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· Original article ·

Effect of various cations on hemolytic activity of tentacle-only extract from jellyfish *Cyanea capillata*

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[**Abstract**] **Objective** To investigate the potential role of the pore-formation in the hemolytic activity of tentacle-only extract (TOE) from the jellyfish *Cyanea capillata*. **Methods** The effects of various cations, including K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, La^{3+} and NH_4^+ on the hemolytic activity of TOE were compared in two different test systems: 1% whole blood and 0.45% erythrocyte suspension with the same erythrocyte concentration. **Results** The hemolytic activities of TOE in both tests were inhibited by Mn^{2+}, Zn^{2+}, La^{3+}, Cu^{2+} and Fe^{2+}, and were promoted to a minor extent by K^+, Ca^{2+}, Mg^{2+} and NH_4^+. The chelating agent EDTA also inhibited the hemolytic activity of TOE. **Conclusion** The pore-formation mechanism might play an important role in the hemolytic activity of TOE.

[**Key words**] jellyfish; *Cyanea capillata*; tentacle-only extract; hemolysis

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In recent years, a marked increase in jellyfish blooms has been observed worldwide in marine ecosystems due to anthropogenic disturbance and climate change[1], and the jellyfish venoms have been demonstrated to possess a wide spectrum of biological activities, including hemolytic, enzymatic, dermonecrotic, myotoxic, neurotoxic and cardiovascular toxic effects [2-4], which arise from a complex mixture of biologically active molecules in the jellyfish venoms[5]. As the jellyfish toxin has the nature of hydrophobicity, thermolability, adhesion, easy breakdown and aggregation[6], its separation and purification are difficult. However, Nagai *et al*. have separated and purified the hemotoxin of *Carybdea rastoni* and obtained the sequence of its amino acid[7]. Today, the amino acid sequences of six jellyfish hemotoxins have been reported [7-11].

As a ubiquitous toxic effect and an initial property for purification or characterization of the jellyfish venom, the hemolytic activity was the most studied among all its biological activities. Meanwhile, increasingly more studies have investigated the factors affecting hemolysis, such as various cations, proteases, antioxidants and osmotic protectants in an attempt to explore the hemolysis mechanism. Three hypotheses have been proposed for hemolysis, including the effect of protease activity, oxidative damage and pore-formation [2,5,12-15]. Marino *et al*. reported that both oxidative damage and pore-formation in cell membrane can help to explain cell lysis after toxins treatment with regard to the action of cnidarian toxins[15]. Batista *et al*. described sea anemone toxins as channel forming toxins affecting biological membranes[16]. Bhakdi and Tranum-Jensen indicated that Portuguese Man-of-war venom produced damage to target cells by a mechanism similar to that of bacterial cytolysins, which involves binding and insertion of toxin molecules into the plasma membrane followed by oligomerization to form transmembrane pores[17]. Ed-

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wards *et al*. detected apparent membrane pore-formation by Portuguese Man-of-war venom in intact cultured cells[12]. Recently, Helmholz analyzed the selective toxin-lipid membrane interactions of natural, hemolytic Scyphozoan toxins by a chip-based technology with immobilized liposomes as artificial cell membrane[18]. Therefore, more attention has been paid to the pore-formation mechanism, though there is no consensus about the cellular mechanism of hemolysis now.

We have used both the erythrocyte suspension and diluted whole blood to detect the hemolytic activity of tentacle-only extract (TOE) from the jellyfish *Cyanea capillata* and found that the hemolysis activity in the erythrocyte suspension was higher than in diluted whole blood, indicating that the plasma may play a protective role against hemolysis of TOE[19-20]. In this study, both tests were used for a comparative study of the effects of various cations on the hemolytic activity of TOE, so as to investigate the potential role of the pore-formation mechanism in the hemolytic activity of TOE.

1　Materials and methods

1.1　TOE isolation from the jellyfish *C. capillata*

Specimens of *C. capillata* were collected in June 2010 on the *Sanmenwan* coast of the East China Sea in Zhejiang Province, China, and identified by Professor Hong Hui-xin from the Fisheries College of Jimei University, Xiamen, China. The removed tentacles were preserved in plastic bags on dry ice and immediately shipped to Shanghai, where the samples were frozen at −70℃ until use. The TOE devoid of nematocysts was prepared following the method described previously[6,21]. Briefly, the frozen tentacles were thawed at 4℃ and immersed in filtered seawater at the mass : volume ratio of 1 : 1 to allow autolysis of the tissues for 4 d. The mixture was stirred for 30 min twice daily. The autolyzed mixture was centrifuged at 10 000×*g* for 15 min thrice. The resultant supernatant was the TOE. All procedures were performed at 4℃ or in an ice bath. Before use, the TOE was centrifuged at 10 000×*g* for 15 min to remove the sediments, followed by dialysis against phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.4) for over 8 h. The protein concentration in the preparations was determined using the method of Bradford [22].

1.2　Hemolytic activity of TOE in both diluted whole blood and erythrocyte suspension

Hemolytic activity of TOE was tested in two systems, namely 1% whole blood and 0.45% erythrocyte suspension. Arterial blood was drawn by a heparinized syringe through a catheter inserted into the left femoral artery of anesthetized (25% urethane, 1.0 g/kg, i. p.) male Sprague Dawley (SD) rats (220±20) g, provided by the Laboratory Animal Center of Second Military Medical University, Shanghai. The erythrocytes were centrifuged from the heparinized blood samples, washed three times with PBS and resuspended in the same buffer to a final concentration of 0.45% (*v*/*v*). A subsample of the whole blood was diluted in PBS to a final concentration (*v*/*v*) of 1%, in which the erythrocyte concentration was approximately 0.45%. Aliquots of both 1% whole blood and 0.45% erythrocyte suspension were incubated with TOE at 37℃ for 30 min and then centrifuged at 2 000×*g* for 10 min to precipitate both the intact erythrocytes and ghosts. Aliquots of the supernatants were then taken and the optical density was spectrophotometrically measured at 414 nm. The hemolytic activity of TOE was expressed as % absorbance compared with that observed after maximal lysis under saponin (25 μg/ml). The supernatant of untreated 1% whole blood or 0.45% erythrocyte suspension was taken as the background and subtracted. All the animal experiments were approved by the Ethics Committee of the Second Military Medical University.

1.3　Effect of various cations on the hemolytic activity of TOE

Various salts, including KCl, $CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, $LaCl_3$, $CuCl_2$, $FeSO_4$ and $(NH_4)_2SO_4$, were separtely added into 1% whole blood or 0.45% erythrocyte suspension. TOE (400 μg/ml) was added and the hemolytic activity was assayed as described above. The 1% whole blood or

0.45% erythrocyte suspension without addition of the salts was used as control. Ethylenediaminetetraacetic acid (EDTA), a widely used chelating agent for metal ions such as Ca^{2+}, was also employed to observe the results caused by decrease of some cations by the complexation reaction on the hemolytic activity of TOE. All the salts and EDTA were used at final concentrations of 20, 50 or 100 mmol/L from 1 mol/L stock solutions.

1.4 Statistical analysis One-way analysis of variance (ANOVA) was used. In all cases, statistical significance was indicated by $P<0.05$. All data were expressed as $\bar{x}\pm s$.

2 Results

2.1 Hemolytic activity of TOE Dose-dependent hemolytic activity of TOE was observed in both 1% whole blood and 0.45% erythrocyte suspension (Fig 1). No difference was observed between the two tests at 100 and 400 μg/ml of TOE. However, the hemolysis in 0.45% erythrocyte suspension was significantly higher than in 1% whole blood ([73.1±13.4]% vs [23.7±8.6]%, $P<0.05$) at 200 μg/ml of TOE.

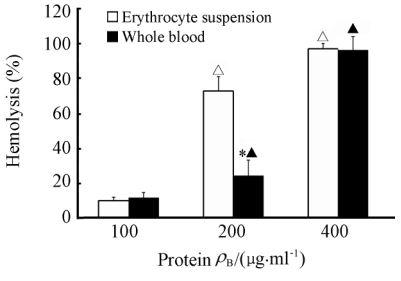

Fig 1 Hemolytic activity of TOE at 100, 200 and 400 μg/ml in both 1% rat whole blood and 0.45% erythrocyte suspension

* $P<0.05$ vs 0.45% erythrocyte suspension. △ $P<0.05$ vs 100 μg/ml TOE group (in 0.45% erythrocyte suspension). ▲ $P<0.05$ vs 100 μg/ml TOE group (in 1% rat whole blood). $n=6$, $\bar{x}\pm s$

2.2 Effect of cations on the hemolytic activity of TOE All the cations and EDTA, in the absence of TOE, did not alter erythrocyte integrity at the concentration chosen for the experiments. As shown in Fig 2A-2C, in the presence of Mg^{2+}, Ca^{2+} and K^+, a dose-dependent increase of the hemolytic activity of TOE was observed in both 1% whole blood and 0.45% erythrocyte suspension, except for that in the presence of K^+ at 100 mmol/L where an inhibition of hemolysis in erythrocyte suspension was observed. Hemolysis was significantly higher in the diluted whole blood than in the erythrocyte suspension at 100 mmol/L of Mg^{2+}, Ca^{2+} and K^+.

Five cations, including Fe^{2+}, La^{3+}, Cu^{2+}, Zn^{2+} and Mn^{2+}, showed an inhibitory effect on the hemolytic activity of TOE, and Zn^{2+} had the strongest inhibition. In the presence of Fe^{2+} or La^{3+}, a dose-dependent decrease of hemolytic activity was observed, and no significant difference was present in both tests. In the presence of Mn^{2+}, a dose-dependent decrease of hemolytic activity was also observed in both tests, and the hemolytic activity of TOE was weaker in the diluted whole blood than in erythrocyte suspension (Fig 2D-2H).

The hemolytic activity of TOE was slightly increased in the presence of NH_4^+ in both tests, and it was higher in the diluted whole blood than in the erythrocyte suspension at 50 mmol/L of NH_4^+ (Fig 3).

As shown in Fig 4, EDTA produced an inhibitory effect on the hemolytic activity of TOE at 50 and 100 mmol/L, and no difference was found between the two tests.

3 Discussion

C. capillata is a moderately toxic jellyfish which produces cardiovascular and hemolytic toxins[21-24]. As an initial approach for purification or characterization of the jellyfish venom, the hemolytic activity has always been a focus of study due to rapid, easily reproducible and quantifiable methods. Before we recommended the diluted whole blood as a valid test system for hemolysis study *in vitro*[19-20], almost all the previous studies utilized erythrocyte suspension as the exclusive test system for hemolysis of jellyfish venoms *in vitro*, but this procedure may not be consistent with the actual hemolytic process either *in vitro* or *in vivo* due to lack of blood plasma. In this article, we used both the diluted whole blood and erythrocyte suspension

with the same erythrocyte concentration to compare the effects of various cations on the hemolytic activity of TOE from *C. capillata*. Our results showed that the hemolytic activity of TOE was dose-dependent in both tests, and the hemolysis in erythrocyte suspension was generally higher than that in the diluted whole blood at 200 μg/ml TOE, indicating that there are certain protective factors against hemolytic activity of TOE in the plasma. So it is more reliable to utilize the diluted whole blood than erythrocyte suspension to examine the hemolysis of TOE.

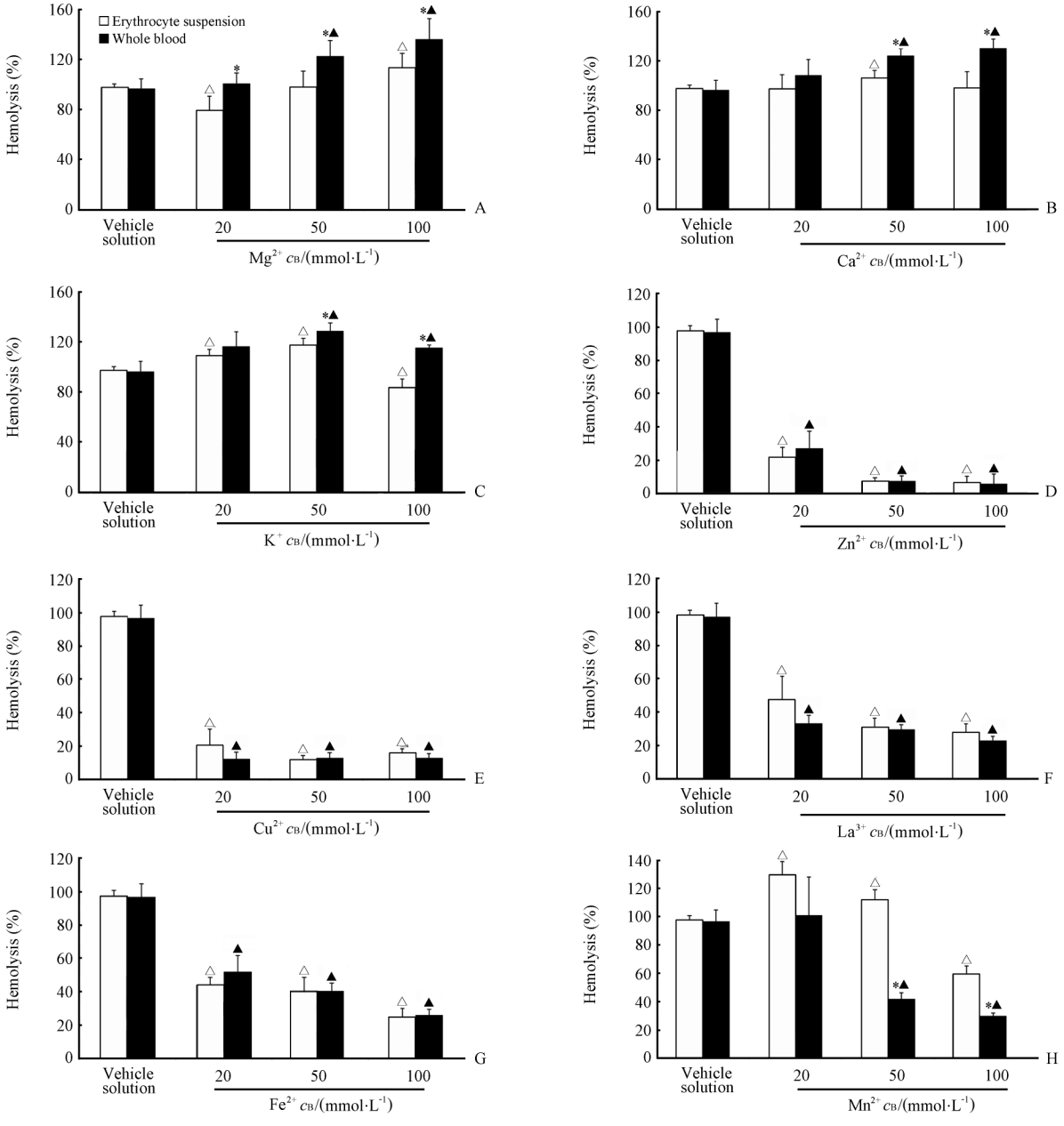

Fig 2　Effect of various cations on the hemolytic activity of TOE

(A) Mg^{2+}, (B) Ca^{2+} and (C) K^{+} showed a dose-dependent increase of the hemolytic activity of TOE; but in the presence of (D) Zn^{2+}, (E) Cu^{2+}, (F) La^{3+}, (G) Fe^{2+} and (H) Mn^{2+} showed an inhibitory effect in both 1% whole blood and 0.45% erythrocyte suspension. * $P<0.05$ vs 0.45% erythrocyte suspension. △$P<0.05$ vs control group (in 0.45% erythrocyte suspension). ▲$P<0.05$ vs control group (in 1% whole blood). $n=6$, $\bar{x}\pm s$

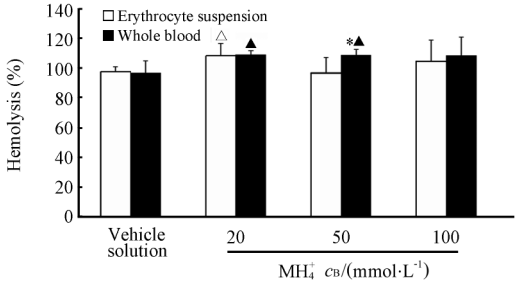

Fig 3　Effect of NH_4^+ on the hemolytic activity of TOE

$^*P<0.05$ vs 0.45% erythrocyte suspension. $^\triangle P<0.05$ vs control group (in 0.45% erythrocyte suspension). $^\blacktriangle P<0.05$ vs control group (in 1% whole blood). $n=6$, $\bar{x}\pm s$

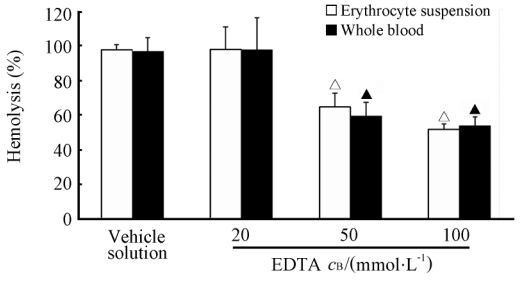

Fig 4　Effect of EDTA on the hemolytic activity of TOE

$^\triangle P<0.05$ vs control group (in 0.45% erythrocyte suspension). $^\blacktriangle P<0.05$ vs control group (in 1% whole blood). $n=6$, $\bar{x}\pm s$

Jellyfish venoms clearly vary in composition and activity[25], and some investigations have been performed to study the role of ionic species on the toxicological characteristics of jellyfish crude extracts [5,13,26]. In this study we used both the diluted whole blood and erythrocyte suspension to investigate the effect of cations on the hemolytic activity of TOE from *C. capillata*, and found that the hemolytic activity was inhibited by Zn^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, La^{3+} and EDTA, but not by Mg^{2+}, Ca^{2+}, K^+ and NH_4^+. One of the possible reasons is that Zn^{2+} and Mn^{2+} can enhance the protease activity and EDTA; as a chelator, EDTA can chelate some heavy metal cations such as Hg^{2+} and Pb^{2+} to reduce the inhibitory effect on protease activity[26], resulting in the reduction of hemolytic activity. However, Mg^{2+}, which can also enhance the protease activity[26], has no inhibitory effect on the hemolytic activity of TOE in the present study. Marino *et al*. reported that Mg^{2+} and K^+ significantly inhibited the hemolytic activity of the venom from *Aiptasia mutabilis*, and Ca^{2+} and Cu^{2+} totally blocked the hemolytic activity. However, the proteases, including trypsin, α-chymotrypsin and collagenase, significantly inhibited the hemolytic activity, whereas papain was ineffective[15]. Therefore, the action of proteases on the hemolytic activity of TOE remains a controversy.

Oxidative damage to the erythrocyte membrane may lead to a reduction in membrane fluidity and an increase in membrane fragility[27]. So the inhibitory effect of Cu^{2+} and Fe^{2+} on the hemolysis may be due to their antioxidant activity. However, the antioxidants such as GSH, cysteine and ascorbic acid did not impair the hemolytic power of the venom from jellyfish *Pelagia noctiluca*[14]. Hence the hemolysis of jellyfish venom may not be due to oxidative damage either. Another hypothetical explanation is that the divalent cations reversibly act on the lipid bilayer, which makes membrane fluidity stable or rigid[28]. This disturbance of membrane fluidity may make it difficult for the cytolysin to move laterally on the cell membrane to form functional transmembrane pores or channels.

Apparent membrane pore-formation has been revealed in cultured cells after exposure to jellyfish venoms[12,25]. Moreover, in myocytes, jellyfish venoms could cause a large, irreversible elevation of cytosolic Ca^{2+} and the effect could be inhibited by La^{3+}, which is well known as a non-specific channel and pore blocker, but not by the L-type Ca^{2+} channel antagonist verapamil[25]. In consistent with these findings, we found La^{3+} also significantly inhibited hemolytic activity in the present study, indicating that erythrocyte lysis is likely due to pore-formation in cell membranes by TOE [13,29-32].

Chung *et al*. reported that the hemolytic activity of the crude venom from *C. capillata* was dependent on the presence of divalent cations Ca^{2+} or Mg^{2+}, while it was irreversibly eliminated when the crude venom was dialysed against the buffer containing EDTA (20 mmol/L)[5]. This is supported by our finding that EDTA displayed an obvious inhibitory effect on the hemolysis in both the diluted whole blood and erythrocyte suspension at 50 and

100 mmol/L. The hemolytic activity of the venoms from the Cnidaria *Aiptasia pallida* and *Actnia equina* was found to be enhanced in the presence of Ca^{2+}[29-30]. In contrast, Rottini *et al*. reported that when Ca^{2+} concentration was raised to 10 mmol/L, an inhibitory effect on the hemolytic activity of the venom from *C. marsupialis* was observed[33]. Recently, it has also been demonstrated that the hemolytic activity of the crude venom from *Pelagia noctiluca* was unaffected by Ca^{2+} at all concentrations tested[14].

In this study, a dose-dependent increase of the hemolytic activity of TOE was observed in both the diluted whole blood and erythrocyte suspension in the presence of Ca^{2+}. Rottini *et al*. reported that a Ca^{2+}-dependent pore formation was required to induce hemolysis under *C. marsupialis* venom[33]. The role of Ca^{2+} in the activation of crude venom toxicity has also been discussed by Iwase *et al*. suggesting that cations may modulate events occurring after the first interaction of toxin with cell membrane[34]. Such interaction may lead to the pore formation and then osmotic lysis of the cell. In addition, it has been reported by Yu *et al*. that the hemolytic activity of the venom from the jellyfish *Rhopilema esculentum* was inhibited by $(NH_4)_2SO_4$ and the inhibitory effect was in a dose-dependent manner[13]. However, our result showed that the hemolytic activity of TOE from *C. capillata* was not markedly affected by NH_4^+.

In conclusion, various cations, including monovalent, divalent or trivalent, can impact the hemolytic activity of TOE to varying degrees. In the hypotheses about the hemolysis of cnidarian toxins, there are contradictions between the theory of protease activity and theory of oxidative damage, so we believe that the pore-formation mechanism might play an important role in the hemolytic activity of TOE.

4　Conflict of interest

The authors declare that there is no conflict of interest.

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不同阳离子对 *Cyanea capillata* 水母触手提取物溶血活性的影响

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[**摘要**] **目的**　探讨血浆对 *Cyanea capillata* 水母触手提取物(TOE)溶血活性的影响和孔道形成在其溶血机制中的作用。**方法**　在具有相同红细胞浓度的 1%稀释的全血和 0.5%红细胞悬液中，检测不同浓度(100、200、400 μg/ml)的 TOE 产生的溶血效应，并在两套体系中分别加入 20、50 或 100 mmol/L 的 K^+、Ca^{2+}、Mg^{2+}、Mn^{2+}、Zn^{2+}、Cu^{2+}、Fe^{2+}、La^{3+}、NH_4^+等阳离子和离子螯合剂 EDTA，观察其对 TOE 溶血活性的影响。**结果**　在两套溶血检测体系中，TOE 均呈现出剂量依赖性的溶血效应，TOE 为 200 μg/ml 时在红细胞悬液产生的溶血强度高于稀释的全血；Mn^{2+}、Zn^{2+}、La^{3+}、Cu^{2+}、Fe^{2+}和 EDTA 均明显降低 TOE 溶血活性($P<0.05$)，其中以 Zn^{2+}的抑制作用最强，而 K^+、Ca^{2+}、Mg^{2+}、NH_4^+可不同程度地增强其溶血活性($P<0.05$)。**结论**　血浆对 TOE 的溶血作用有一定的保护作用，而孔道形成可能在 TOE 的溶血机制中具有重要作用。

[**关键词**] 水母；霞水母；触手提取物；溶血
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