum, then decreased with the increase of the concentration of VC-12. It has been pointed out that $t_{lag}$ is generated by the endogenous antioxidants in erythrocyte membrane and is prolonged by the exogenous antioxidants or low concentration of AAPH (Sato et al 1995). Figure 4 revealed that $t_{lag}$ became longer with the decrease of the concentration of AAPH, whereas, at the same concentration of AAPH, $t_{lag}$ decreased rapidly with increased concentration of VC-12, even lower than in the absence of VC-12. This implied that high concentrations of VC-12 accelerated the depletion of the endogenous antioxidants in the erythrocyte membrane, resulting in the decrease of $t_{lag}$. This was direct evidence for the prooxidative property of VC-12, and could be regarded as a VC-12-mediated peroxidation mechanism similar to the $\alpha$-tocopherol-mediated peroxidation in the same experimental system (Liu 2006b), as shown in equations 1–3:

$$\text{R-N=N-R} \xrightarrow{O_2} 2\text{ROO}^{\cdot} + N_2 \quad (1)$$

$$\text{VC-12} + \text{ROO}^{\cdot} \rightarrow \text{VC-12}^{\cdot} + \text{ROOH} \quad (2)$$

$$\text{VC-12}^{\cdot} + \text{LH} \xrightarrow{O_2} \text{VC-12} + \text{LOO}^{\cdot} \quad (3)$$

The low concentration of initiating radical ($ROO^{\cdot}$) derived from the decomposition of AAPH (R-N=N-R) may have more opportunity to attack VC-12 with a high concentration, and generate VC-12 radical (VC-12$^{\bullet}$). VC-12$^{\bullet}$ then subtracts a hydrogen atom from the polyunsaturated fatty acid in membrane (LH), which leads to the formation of a peroxyl radical ($LOO^{\cdot}$) in the presence of oxygen. Thus, the haemolysis is accelerated because of the additional propagation initiated by VC-12$^{\bullet}$. The reason why vitamin C cannot promote haemolysis may be because, if vitamin C is oxidized by AAPH to generate vitamin C radical, its water-solubility makes the radical exist in the aqueous solution and so has fewer opportunities to penetrate the membrane. The lipophilicity of VC-12$^{\bullet}$, however, makes it easier to approach and penetrate the membrane, and finally initiate peroxidation of LH in the membrane.

### Acting as a surfactant, vitamin C-12 promoted haemolysis in the absence of AAPH

Figure 5 illustrates the haemolysis behaviour correlated with VC-12 concentration in the absence of AAPH. Haemolysis still took place and even got more serious with the increase of incubation time when the concentration of VC-12 was higher than 60 $\mu$M. Two reasons could be ascribed for the haemolysis initiated by VC-12. The first relates to its structure, in which a nonpolar carbon chain attached to a polar ascorbyl group makes VC-12 a surfactant. Erythrocytes may be destroyed by a surfactant because of the unbalance of osmotic pressure within and outside the membrane. Haemolysis in the presence of a high concentration of VC-12 may result from the surfactant function of VC-12. The second reason correlates with the autooxidation of VC-12 under high concentration. It was found that high concentrations of vitamin C were capable of initiating peroxidation of glycerol trioleate in micelles via autooxidation to generate vitamin C radical (Liu 2006a). Thus, although VC-12 itself was stable in the solid state, the ascorbyl group in VC-12 may have been autoxidized to generate VC-12$^{\bullet}$ in this experimental system, and VC-12$^{\bullet}$ within the erythrocyte membrane may have induced haemolysis.

### Conclusion

Vitamin C protected erythrocytes against AAPH-induced haemolysis regardless of the concentrations of vitamin C and AAPH used. However, its lipophilic derivative VC-12 could act not only as an antioxidant, but also could accelerate haemolysis by means of VC-12-mediated peroxidation and surfactant function. In particular, when the concentration of VC-12 exceeded 150 $\mu$M, the surfactant function of VC-12 promoted haemolysis, and high concentrations of VC-12 may also generate radical via autooxidation and result in haemolysis. This information may be useful for the safe usage of lipophilic vitamin C.

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