**The metabolites of glutamine prevent hydroxyl radical-induced
apoptosis through inhibiting mitochondria and calcium ion involved
pathways in fish erythrocytes**

Huatao Li^{a, e}, Weidan Jiang^{a, b, c}, Yang Liu^{a, b, c}, Jun Jiang^{a, b, c}, Yongan Zhang^d, Pei Wu^{a, b, c}, Juan Zhao^{a, b, c}, Xudong Duan^{a, b, c}, Xiaoqiu Zhou^{a, b, c, *}, Lin Feng^{a, b, c, *}

^a *Animal Nutrition Institute, Sichuan Agricultural University, Sichuan, Chengdu, 611130, China*

^b *Fish Nutrition and Safety Production University Key Laboratory of Sichuan Province, Sichuan Agricultural University, Sichuan, Chengdu, 611130, China*

^c *Key Laboratory for Animal Disease-Resistance Nutrition of Ministry of Education, Sichuan Agricultural University, Sichuan, Chengdu, 611130, China*

^d *Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China*

^e *Conservation and Utilization of Fishes resources in the Upper Reaches of the Yangtze River Key Laboratory of Sichuan Province, Neijiang Normal University, Sichuan, Neijiang, 641000, China*

* Corresponding authors. Tel.: +86 835 2885157; fax: +86 835 2885968.

E-mail addresses: xqzhouqq@tom.com, zhouxq@sicau.edu.cn (Xiaoqiu Zhou).

fenglin@sicau.edu.cn (L. Feng)

Postal address: Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, 611130, Sichuan, China

Abbreviations

Cit, citrulline; CAD, caspase-activated DNase; PS, phosphatidylserine; ROS, reactive oxygen species; PUFAs, polyunsaturated fatty acids; EGTA, ethylene glycol tetraacetic acid; PI, propidium iodide; DCFH-DA, 2', 7'-dichlorofluorescein diacetate; PCS, physiological carp saline; Hb, hemoglobin; AHR, anti-hydroxyl radical; Ala₁₀Pro₄Cit₁, the combination of Ala, Pro and Cit; TUNEL, the terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labeling; LMWPs, the low-molecular-weight proteins; HMWPs, the high-molecular-weight proteins;

Anti-Cyt C, the murine monoclonal anti-cytochrome c.

Abstract

The present study explored the apoptosis pathways in hydroxyl radicals ($\bullet\text{OH}$)-induced carp erythrocytes. Carp erythrocytes were treated with the caspase inhibitors in physiological carp saline (PCS) or Ca^{2+} -free PCS in the presence of 40 μM FeSO_4 /20 μM H_2O_2 . The results showed that the generation of reactive oxygen species (ROS), the release of cytochrome c and DNA fragmentation were caspase-dependent, and Ca^{2+} was involved in calpain activation and phosphatidylserine (PS) exposure in $\bullet\text{OH}$ -induced carp erythrocytes. Moreover, the results suggested that caspases were involved in PS exposure, and Ca^{2+} was involved in DNA fragmentation in $\bullet\text{OH}$ -induced fish erythrocytes. These results demonstrated that there might be two apoptosis pathways in fish erythrocytes, one is the caspase and cytochrome c-dependent apoptosis that is similar to that in mammal nucleated cells, the other is the Ca^{2+} -involved apoptosis that was similar to that in mammal non-nucleated erythrocytes. So, fish erythrocytes may be used as a model for studying oxidative stress and apoptosis in mammal cells. Furthermore, the present study investigated the effects of glutamine (Gln)'s metabolites [alanine (Ala), citrulline (Cit), proline (Pro) and their combination ($\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$)] on the pathways of apoptosis in fish erythrocytes. The results displayed that Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ effectively suppressed ROS generation, cytochrome c release, activation of caspase-3, caspase-8 and caspase-9 at the physiological concentrations, prevented Ca^{2+} influx, calpain activation, PS exposure, DNA fragmentation and the degradation of the cytoskeleton and oxidation of membrane and hemoglobin (Hb) and increased activity of anti-hydroxyl radical (AHR) in $\bullet\text{OH}$ -induced carp erythrocytes. $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ produced a synergistic effect of inhibited oxidative stress and apoptosis in fish erythrocytes. These results demonstrated that Ala, Cit, Pro and their combination can protect mammal erythrocytes and nucleated cells against oxidative stress and apoptosis. The studies supported the use of Gln, Ala, Cit and Pro as oxidative stress and apoptosis inhibitors in mammal cells and the hypothesis that the inhibited effects of Gln on oxidative stress and apoptosis are at least partly dependent on that of its

60 metabolites in mammalian.

61 **Keywords:** Hydroxyl radicals; fish erythrocytes; apoptosis; glutamine; metabolites

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Introduction

Glutamine (Gln) plays an important role in promoting and maintaining the functions of various organs and cells in mammalian [1, 2]. Studies demonstrated that Gln improves clinical antioxidant outcome in patients [3-5] and attenuates apoptosis in experimental rat [6-8]. The anti-oxidative and anti-apoptotic effects of Gln may be closely associated with its metabolites in mammalian. Studies showed that the small intestine converts the most of Gln in diet and plasma to alanine (Ala), citrulline (Cit) and proline (Pro), which released into the portal circulation in mammalian [9-12]. Ala exerts cytoprotection against oxidative stress in human endothelial cells [13] and rat hepatocytes [14]. Pro protects against oxidative stress and apoptosis in mammalian cells [15]. Therefore, the metabolites may be the real inhibitors of oxidative stress and apoptosis in Gln-supplementation mammalian. However, the information regarding the anti-oxidative and anti-apoptotic effects of Cit and the combination Ala, Cit and Pro and anti-apoptotic effects of Ala are scarce in mammal cells.

After entering the circulation, amino acids can enter the human erythrocytes [16]. Erythrocytes carry hemoglobin (Hb) to supply oxygen to all tissues and organs [17]. However, erythrocytes are continuously exposed to both endogenous and exogenous sources of reactive oxygen species (ROS) [18]. Studies indicated that ROS released from neutrophils, macrophages and endothelial cells in the vasculature are taken up by mammal erythrocytes [19-21]. Endogenous ROS are continuously generated by the autoxidation of Hb in mammal erythrocytes [22-24]. Erythrocytes are very sensitive to ROS due to the high poly-unsaturated fatty acids (PUFAs) content in the membrane [25]. The pool of ROS can induce oxidative stress and eventually trigger apoptosis that impairs the function of erythrocytes [24, 26] and leads to a wide variety of further diseases or clinical conditions [27]. Thus, it is important to protect erythrocytes against oxidative stress and apoptosis. Because ROS and oxidative stress is an important factor leading to apoptosis in mammal erythrocytes, it is possible that Ala, Cit and Pro could protect mammal erythrocytes from apoptosis due to their antioxidant effects [13, 15, 28]. However, very little information is available to date

on the inhibited effects of Ala, Cit or Pro on oxidative stress in mammal erythrocytes.

In our previous studies, hydroxyl radicals ($\bullet\text{OH}$) induced apoptosis in fish erythrocytes [29], which provides a good experimental model for studying oxidative stress and apoptosis in fish cells [30]. Fish erythrocytes could be used as the model in mammal non-nucleated erythrocytes or nucleated cells. As the mammal erythrocytes, fish erythrocytes contain the high Hb concentrations in the cytoplasm, which can continuously produce ROS, and the high PUFAs content in the membrane, which is very sensitive to ROS [30-32]. ROS can induce the influx of Ca^{2+} that activates scramblase with subsequent phosphatidylserine (PS) externalization at the cell surface (a biomarker of apoptosis) in mammal erythrocytes [33]. Fish erythrocytes retain nucleus, mitochondria and other organelles, which are most like the mammal nucleated cells in structure [32, 34]. Studies have indicated that oxidative stress can initiate the aggregation of death receptors (e.g. CD95) with subsequent caspase-8 activation, and stimulate the release of mitochondrial cytochrome c with subsequent caspase-9 activation [35], both caspases converge finally at the activation of effector caspases (e.g. caspase-3) that results in nucleus DNA fragmentation (a biomarker of apoptosis) in mammal nucleated cells [36-38]. It has been reported that the mechanisms of antioxidant defences in fish cells are similar to those in mammal nucleated cells [39-41]. However, the pathways of apoptosis still remains unknown in fish erythrocytes. Our previous study indicated that Ala, Cit and Pro inhibit the $\bullet\text{OH}$ -induced apoptosis in fish erythrocytes [30]. Nevertheless, the information regarding the effects of Ala, Cit and Pro on the mechanisms of apoptosis is scarce in fish erythrocytes.

In this study, the apoptosis was induced by $\bullet\text{OH}$ in fish erythrocytes. $\bullet\text{OH}$ was generated by the Fenton reaction. We investigated the pathways of apoptosis in fish erythrocytes, which may give a basis for applications of fish erythrocytes as a model of oxidative stress and apoptosis in mammalian cells. We researched the effects of Gln's metabolites (Ala, Cit, Pro and their combination) on the pathways of apoptosis in fish erythrocytes, which could support the use of Ala, Cit and Pro as oxidative stress and apoptosis inhibitors in mammal cells, and reveal the underlying

mechanisms of antioxidation and anti-apoptosis of Gln in mammalian.

Materials and methods

Chemicals

L-Ala, L-Cit and Pro were purchased from Sigma (St. Louis, MO, USA). The aqueous solution of H₂O₂ (30%) and FeSO₄ (analytical pure) were purchased from Shanghai Chemical Reagent Factory (Shanghai, China). Heparin sodium and ethylene glycol tetraacetic acid (EGTA) were purchased from Beijing Biodee Biotechnology Co. Ltd (Beijing, China). Z-VAD-fmk (a broad range inhibitor of caspases) and a murine monoclonal anti-cytochrome c (Anti-Cyt C) were purchased from Beyotime Institute of Biotechnology (Nantong, Jiangsu, China). The paraformaldehyde, Triton X-100, propidium iodide (PI), Ac-IETD-fmk (caspase-8 inhibitor) and Z-LEHD-fmk (caspase-9 inhibitor) were purchased from Nanjing KeyGEN Biotech. CO., LTD (Nanjing, Jiangsu, China). The Griess reagent and the coomassie brilliant blue were purchased from Jiancheng Institute of Biotechnology (Nanjing, Jiangsu, China). All other chemicals were analytical grade. All water used was Milli-Q grade. Physiological carp saline (PCS) (contained 141.1 mM NaCl, 1.43 mM KCl, 0.99 mM CaCl₂, 2.64 mM NaHCO₃ and 6.16 mM glucose) and Ca²⁺-free PCS (CaCl₂ was substituted for EGTA in PCS) modified to give a total osmolarity of 280 mosm L⁻¹ and pH 7.9 were prepared in our laboratory.

Erythrocyte isolation

The procedures of cell isolation were based on that described by Phillips et al. (2000) [42] with slight modifications. Individual healthy carp (*Cyprinus carpio* var. Jian) (100–110g) was anaesthetized, and the blood (approximately 1 ml) was drawn into a syringe via caudal puncture. The blood was placed in a chilled centrifuge tube containing heparinized (40 i.u.ml⁻¹) PCS. The blood from three different carp was pooled and centrifugated (Thermo, Waltham, MA, USA) at 900 × g and 4 °C for 10 min. Then, the plasma and buffy coat of pooled blood were removed in the centrifuge tube. After washing three times with PCS or Ca²⁺-free PCS, the isolated erythrocytes were resuspended in PCS or Ca²⁺-free PCS at a 1% hematocrit in following experiments. All procedures were approved by the Institutional Animal Care and Use

Committee of Sichuan Agricultural University in accordance with the Institutional Ethics Committee of the Chinese Institute of Chemical Biology guidelines.

Erythrocyte treatments

Erythrocyte treatments were based on the method described by Li et al. (2013) [30] with slight modifications. Alanine, Cit and Pro were combined in the molar ratio 10: 4: 1 (Ala₁₀Pro₄Cit₁) for investigating their mixture effects on apoptosis. Briefly, the isolated erythrocytes were suspended in PCS (control), Ca²⁺-free PCS and PCS containing 0.00 or 1.40 mM of Ala, Cit, Pro or Ala₁₀Pro₄Cit₁ or 50 μM of zVAD-fmk at a 1% hematocrit, respectively. All erythrocyte suspensions above were pre-incubated at 37 °C for 1.5 h. FeSO₄/H₂O₂ was then added at a final concentration of 40 μM/20 μM for the induction of apoptosis, except in the control. After incubation at 37 °C for 9 h, the erythrocytes were collected for measurement of the DNA fragmentation, PS exposure, the modification of membrane proteins, the oxidation of hemoglobin (Hb), the levels of ROS, cytochrome c and Ca²⁺ and the activities of caspase-8, caspase-9, caspase-3, calpain and anti-hydroxyl radical (AHR). The experiment was performed with 4 replicates per treatment and the control.

To further determine the effects of Ala, Cit and Pro on caspases, ROS and cytochrome c, a series of experiments were carried out as follows. The isolated erythrocytes were resuspended in PCS at a 1% hematocrit. FeSO₄/H₂O₂ was then added to positive control at a final concentration of 40 μM/20 μM, except in the control. After incubation at 37 °C for 7 h, the erythrocytes in positive control and control were collected for measurement of the activities of caspase-8, caspase-9 and caspase-3 and the levels of ROS and cytochrome c. After washing three times with PCS, the erythrocytes in positive control were suspended in PCS and PCS containing 100 μM of Ac-IETD-fmk or Z-LEHD-fmk, 1.40 mM of Ala, Cit or Pro, 100 μM of Ac-IETD-fmk and 1.40 mM of Ala, Cit or Pro, 100 μM of Ac-IETD-fmk and Z-LEHD-fmk, or 100 μM of Ac-IETD-fmk and Z-LEHD-fmk and 1.40 mM of Ala, Cit or Pro at a 1% hematocrit, respectively. Or, a cell-free assays were performed as described by Berg et al. (2001) [43] with slight modifications. Briefly, after washing with PCS, 5×10⁷ erythrocytes in positive control were lysed in 100 μl of lysis buffer

(Beyotime, Nantong, China) and lysis buffer containing 100 μ M of Z-LEHD-fmk, 2.0 μ g/ml of Anti-Cyt C [44] or 2.0 μ g/ml of Anti-Cyt C and 1.40 mM of Ala, Cit or Pro, respectively. After incubation at 37 °C for 2 h, the activities of caspase-8, caspase-9 and caspase-3 and the levels of ROS and cytochrome c were measured and expressed as the fold of the activities of caspase and the levels of ROS and cytochrome c compared to that of control cells. All experiment above was performed with 4 replicates per treatment and the control.

To further determine the effects of Ala, Cit and Pro at the physiological concentrations on the caspases, ROS and cytochrome c, a series of experiments and statistical analysis were carried out as in Ref [45].

Measurement of caspases

The activities of caspase-3, caspase-8 and caspase-9 were determined by measuring cleavage of the Ac-DEVD-pNA (caspase-3 substrate), Ac-IETD-pNA (caspase-8 substrate) and Ac-LEHD-pNA (caspase-9 substrate) to pNA using the detection kits (Beyotime, Nantong, China) as described previously [46, 47]. The caspase activity in the samples was quantified with a Microplate reader (Thermo, Waltham, USA) at 405 nm.

Measurement of ROS

ROS levels in the erythrocytes were determined by measuring the oxidative conversion of 2', 7'-dichlorofluorescein diacetate (DCFH-DA) to fluorescent compound DCF using a detection kit (Beyotime, Nantong, China) as described previously [48-50]. DCF fluorescence in the erythrocytes was determined at 485 nm excitation and 520 nm emission using fluorescence spectrophotometer (Thermo, Waltham, USA). The images of the cells were captured by a Nikon TE2000E inverted fluorescent microscope (Tokyo, Japan).

Measurement of cytochrome c

The cytosolic proteins and mitochondria in fish erythrocytes were isolated by a commercial extraction kit (Beyotime, Nantong, China) as described previously [51]. The cytochrome c release from mitochondria in cytosol was evaluated by an enzyme-linked immunosorbent assay (ELISA) kit (ElabScience, Wuhan, China) [52].

Absorbance value in the samples was measured with the Microplate reader at 450 nm.

Measurement of Ca^{2+}

Intracellular Ca^{2+} concentration was measured by detection of the fluorescent Ca^{2+} chelator Fura-2 AM using a detection kit (Beyotime, Nantong, China) as described previously [53, 54]. The fluorescent intensity in the cells was determined at 340 nm excitation and 510 nm emission using the fluorescence spectrophotometer.

Measurement of calpain

Calpain activity was measured by detection of cleavage of the Ac-LLY-AFC substrate to AFC using a detection kit (GenMed Co. Ltd. USA) as described by Wang et al. (2011) [55]. The fluorescent intensity of the sample was measured at 400 nm excitation and 505 nm emissions using the fluorescence spectrophotometer.

Measurement of AHR

AHR activity was determined by Hydroxyl Radical Detection Kit (Jiancheng, Nanjing, China) as described by Jiang et al. (2009) [56].

Measurement of apoptosis

DNA fragmentation in the erythrocytes was assessed by the terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labeling (TUNEL) kit (KeyGEN, Nanjing, China) as described by Zhou et al., (2009) [57]. The number of TUNEL-positive cells was detected by flow cytometry (Becton Dickinson, Germany). The amount of cells was 10000 in each case of flow cytometry analysis.

PS exposure in the erythrocytes was assessed by the Annexin V/PI detection kit (Beyotime, Nantong, China) as described previously [58, 59]. The images of the annexin binding cells were captured by the fluorescent microscope. The amount of cells was 10000 in each case for analysis.

Analysis of membrane proteins

The modifications of the membrane proteins were determined by SDS-PAGE as described previously [60, 61]. The membrane proteins were isolated by Membrane and Cytosol Protein Extraction Kit (Beyotime, Nantong, China). SDS-PAGE was performed on a slab gel of 1.5 mm thick using 5% and 7.5% gels for condensation and separation, respectively. The appropriate controls were run along with the test samples.

The gel was then stained with the coomassie brilliant blue and photographed using a Gene Genius Bio-Imaging system (Syngene, Frederick, USA). The molecular weight of the membrane proteins was calibrated by migration of the standard proteins in the gel system.

Measurement of Hb oxidation

Hb absorption spectrum was determined as described by Takebayashi et al. (2007) [62]. Briefly, the isolated erythrocytes were suspended in PCS (control) and PCS containing 0 or 1.40 mM of Ala, Cit, Pro or Ala₁₀Pro₄Cit₁ at a 1% hematocrit, respectively. FeSO₄/H₂O₂ was then added at final concentration of 40 μM/20 μM, except for the control. After incubation at 37 °C for 90 min, the erythrocytes were collected and hemolyzed with 400 vol of deionized water. The hemolysate was then centrifuged at 4°C and 3000× g for 10 min. The absorption of the supernatant was measured by the Microplate reader at 600-700 nm. The oxidation of Hb was determined as described by Pietraforte et al. (2007) [63]. Briefly, the isolated erythrocytes were treated as that above. Once FeSO₄/H₂O₂ was added to the erythrocyte suspensions, the kinetics of Hb oxidation were measured using the Microplate reader at 632 nm and 37°C (Kinetic interval, 1 min).

Protein measurements and statistical analysis

Protein concentration in the hemolysate was measured by Darbkin method [64]. All data are presented as means ± S.D. (n = 4). The data were subjected to one-way analysis of variance (ANOVA). If significant differences were found (P<0.05), Duncan's multiple range tests were used to rank the groups [65]. All statistical analyses were performed using the SPSS 13.0 for Window software (SPSS, Chicago, IL, USA) [66].

Results

The effects of inhibitor, Ala, Cit and Pro on the caspase activity in carp erythrocyte

The effects of zVAD-fmk, Ca²⁺-free PCS, Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on the caspases activities in the •OH-induced carp erythrocytes were presented in Table 1. Compared with the control, the activities of caspase-3, caspase-8 and caspase-9 were significantly increased by 112.19 %, 254.27% and 225.12% in carp erythrocytes

exposed to •OH for 9h, respectively. However, zVAD-fmk, Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ significantly inhibited the •OH-induced increase in the activities of caspase-3, caspase-8 and caspase-9 in carp erythrocytes. The activities of caspase-3, caspase-8 and caspase-9 were gradually decreased with increasing concentrations of Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ (0.175-1.400 mM) in the •OH-induced carp erythrocytes (see Fig. 1, 2 and 3 in Ref [45]). Especially, when the erythrocytes were treated with Ala₁₀Pro₄Cit₁ in the presence of •OH, the 50% inhibitory doses (ID₅₀) on the activities of caspase-8, caspase-9 and caspase-3 were respectively estimated to be 0.60, 0.61 and 0.35 mM that are the minimum values among amino acids examined so far (see Table 1 in Ref [45]). The 5% inhibitory doses (ID₅) of Ala₁₀Pro₄Cit₁ on the activities of caspase-8 and caspase-3 were respectively estimated to be 0.026 and 0.024 mM, the ID₅ of Cit on the activities of caspase-3 and caspase-9 were respectively estimated to be 0.025 and 0.021 mM, which are the minimum values among amino acids examined so far. (see Table 2 in Ref [45]). Z-VAD-fmk completely prevented the increase in the activities of the caspase-9 and caspase-3 in the •OH-induced carp erythrocytes. Little inhibition on the activities of caspase-8, caspase-9 and caspase-3 in •OH-induced carp erythrocytes was observed in the presence of Ca²⁺-free PCS.

(Table 1 inserted here)

Effects of Ac-IETD-fmk, Z-LEHD-fmk, Anti-Cyt C, Ala, Cit and Pro on caspases activities in the erythrocyte after exposing to •OH for 7h were shown in Fig. 1. Compared with the control, the activities of caspase-8, caspase-9 and caspase-3 were significantly increased by 227.74%, 195.99% and 107.40% in carp erythrocytes exposed to •OH for 7h, respectively. After continuing incubation for 2 h in the absence of •OH, the activity of caspase was still higher than the control in the erythrocytes. However, when treatment with Ac-IETD-fmk and Z-LEHD-fmk in carp erythrocytes, the •OH-induced increases in the activities of the caspase-8 and caspase-9 were completely prevented in the absence of •OH, respectively (Fig. 1 A and B). Little inhibition on the activity of caspase-8 in the presence of Ala, Cit or Pro was observed in carp erythrocytes in the absence of •OH (Fig. 1 A). Though Anti-Cyt

C significantly decreased the activity of the caspase-9 in the absence of •OH, the combination of Anti-Cyt C and Ala, Cit or Pro didn't further reduce the activity in carp erythrocytes (Fig. 1 B). Ac-IETD-fmk and Z-LEHD-fmk all effectively relieved the activity of the caspase-3 in the absence of •OH. Moreover, the combination of Ac-IETD-fmk and Z-LEHD-fmk further enhanced inhibited effects on the activity of the caspase-3. Nevertheless, addition Ala, Cit or Pro to Ac-IETD-fmk and Z-LEHD-fmk didn't improve inhibition on caspase-3 in the erythrocyte (Fig. 1 C).

(Fig. 1 inserted here)

The effects of inhibitor, Ala, Cit and Pro on the cytochrome c level in carp erythrocytes

The effects of zVAD-fmk, Ca^{2+} -free PCS, Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ on the cytochrome c levels in the •OH-induced carp erythrocytes were presented in Table 1. Compared to the controls, the level of cytochrome c was significantly increased by 248.68% in carp erythrocytes exposed to •OH for 9h. However, zVAD-fmk, Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ significantly inhibited the •OH-induced increase in the cytochrome c level in carp erythrocytes. When treatment with Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ in the •OH-induced carp erythrocytes, the levels of cytochrome c were gradually decreased with the increasing concentrations (0.175-1.400 mM) in comparison to that treated with •OH alone (see Fig. 4 in Ref [45]). Especially, when treatment with $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$, the ID_{50} on the cytochrome c was estimated to be 1.01 mM that is the minimum value among amino acids examined so far (see Table 1 in Ref [45]). The ID_5 of Ala, Cit, Pro or $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ on the levels of cytochrome c were estimated to be 0.102, 0.064, 0.068 or 0.028 mM, respectively (see Table 2 in Ref [45]). Z-VAD-fmk showed the strongest protective effects among compound examined. Little inhibition on the cytochrome c was observed in the presence of Ca^{2+} -free PCS in •OH-induced carp erythrocytes.

Effects of Ac-IETD-fmk, Ala, Cit and Pro on the cytochrome c level in the erythrocyte after exposing to •OH for 7h were shown in Fig. 1 D. Compared with the control, the cytochrome c level was significantly increased by 206.84% in carp erythrocytes exposed to •OH for 7h. After continuing incubation for 2 h in the absence

of •OH, the level of cytochrome c was still higher than the control in the erythrocytes. However, when treatment with Ac-IETD-fmk in carp erythrocytes, the •OH-induced increase in the level of cytochrome c was markedly decreased in the absence of •OH. Moreover, the combination of Ac-IETD-fmk and Ala, Cit or Pro further reduced the level of cytochrome c in carp erythrocytes. Especially, the combination of Ac-IETD-fmk and Pro showed the strongest inhibition efficacy on the cytochrome c level in the absence of •OH.

The effects of inhibitor, Ala, Cit and Pro on the ROS level in carp erythrocytes

The effects of zVAD-fmk, Ca²⁺-free PCS, Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on the levels of ROS in •OH-induced carp erythrocytes were presented in Fig. 2 and Table 1. Compared to the controls, the level of ROS was significantly increased by 715.86% in carp erythrocytes exposed to •OH for 9h. However, zVAD-fmk, Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ significantly inhibited the •OH-induced increase in the levels of ROS in carp erythrocytes. The levels of ROS were gradually decreased with increasing concentrations of Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ (0.175-1.400 mM) in the •OH-induced carp erythrocytes (see Fig. 5 in Ref [45]). Especially, when treatment with 1.400 mM of Ala₁₀Pro₄Cit₁, the level of ROS was the minimum value (256.21% of control) among Gln's metabolites examined, which is equivalent to that of zVAD-fmk (280.65% of control). The ID₅₀ of Ala₁₀Pro₄Cit₁ on ROS was estimated to be 0.53 mM that is the minimum value among amino acids examined so far (see Table 1 in Ref [45]). The ID₅ of Ala, Cit, Pro or Ala₁₀Pro₄Cit₁ on ROS were estimated to be 0.092, 0.047, 0.107 or 0.041 mM, respectively (see Table 2 in Ref [45]). Little inhibition on ROS was observed in the presence of Ca²⁺-free PCS in •OH-induced carp erythrocytes.

(Fig. 2 inserted here)

Effects of Ac-IETD-fmk, Ala, Cit and Pro on the level of ROS in carp erythrocyte after exposing to •OH for 7h were shown in Fig. 1 E. Compared with the control, the level of ROS was significantly increased by 486.43% in the erythrocytes exposed to •OH for 7h. After continuing incubation for 2 h in the absence of •OH, the level of ROS was still higher than the control in carp erythrocytes. However, Ac-IETD-fmk

effectively suppressed the •OH-induced increase in the level of ROS in the absence of •OH. Moreover, the combination of Ac-IETD-fmk and Ala, Cit or Pro further improve inhibition on ROS in the erythrocyte. In particular, the combination of Ac-IETD-fmk and Pro showed the strongest inhibition efficacy on the ROS level in the absence of •OH.

The effects of inhibitor, Ala, Cit and Pro on TUNEL in carp erythrocytes

The effects of zVAD-fmk, Ca²⁺-free PCS, Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on TUNEL in •OH-induced carp erythrocytes were showed in Fig. 3. •OH caused a significantly increase in the number of TUNEL-positive cells in comparison to the control, suggesting the increase of DNA fragmentation in carp erythrocytes. However, zVAD-fmk, Ca²⁺-free PCS, Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ significantly relieved the •OH-induced increase in the number of TUNEL-positive erythrocytes, suggesting the decrease of DNA fragmentation in carp erythrocytes. Especially, when incubation with zVAD-fmk in the •OH-induced carp erythrocytes, the number of TUNEL-positive erythrocytes was decreased to the lowest level among these compounds examined so far. When the erythrocytes were treated with Ala₁₀Pro₄Cit₁ in the presence of •OH, the number of TUNEL-positive cells was estimated to be 4.67 %, which is the lowest level among Gln's metabolites examined so far.

(Fig. 3 inserted here)

The effects of inhibitor, Ala, Cit and Pro on the levels of Ca²⁺ in carp erythrocytes

The effects of zVAD-fmk, Ca²⁺-free PCS, Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on the levels of Ca²⁺ in •OH-induced carp erythrocytes were presented in Table 2. The levels of Ca²⁺ were significantly increased by 126.76% in carp erythrocytes exposed to •OH compared to the controls. However, when treatment with Ca²⁺-free PCS, Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ the levels of Ca²⁺ in •OH-induced carp erythrocytes were significantly decreased in comparison to that treated with •OH alone. Especially, when incubation with Ala₁₀Pro₄Cit₁ in the •OH-induced carp erythrocytes, the levels of Ca²⁺ were estimated to be 114.90 % of control, which are equivalent to the controls and that treated with Ca²⁺-free PCS (104.68 % of control). Little inhibition on the levels of Ca²⁺ was observed in the presence of zVAD-fmk in •OH-induced carp

erythrocytes.

(Table 2 inserted here)

The effects of inhibitor, Ala, Cit and Pro on the calpain activity in carp erythrocytes

The effects of zVAD-fmk, Ca^{2+} -free PCS, Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ on the calpain activity in $\bullet\text{OH}$ -induced carp erythrocytes were presented in Table 2. Compared with the control, the calpain activity was significantly increased by 125.49 % of control in carp erythrocytes exposed to $\bullet\text{OH}$. However, Ca^{2+} -free PCS, Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ significantly prevented the $\bullet\text{OH}$ -induced increase in the calpain activity in carp erythrocytes. Especially, when the erythrocytes were treated with $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ in the presence of $\bullet\text{OH}$, the calpain activity was estimated to be 112.94 % of control that is the minimum among amino acids examined so far. Ca^{2+} -free PCS completely prevented the $\bullet\text{OH}$ -induced increase of the calpain activity in carp erythrocytes. Little inhibition on the calpain activity was observed in the presence of zVAD-fmk in $\bullet\text{OH}$ -induced carp erythrocytes.

The effects of inhibitor, Ala, Cit and Pro on Annexin binding in carp erythrocytes

The effects of zVAD-fmk, Ca^{2+} -free PCS, Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ on the annexin binding in $\bullet\text{OH}$ -induced carp erythrocytes were shown in Fig. 4. Compared with the control, the levels of annexin binding cells were significantly increased in the presence of $\bullet\text{OH}$, suggesting the increase of PS exposure in carp erythrocytes. However, zVAD-fmk, Ca^{2+} -free PCS, Ala, Cit, Pro, and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ markedly suppressed the $\bullet\text{OH}$ -induced annexin binding, suggesting the decrease of PS exposure in carp erythrocytes. Especially, $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ showed the strongest inhibition efficacy on annexin binding among Gln's metabolites examined, which were equivalent to that of zVAD-fmk and Ca^{2+} -free PCS in $\bullet\text{OH}$ -induced carp erythrocytes.

(Fig. 4 inserted here)

The effects of Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ on the modification of membrane proteins in carp erythrocytes

The effects of Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ on the modification of membrane proteins in $\bullet\text{OH}$ -induced carp erythrocytes were showed in Fig. 5. Calpain can result in the degradation of the cytoskeleton in the apoptotic cells [67]. Compared with the

control (lane 1), the disappearance of band 2 were observed, suggesting the degradation of the cytoskeleton in the membrane proteins exposed to $\bullet\text{OH}$ alone (lane 2). However, only $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ (lane 7) effectively prevent the degradation of the cytoskeleton in band 2 in carp erythrocytes induced by $\bullet\text{OH}$. Oxidation can produce the decrease in the low-molecular-weight proteins (LMWPs) and the increase in the high-molecular-weight proteins (HMWPs) in the cells membrane [68, 69]. Compared with the control (lane 1), the increase of HMWPs at the upside of band 1, and the decrease of LMWPs in band 5 were clearly detected in the erythrocytes exposed to $\bullet\text{OH}$ alone (lane 2). However, Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ attenuated the $\bullet\text{OH}$ -induced alterations of the membrane protein in the carp erythrocytes. Especially, when incubation with $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$, the pattern of membrane proteins in the $\bullet\text{OH}$ -induced carp erythrocytes was maintained at a background level similar to that in the $\bullet\text{OH}$ -untreated samples.

(Fig. 5 inserted here)

The effects of Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ on the oxidation of Hb in carp erythrocytes

The oxidation of Hb is manifested in the increase of the absorption at 630 nm in the mammalian erythrocytes [63]. To determine the exact oxidation of Hb in carp erythrocytes, we measured the absorption spectra of Hb at 600-700 nm. As shown in Fig. 6 A, Hb in all treatment showed an absorption peak at 632 nm in carp erythrocytes. $\bullet\text{OH}$ caused a significantly increase in the absorbance at 632 nm in comparison to the control, suggesting the increase of Hb oxidation in carp erythrocytes. However, Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ significantly relieved the $\bullet\text{OH}$ -induced increase in the absorbance at 632 nm, suggesting the decrease of Hb oxidation in carp erythrocytes.

The effects of Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ on the kinetics of Hb oxidation at 632 nm were presented in Fig 6 B. The oxidation of Hb reached the maximum level within average 104 min and further incubation did not significantly increase the oxidation of Hb in the carp erythrocytes exposed to $\bullet\text{OH}$ alone. However, Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ significantly delayed the time reached the maximum level of

the Hb oxidation. The inhibitory effects of Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on the oxidation of Hb are expressed as the delayed time of the Hb oxidation (ΔT) in this study, which was calculated by

$$\Delta T = MFT_{50} (\text{Ala, Cit, Pro or Ala}_{10}\text{Pro}_4\text{Cit}_1) - MFT_{50} (\bullet\text{OH})$$

where MFT₅₀ is 50% of the time that the Hb oxidation reached the maximum level.

The average MFT₅₀ ($\bullet\text{OH}$) was 73 ± 6 min. The average MFT₅₀ (Ala, Cit, Pro and Ala₁₀Pro₄Cit₁) were greater than the average MFT₅₀ ($\bullet\text{OH}$). The order of the ΔT values was Ala₁₀Pro₄Cit₁ > Ala = Pro > Cit (Fig. 6 C).

(Fig. 6 inserted here)

The effects of Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on the anti-hydroxyl radical (AHR) activity in carp erythrocytes

The effects of Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on the AHR activity in $\bullet\text{OH}$ -induced carp erythrocytes were presented in Fig. 7. Compared with the control, the AHR activity significantly decreased by about 49.0 % in the carp erythrocytes exposed to $\bullet\text{OH}$. However, Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ significantly inhibited the $\bullet\text{OH}$ -induced decrease in the AHR activity in carp erythrocytes. Especially, when treatment with Ala₁₀Pro₄Cit₁ in the $\bullet\text{OH}$ -induced erythrocytes, the AHR activity was estimated to be 3.87 U mg⁻¹ proteins that is the maximum value among these compounds examined so far.

(Fig. 7 inserted here)

Discussion

Compared with the mammal erythrocytes, fish erythrocytes retain their nuclei and other organelles [32]. So, the pathway of apoptosis in fish erythrocytes may be different to that in mammal non-nucleated erythrocytes. However, information regarding the apoptotic mechanism is scarce in fish erythrocytes. Thus, we researched the pathway of apoptosis in fish erythrocytes. In previous study, the incubation with 40 μM FeSO₄/20 μM H₂O₂ at 37 °C induced apoptosis in carp erythrocytes [30]. In the present study, the apoptosis was induced by the same condition in carp erythrocytes.

$\bullet\text{OH}$ -induced apoptosis in fish erythrocytes

DNA fragmentation is a biomarker of apoptosis in mammal cells [70, 71]. The number of TUNEL-positive cells is a widely used index of DNA fragmentation [72, 73]. DNA fragmentation may be closely associated with caspases in fish erythrocytes. Studies indicated that active caspase-3 allows the caspase-activated DNase (CAD) to degrade chromosomal DNA, which leads to DNA fragmentation during apoptosis in mammal cells [74]. However, information regarding the connection between DNA fragmentation and caspases is scarce in fish erythrocytes. In the present study, exposure to $\bullet\text{OH}$ significantly increased caspase-8, caspase-9 and caspase-3 activities in carp erythrocytes. This suggested that $\bullet\text{OH}$ can induce caspase-8, caspase-9 and caspase-3 activation in fish erythrocytes. This is in good agreement with the report that H_2O_2 induces caspase-8, caspase-9 and caspase-3 activation in rat pheochromocytoma cells [47]. Moreover, exposure to $\bullet\text{OH}$ markedly increased the number of TUNEL-positive erythrocytes in this study. This indicated that $\bullet\text{OH}$ induced DNA fragmentation in fish erythrocytes. This is consistent with the report that $\bullet\text{OH}$ induces DNA fragmentation in mice thymocytes [75]. However, zVAD-fmk conferred simultaneous protection against caspase-8, caspase-9 and caspase-3 activation and DNA fragmentation in carp erythrocytes induced by $\bullet\text{OH}$. These displayed that $\bullet\text{OH}$ -induced DNA fragmentation was caspases dependent in fish erythrocytes. This is in line with the report that caspases activity is involved in the H_2O_2 -induced DNA fragmentation in human lens epithelial cells [76].

There may be a positive connection between caspases activation and cytochrome c and ROS in fish erythrocytes. Studies demonstrated that ROS functions as an upstream signal that initiates cytochrome c release and caspases activation in the benzodiazepine-induced Ramos cells [77]. However, there are few papers describing the connection between caspases and cytochrome c and ROS in fish erythrocytes. In this study, exposure to $\bullet\text{OH}$ significantly increased intracellular cytochrome c and ROS levels in carp erythrocytes. This suggested that $\bullet\text{OH}$ -induced cytochrome c release and ROS generation in fish erythrocytes. This is consistent with the report that H_2O_2 induces cytochrome c release in HeLa cells [36] and ROS production in rat neuronal cells [78]. The cytochrome c release and ROS generation may be related to

mitochondrion in fish erythrocytes. Rothmann et al. (2000) reported that fish erythrocytes contain mitochondrial organelles [34]. Studies indicated that mitochondria is the source of cytochrome c release and ROS generation in mammal cells [79]. Dumont et al. (1999) reported that H₂O₂ induces the release of mitochondria cytochrome c and ROS in human T-cells [37]. Interestingly, zVAD-fmk reduced the •OH-induced cytochrome c release and ROS generation in carp erythrocytes. This revealed that the •OH-induced cytochrome c release and ROS generation was caspases dependent in fish erythrocytes. This is not in line with the report that ROS generation and cytochrome c release occurs upstream of caspase activation in Ramos cells [80]. It may be because that caspase-8 mediates ROS generation and cytochrome c release in fish erythrocytes. Studies showed that caspase-8 activates Bid [81] and Bax [82] that trigger the membrane potential breakdown accompanied by an elevation of ROS production [83] and the membrane pore opening accompanied by the release of cytochrome c in mammal mitochondria [84]. Consistent with the hypothesis above, Ac-IETD-fmk simultaneously suppressed the activation of caspase-8 and the release of cytochrome c and ROS in the erythrocytes. This revealed that •OH-induced cytochrome c release and ROS generation were caspases-8 dependent in fish erythrocytes. The cytochrome c can activate caspase-9 with subsequent caspase-3 that results in DNA fragmentation in mammalian cells [38]. It has been reported that the antibody of specific protein suppresses the function of corresponding antigen by blocking access to the catalytic site [85, 86]. In this study, Anti-Cyt C inhibited the •OH-induced activation of caspase-9 in the erythrocytes. This demonstrated that the •OH-induced caspase-9 activation was cytochrome c dependent in fish erythrocytes. Furthermore, Z-LEHD-fmk simultaneously inhibited the •OH-induced activation of caspase-9 and caspase-3, and Ac-IETD-fmk effectively protected against the caspase-3 activity in the erythrocytes. These displayed that the •OH-induced caspase-3 activation was caspase-8 and caspase-9 dependent in fish erythrocytes. This was consistent with the reports that H₂O₂ induces the mitochondria cytochrome c release that initiates the activation of caspase-9 with subsequent caspases-3 in HeLa cells [36]. Although no

reports have been published on that caspase-8 activation is death receptors (CD95) dependent in fish erythrocytes, it is speculated that $\bullet\text{OH}$ may activate caspase-8 by triggering CD95. This is not only because caspase-8 is a key component of the CD95-triggered apoptosis pathway [87, 88], but also because many other apoptotic pathways initiated by distinct stimuli require CD95 involvement [44]. Moreover, studies indicated that oxidative stress causes CD95 ligand expression in mouse microglial cells [89]. So, it is possible that $\bullet\text{OH}$ triggers CD95 that stimulates caspase-8 with subsequent the mitochondrial generation of ROS and the release of cytochrome c, which further activates caspase-9 and caspase-3, and finally results in DNA fragmentation in fish erythrocytes. This is in agreement with the apoptotic pathways in mammalian cells [38].

In addition DNA fragmentation, PS exposure on the cell surface is another biomarker of apoptosis [26, 90]. The number of annexin binding cells is a widely used index of PS exposure [59]. There may be a positive connection between PS exposure and Ca^{2+} levels in fish erythrocytes. Lang et al. (2005) displayed that the increases in cytosolic Ca^{2+} concentration stimulate scramblase shifting PS from the inner leaflet to the outer leaflet of membrane in human erythrocyte [91]. However, information regarding the connection between PS externalization and Ca^{2+} is scarce in fish erythrocytes. In this study, exposure to $\bullet\text{OH}$ significantly increased intracellular Ca^{2+} levels in carp erythrocytes. However, Ca^{2+} -free PCS effectively prevented the increase in intracellular Ca^{2+} levels in the $\bullet\text{OH}$ -induced carp erythrocytes. This suggested that $\bullet\text{OH}$ induced an influx of Ca^{2+} in fish erythrocytes. This is in accordance with the report that oxidative stress opens Ca^{2+} -permeable cation channels, thus increases cytosolic Ca^{2+} concentration in human erythrocytes [91]. In addition the influx of Ca^{2+} , $\bullet\text{OH}$ induced an increase in the number of annexin binding erythrocytes. This displayed that $\bullet\text{OH}$ induced PS exposure in fish erythrocytes. It is worthwhile to note that Ca^{2+} -free PCS simultaneously decreased the PS exposure and Ca^{2+} levels in the $\bullet\text{OH}$ -induced carp erythrocytes. This is consistent with the report that removal of extracellular Ca^{2+} inhibits PS exposure in human erythrocytes [26]. These results demonstrated that $\bullet\text{OH}$ -induced PS exposure was Ca^{2+} dependent in fish erythrocytes.

This is in line with the report that Ca^{2+} is involved in PS exposure in human erythrocytes [33]. Interestingly, zVAD-fmk inhibited the $\bullet\text{OH}$ -induced PS exposure in carp erythrocytes. This suggested that $\bullet\text{OH}$ -induced PS exposure was caspase-dependent in carp erythrocytes. This is in accordance with the report that oxidative stress induces death receptors (Fas) with subsequent caspase-8 and caspase-3 activation that triggers loss of aminophospholipid translocase activity with subsequent PS externalization in human erythrocytes [92, 93]. The caspases-involved PS exposure may be closely associated with intracellular ROS and cytochrome c in fish erythrocyte. Studies indicated that ROS can oxidize PS in the inner leaflet, which results in PS exposure in the outer leaflet of cell membrane in HL-60 cells [94]. The cytochrome c released from mitochondria binds to PS located at the cytoplasmic surface of the plasma membrane and induces its oxidation that causes inhibition of aminophospholipid translocase relevant to PS externalization [95]. It is speculated that caspase-8 stimulates the mitochondrial generation of ROS and release of cytochrome c that triggers PS oxidation and impairs its translocase eventually leading to its exposure in fish erythrocytes. No reports have been published that active caspase causes PS exposure in fish erythrocyte. The detail mechanism needs further research.

The influx of Ca^{2+} may be closely associated with calpain activation in fish erythrocytes. Calpain is the calcium-dependent cysteine proteases [96], which can result in the degradation of the cytoskeleton in human erythrocytes [97]. However, information regarding the connection between cytosolic Ca^{2+} and calpain and cytoskeleton is scarce in fish erythrocytes. In this study, exposure to $\bullet\text{OH}$ markedly increased calpain activity and led to the disappearance of band 2 in carp erythrocytes. This suggested that $\bullet\text{OH}$ induced calpain activation and the degradation of the cytoskeletal proteins in fish erythrocytes. This is in good agreement with the report that oxidative stress stimulates calpain activation and the degradation of the cytoskeleton in human erythrocytes [91]. Interestingly, Ca^{2+} -free PCS inhibited the $\bullet\text{OH}$ -induced calpain activation in carp erythrocytes. These results demonstrated that $\bullet\text{OH}$ -induced calpain activation was Ca^{2+} dependent in fish erythrocytes. This is line

with the report that the increase in cytosolic Ca^{2+} levels stimulates calpain activation with subsequent the degradation of the cytoskeleton in human erythrocytes [97]. So, it is likely that $\bullet\text{OH}$ causes the influx of Ca^{2+} that stimulates scramblase with subsequent PS exposure and trigger calpain activation with subsequent the degradation of the cytoskeleton in fish erythrocytes. This is in good consistent with the apoptosis pathway in human erythrocytes [33, 91].

It is worthwhile to note that Ca^{2+} -free PCS inhibited the $\bullet\text{OH}$ -induced DNA fragmentation in carp erythrocytes. This suggested that $\bullet\text{OH}$ -induced DNA fragmentation was Ca^{2+} dependent in fish erythrocytes. Little is known about the connection between Ca^{2+} and DNA fragmentation in fish erythrocyte. The Ca^{2+} -mediated DNA fragmentation may be closely associated with calpain activation in fish erythrocytes. Studies demonstrated that calpain cleaves both Bid and Bax that have a cytochrome c releasing activity in the mitochondria [98]. An hypothetical model for the pathway of apoptosis could be that $\bullet\text{OH}$ stimulates Ca^{2+} influx that triggers calpain activities, which in turn cleaves Bid or Bax that induces the release of cytochrome c with subsequent caspase-9 and caspase-3 activation that results in DNA fragmentation in fish erythrocytes. Nevertheless, Nandi et al. (2010) revealed that H_2O_2 induces a distinct calpain-dependent DNA fragmentation without the involvement of caspases in *E. histolytica* trophozoites [99]. Furthermore, our results showed that zVAD-fmk completely prevented the caspase-9 and caspase-3 activation, but did not fully inhibited DNA fragmentation in the $\bullet\text{OH}$ -induced carp erythrocytes. So, there may be a caspase-independent but calpain-dependent DNA fragmentation in fish erythrocytes. No reports have been published the calpain involved DNA fragmentation in fish cells. The detail mechanism needs further research.

According to the study above, there might be two distinct apoptosis pathways in fish erythrocytes. One is that $\bullet\text{OH}$ activates the caspase-8 by triggering CD95, which results in the generation of ROS and the release of cytochrome c with subsequent caspase-9 and caspase-3 activation that stimulates PS exposure and DNA fragmentation. The other is that $\bullet\text{OH}$ causes the influx of Ca^{2+} that activates scramblase with subsequent PS exposure, and initiates calpain activity with

subsequent the cytoskeleton degradation and DNA fragmentation in fish erythrocytes. This is in good agreement with our previous reports that apoptosis might be caspase- and Ca^{2+} -dependent in the young and mature erythrocytes of fish, respectively [29]. The present results showed that the mechanisms of apoptosis in fish erythrocytes were similar to those in mammal erythrocytes and nucleated cells. It has been demonstrated that the mechanisms of oxidative stress in fish cells are similar to those in mammal cells [31, 39-41]. Moreover, as the mammal erythrocytes, fish erythrocytes contain the high concentrations of Hb that transports O_2 and CO_2 for respiration and continuously produce ROS [24, 30]. Fish erythrocytes retain nucleus, mitochondria and other organelles, which are most like the mammal nucleated cells in structure [32, 34]. In our experience, fish erythrocytes are easy to harvest and culture *in vitro* [30]. So, fish erythrocytes may be used as a good model for studying oxidative stress and apoptosis in mammal erythrocytes or nucleated cells.

Effects of Ala, Cit and Pro on •OH-induced apoptosis

In the present study, Ala, Cit and Pro significantly inhibited •OH-induced PS exposure and DNA fragmentation in fish erythrocytes. This suggested that Ala, Cit and Pro protect fish erythrocytes against apoptosis. Furthermore, Ala, Cit and Pro conferred protection against caspase-8 activation in carp erythrocytes induced by •OH. However, Ala, Cit and Pro didn't protect against caspase-8 activity in carp erythrocytes in the absence of •OH. This displayed that the inhibition efficacy of Ala, Cit and Pro on caspase-8 was •OH dependent in the erythrocytes. It is possible that Ala, Cit and Pro inhibit the Fenton reaction or quench •OH *in vitro*. Akashi et al. (2001) reported that Cit can effectively scavenge •OH *in vitro* due to its long side chain with multiple sites for attacks by •OH [28]. It has been reported that Pro is an efficient •OH scavenger *in vitro* [100]. The low ionization potential and the secondary amine of the pyrrolidine ring are the key factors in the quenching •OH of Pro [101]. It has been demonstrated that Ala stimulates the expression of ferritin and heme oxygenase-1 with subsequent a accumulation of bilirubin in human endothelial cells [13, 102]. The ferritin can rapidly sequester free cytosolic iron that is the crucial catalyst of •OH formation [103, 104]. The bilirubin can act as a direct antioxidant in

biological systems [105]. Our results displayed that Ala, Cit and Pro markedly increased AHR activity in carp erythrocytes. So, Ala, Cit and Pro couldn't directly inhibit caspase-8 activation but quench $\bullet\text{OH}$ in the erythrocytes.

In this study, Ala, Cit and Pro effectively inhibited the $\bullet\text{OH}$ -induced generation of ROS and release of cytochrome c in fish erythrocytes. Ac-IETD-fmk suppressed the generation of ROS and release of cytochrome c in the erythrocytes in the absence of $\bullet\text{OH}$. Moreover, the combination of Ac-IETD-fmk and Ala, Cit or Pro further improve inhibited efficacy on ROS generation and cytochrome c release in the erythrocytes in the absence of $\bullet\text{OH}$. Our results showed that Ala, Cit and Pro couldn't directly inhibit caspase-8, and 100 μM of Ac-IETD-fmk could completely prevent the caspases-8 activation in the erythrocytes. So, it is impossible that Ala, Cit or Pro inhibits ROS generation and cytochrome c release by protecting against caspase-8 activation in carp erythrocytes in the absence of $\bullet\text{OH}$. These data revealed that Ala, Cit and Pro could directly inhibit cytochrome c release and ROS generation in fish erythrocytes. This is in accordance with the reports that Ala, Cit and Pro effectively blocked the increase in ROS levels in fish erythrocytes [30]. The reasons of quenching ROS might be that Ala, Cit and Pro maintains the activities of enzymatic and non-enzymatic antioxidant. The key enzymes for the detoxification of ROS are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [106, 107]. Glutathione (GSH) is the major non-enzymatic antioxidant [108], which can directly scavenge intracellular ROS [109]. It has been reported that SOD and CAT can inhibit apoptosis in human neutrophils [110]. GSH can protect human T cells from apoptosis [111, 112]. Previous studies from our laboratory showed that Ala, Cit and Pro restores the activities of SOD, CAT, GPx and levels of GSH in carp erythrocytes [30]. This is in good line with the report that Pro maintains the activity of CAT in the ROS-induced yeast cells [113]. These results revealed that except for quenching ROS and preventing cytochrome c release, the way Ala, Cit and Pro affect apoptosis may be explained by their ability to elevate the enzymatic and non-enzymatic antioxidant activity.

In this study, Ala, Cit and Pro conferred protection against caspase-9 activation in carp erythrocytes induced by $\bullet\text{OH}$. Anti-Cyt C suppressed the activation of caspase-9

in the erythrocytes in the absence of •OH. It is worthwhile to note that the combination of Anti-Cyt C and Ala, Cit or Pro didn't further enhanced inhibited effects on caspase-9 in the erythrocytes in the absence of •OH. These displayed that the inhibition of Ala, Cit and Pro on caspase-9 activation was cytochrome c and •OH dependent in the erythrocytes. It has been demonstrated that Ala, Cit and Pro could inhibit cytochrome c release and quench •OH as expression above. So, Ala, Cit and Pro couldn't directly inhibit caspase-9 activation but prevent cytochrome c release and quench •OH in fish erythrocytes. The present results suggested Ala, Cit and Pro prevented caspase-3 activation in carp erythrocytes induced by •OH. The combination of Ac-IETD-fmk and Z-LEHD-fmk suppressed caspase-3 activation in the erythrocytes in the absence of •OH. Interestingly, the combination of Ac-IETD-fmk, Z-LEHD-fmk and Ala, Cit or Pro didn't further enhanced the inhibition on caspase-3 in the erythrocytes in the absence of •OH. Our results showed that 100 µM of Ac-IETD-fmk and Z-LEHD-fmk can completely suppress the activities of caspases-8 and caspase-9 in the erythrocytes. So, the inhibition efficacy of Ala, Cit and Pro on caspase-3 activation was caspase-8, caspase-9 or •OH dependent in the erythrocytes. Nevertheless, it has been indicated that Ala, Cit and Pro couldn't directly inhibit the activation of caspase-8 and caspase-9 in the erythrocytes. So, Ala, Cit or Pro is incapable of inhibiting caspase-3 by protecting against the activation of caspases-8 and caspase-9 in the erythrocytes. But, it has been demonstrated that •OH activates caspases-8, and cytochrome c triggers caspase-9 in fish erythrocytes. And, Ala, Cit and Pro could quench •OH and inhibits cytochrome c release in the erythrocytes as expression above. So, it is possible that Ala, Cit and Pro indirectly inhibit caspase-3 through preventing cytochrome c release and quenching •OH in fish erythrocytes.

In the present study, Ala, Cit and Pro effectively inhibited •OH-induced influx of Ca^{2+} and activation of calpain in fish erythrocytes. It has been shown that amino acids can chelate Ca^{2+} by composing a divalent metal-amino acid complexes [114]. The bidentate salt-bridge structure is the most favorable for all the twenty kinds of amino acids to chelate Ca^{2+} to both the oxygen atoms of the negatively carboxylate group in

the backbone [115]. These results demonstrated that Ala, Cit and Pro could protect erythrocytes against apoptosis through quenching $\bullet\text{OH}$, inhibiting the generation of ROS or release of cytochrome c or chelating Ca^{2+} with subsequent preventing influx of Ca^{2+} and activation of calpain, caspase-8, caspase-9 and caspase-3. This is consistent with the report that Pro suppresses apoptosis in yeast cells and human embryonic kidney cells by decreasing ROS levels [15, 113]. No pervious study has addressed the inhibition of Ala, Cit and Pro on the release of cytochrome c, influx of Ca^{2+} and activation of calpain, caspase-8, caspase-9 and caspase-3 in apoptotic cells.

Hb can generate $\bullet\text{OH}$ by the formation of H_2O_2 and releasing Fe^{2+} in human erythrocytes [24, 116]. Hydroxyl radical has the propensity to oxidize crucial cellular components, such as lipids and proteins [117]. The oxidation of cellular components can cause apoptosis [118]. It has been thought that the HMWPs are formed by the cross-linking of LMWPs and oxidized lipids in membranes [119, 120]. In the present study, Ala, Cit and Pro markedly prevented the $\bullet\text{OH}$ -induced formation of HMWPs and decrease of LMWPs in carp erythrocytes. This demonstrated that Ala, Cit and Pro inhibited the $\bullet\text{OH}$ -induced oxidation of membrane components in fish erythrocytes. $\bullet\text{OH}$ can oxidize Hb in the cytoplasm, which produces methemoglobin (met-Hb) and impairs the function of erythrocytes [24]. The present study exhibited that Ala, Cit and Pro significantly suppressed the $\bullet\text{OH}$ -induced oxidation of Hb in carp erythrocytes. This suggested that Ala, Cit and Pro can quench the oxidation of cytoplasm components in the erythrocytes. This is in good agreement with the previous report that Ala, Cit and Pro inhibited the formation of malondialdehyde (MDA, the oxidative products of lipids), protein carbonyl (PC, the oxidative products of protein) and met-Hb in fish erythrocytes [30]. The oxidative products of lipids and proteins play an important role in the induction of apoptosis in mammalian cells [121-123]. These results indicated that Ala, Cit and Pro could inhibit apoptosis by preventing the oxidation of cellular components in apoptotic cells.

Effects of the combination of Ala, Cit and Pro on $\bullet\text{OH}$ -induced apoptosis

Usually, Ala, Pro and Cit in mixture form are present simultaneously in animal blood [12]. However, there are few studies on the combinative effects of these

nutrients. In this study, the combination of Ala, Pro and Cit showed the strongest effects on decreasing the levels of ROS, cytochrome c and Ca^{2+} , preventing the activation of caspase-3, caspase-8, caspase-9 and calpain, inhibiting PS exposure, DNA fragmentation, cytoskeleton degradation and the oxidation of cellular components and scavenging $\bullet\text{OH}$ among amino acids examined in fish erythrocytes. The results demonstrated that the combination of Ala, Cit and Pro produced a synergistic effect on inhibiting apoptosis and oxidative stress, which was stronger than their individual effects at the same concentrations. No study has addressed the anti-apoptotic synergism of amino acids. As everyone knows, oxidative stress can induce apoptosis [124, 125]. It may be ascribed to the synergistic effects on preventing oxidative stress. Marcuse (1960) have indicated that amino acids are synergistic antioxidants, and the mechanism of synergistic effects can be explained by the chelation of pro-oxidative metal traces and by regeneration of oxidized primary antioxidants [126]. Nevertheless, the reason for the anti-apoptotic synergism of Ala, Cit and Pro is still unclear. The detailed mechanism needs further research.

The previous studies have shown that Gln improves the antioxidant defenses in patients [3-5, 127] and attenuates apoptosis in experimental rat [6-8, 128]. The enterocytes in the small intestine convert the most of Gln in diet and plasma to Ala, Cit and Pro that are released into the blood circulation in Gln-supplementation mammalian [9-12]. After entering the circulation, Cit is transported to the kidneys where it is converted to arginine (Arg) in human [129, 130]. About 80-90% of Cit is derived from Gln in the gut, and about 15% of Arg appearance is via the conversion of Cit in the kidneys [131, 132]. Citrulline is the precursor of Arg [133]. Studies suggested that Arg can scavenge ROS in rat hearts [134] and abrogate oxidative stress in experimental mice [135, 136] and patient [137]. Fujita and Yanaga (2007) reported that the levels of Ala, Cit and Pro are 0.258-0.327 mM, 0.019-0.056 mM and 0.124-0.141 mM in human plasma, respectively [12]. The present studies displayed that Ala, Cit, Pro and their combination at the physiological concentrations effectively protected against the release of cytochrome c, generation of ROS and activation of caspases in fish erythrocytes. The combination of Ala, Cit and Pro produced a

synergistic effect of inhibited oxidative stress and apoptosis in fish erythrocytes. Moreover, the anti-oxidative and anti-apoptotic effects of the combination of Ala, Pro and Cit are equivalent to or stronger than that of Gln in fish erythrocytes [30]. So, it is reasonable to conclude that the anti-oxidative and anti-apoptotic effects of Gln are at least partly depended on its metabolites *in vivo*, which provide one novel explanation for the working mechanism of glutamine in mammalian.

Conclusion

In summary, our study firstly showed two distinct apoptosis pathways in fish erythrocytes. One is that •OH activates the caspase-8 by triggering CD95, which results in the generation of ROS and the release of cytochrome c with subsequent caspase-9 and caspase-3 activation that stimulates PS exposure and DNA fragmentation. The other is that •OH causes the influx of Ca^{2+} that activates scramblase with subsequent PS exposure, and initiates calpain activity with subsequent the cytoskeleton degradation and DNA fragmentation in fish erythrocytes. Meanwhile, we found that the apoptotic pathways in fish erythrocytes were similar to those in mammal erythrocytes and nucleated cells. This revealed that fish erythrocytes may be used as a novel model for studying oxidative stress and apoptosis in mammal cells. Moreover, our data demonstrated for the first time that Gln's metabolites, Ala, Cit and Pro, protected erythrocytes against apoptosis by scavenging •OH, decreasing levels of ROS and Ca^{2+} and the release of cytochrome c, with subsequent inhibiting activation of caspase-3, caspase-8, caspase-9 and calpain and preventing the oxidation of cellular components. The combination of Ala, Cit and Pro produced a synergistic effect of inhibited oxidative stress and apoptosis. This study firstly indicated that the anti-oxidative and anti-apoptotic effects of Gln were at least partly depended on its metabolites *in vivo*, which gave a basis for revealing the underlying mechanisms of Gln in mammalian, and supported the use of Gln, Ala, Cit and Pro as oxidative stress and apoptosis inhibitors in mammal cells.

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Data article

Title: Data in the activities of caspases and the levels of reactive oxygen species and cytochrome c in the •OH-induced fish erythrocytes treated with alanine, citrulline, proline and their combination

Authors: Huatao Li ^{a, e}, Lin Feng ^{a, b, c}, Weidan Jiang ^{a, b, c}, Yang Liu ^{a, b, c}, Jun Jiang ^{a, b, c}, Yongan Zhang ^d, Pei Wu ^{a, b, c}, Juan Zhao ^{a, b, c}, Xudong Duan ^{a, b, c}, Xiaoqiu Zhou ^{a, b, c, *}

Affiliations:

^a Animal Nutrition Institute, Sichuan Agricultural University, Sichuan, Chengdu, 611130, China

^b Fish Nutrition and Safety Production University Key Laboratory of Sichuan Province, Sichuan Agricultural University, Sichuan, Chengdu, 611130, China

^c Key Laboratory for Animal Disease-Resistance Nutrition of Ministry of Education, Sichuan Agricultural University, Sichuan, Chengdu, 611130, China

^d *Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China*

^e *Conservation and Utilization of Fishes resources in the Upper Reaches of the Yangtze River Key Laboratory of Sichuan Province, Neijiang Normal University, Sichuan, Neijiang, 641000, China*

* Corresponding authors.

Contact email: xqzhouqq@tom.com, zhouxq@sicau.edu.cn (Xiaoqiu Zhou).

Abstract

The present study explored the effects of alanine (Ala), citrulline (Cit), proline (Pro) and their combination (Ala₁₀Pro₄Cit₁) on the activities of caspases and levels of reactive oxygen species (ROS) and cytochrome c in hydroxyl radicals (•OH)-induced carp erythrocytes. The data displayed that •OH induced the increases in the activities of caspase-3, caspase-8 and caspase-9 and the levels of ROS and cytochrome c in carp erythrocytes. However, Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ effectively suppressed the •OH-induced increases in the activities of caspase-3, caspase-8 and caspase-9 and the levels of ROS and cytochrome c in carp erythrocytes. Furthermore, the activities of caspase-3, caspase-8 and caspase-9 and the levels of ROS and cytochrome c were gradually decreased with increasing concentrations of Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ (0.175-1.400 mM) in the •OH-induced carp erythrocytes. These data demonstrated that the 50% inhibitory doses (ID₅₀) of Ala₁₀Pro₄Cit₁ on the activities of caspase-8, caspase-9 and caspase-3 and levels of ROS and cytochrome c were respectively estimated to be the minimum values among amino acids examined so far. The 5% inhibitory doses (ID₅) of Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on the activities of caspase-8, caspase-9 and caspase-3 and levels of ROS and cytochrome c were estimated to be at their physiological concentrations in mammalian. Our research article for further interpretation and discussion from these data in Ref.[1].

Specifications Table

Subject area	<i>Biology</i>
More specific subject area	<i>Nutritional Medicine</i>
Type of data	<i>Table, figure</i>
How data was	<i>The commercial kit, microplate reader, fluorescence</i>

acquired	<i>spectrophotometer, SPSS 13.0 for Window software</i>
Data format	<i>Analyzed</i>
Experimental factors	<i>The isolated carp erythrocytes were suspended in physiological carp saline (PCS) (control) and PCS containing 0.000, 0.175, 0.350, 0.700 or 1.400 mM of alanine, citrulline, proline and their combination at a 1% hematocrit, respectively. All erythrocyte suspensions above were pre-incubated at 37 °C for 1.5 h. FeSO₄/H₂O₂ was then added at a final concentration of 40 μM/20 μM for incubation at 37 °C for 9 h, except in the control.</i>
Experimental features	<i>The measurement of the activities of caspase-3, caspase-8 and caspase-9 and the levels of reactive oxygen species and cytochrome c were performed using the commercial kits.</i>
Data source location	<i>Chengdu, Sichuan, China</i>
Data accessibility	<i>Data are presented in this article</i>

Value of the data

1. The data provide the information of the effect of different concentrations of alanine (Ala), citrulline (Cit), proline (Pro) and their combination (Ala₁₀Pro₄Cit₁) on the activities of caspases and levels of reactive oxygen species and cytochrome c in hydroxyl radicals-induced carp erythrocytes.
2. This data would be valuable for further studies of oxidative stress and apoptosis in fish erythrocytes.
3. The data support the development of further experiments on the use of Ala, Cit and Pro as oxidative stress and apoptosis inhibitors in fish cells.
4. This data could give a basis for further experiments on revealing the underlying mechanisms of anti-oxidation and anti-apoptosis of glutamine in mammalian.

Data

In the data, the effects of alanine (Ala), citrulline (Cit), proline (Pro) and their combination (Ala₁₀Pro₄Cit₁) at different concentrations on caspases activities and reactive oxygen species (ROS) and cytochrome c levels in •OH-induced carp erythrocytes were presented in Fig. 1, 2, 3, 4 and 5. Compared with the control, the activities of caspase-3, caspase-8 and caspase-9 and levels of ROS and cytochrome c

was significantly increased in carp erythrocytes exposed to $\bullet\text{OH}$. However, Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ significantly inhibited the $\bullet\text{OH}$ -induced increase in the activities of caspases and levels of ROS and cytochrome c in carp erythrocytes. The activities of caspases and levels of ROS and cytochrome c were gradually decreased with increasing concentrations of Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ (0.175-1.400 mM) in the $\bullet\text{OH}$ -induced carp erythrocytes.

Fig. 1. Effects of Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ on the activity of caspase-3 in the $\bullet\text{OH}$ -induced carp erythrocyte. The data represent the means $\pm$ S.D. of four replicates.

Fig. 2. Effects of Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ on the activity of caspase-8 in the $\bullet\text{OH}$ -induced carp erythrocyte. The data represent the means $\pm$ S.D. of four replicates.

Fig. 3. Effects of Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on the activity of caspase-9 in the •OH-induced carp erythrocyte. The data represent the means $\pm$ S.D. of four replicates.

Fig. 4. Effects of Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on the levels of cytochrome c in the •OH-induced carp erythrocyte. The data represent the means $\pm$ S.D. of four replicates.

Fig. 5. Effects of Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on the levels of ROS in the •OH-induced carp erythrocyte. The data represent the means $\pm$ S.D. of four replicates.

The data in Table 1 showed that when the erythrocytes were treated with Ala₁₀Pro₄Cit₁ in the presence of •OH, the 50% inhibitory doses (ID₅₀) on the activities

of caspase-3, caspase-8 and caspase-9 and levels of ROS and cytochrome c were estimated to be 0.35, 0.60, 0.61, 0.53 and 1.01 mM that are the minimum values among amino acids examined so far.

Table 1

ID₅₀ of Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on the activities of caspase-8, caspase-9 and caspase-3 and the levels of cytochrome c and ROS in the •OH-induced carp erythrocytes.

<i>Treatment</i>	<i>Caspase-8 (mM)</i>	<i>Caspase-9 (mM)</i>	<i>Caspase-3 (mM)</i>	<i>Cytochrome c (mM)</i>	<i>ROS (mM)</i>
Ala	3.39±0.24 ^c	3.97±0.22 ^d	0.95±0.06 ^c	2.35±0.16 ^d	2.06±0.13 ^c
Cit	1.36±0.06 ^b	2.52±0.13 ^c	0.67±0.04 ^b	2.16±0.12 ^c	1.28±0.06 ^b
Pro	1.37±0.08 ^b	1.36±0.07 ^b	1.24±0.09 ^d	1.33±0.09 ^b	4.78±0.23 ^d
Ala ₁₀ Pro ₄ Cit ₁	0.60±0.04 ^a	0.61±0.04 ^a	0.35±0.02 ^a	1.01±0.06 ^a	0.53±0.03 ^a

Data represent means ± S.D. of four replicates. Values within the same column with different superscripts are significantly different ($p<0.05$).

The data in Table 2 showed that the 5% inhibitory doses (ID₅) of Ala₁₀Pro₄Cit₁ on the activity of caspase-8 and level of cytochrome c were respectively estimated to be 0.026 and 0.028 mM, the ID₅ of Ala₁₀Pro₄Cit₁ and Cit on the activity of caspase-3 or level of ROS were respectively estimated to be 0.024 and 0.025 mM or 0.041 and 0.047 mM, the ID₅ of Cit on the activity of caspase-9 was estimated to be 0.021 mM, which are the minimum values among amino acids examined so far.

Table 2

ID₅ of Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on the activities of caspase-8, caspase-9 and caspase-3 and the levels of cytochrome c and ROS in the •OH-induced carp erythrocytes.

<i>Treatment</i>	<i>Caspase-8 (mM)</i>	<i>Caspase-9 (mM)</i>	<i>Caspase-3 (mM)</i>	<i>Cytochrome c (mM)</i>	<i>ROS (mM)</i>
Ala	0.064±0.004 ^c	0.079±0.005 ^d	0.044±0.003 ^c	0.102±0.006 ^c	0.092±0.006 ^b
Cit	0.048±0.003 ^b	0.021±0.001 ^a	0.025±0.002 ^a	0.064±0.004 ^b	0.047±0.003 ^a
Pro	0.100±0.005 ^d	0.071±0.004 ^c	0.030±0.002 ^b	0.068±0.005 ^b	0.107±0.008 ^c
Ala ₁₀ Pro ₄ Cit ₁	0.026±0.002 ^a	0.032±0.002 ^b	0.024±0.001 ^a	0.028±0.002 ^a	0.041±0.003 ^a

Data represent means ± S.D. of four replicates. Values within the same column with different superscripts are significantly different ($p<0.05$).

Experimental Design, Materials and Methods

Chemicals

L-Ala, L-Cit and Pro were purchased from Sigma (St. Louis, MO, USA). The aqueous solution of H₂O₂ (30%) and FeSO₄ (analytical pure) were purchased from Shanghai Chemical Reagent Factory (Shanghai, China). All other chemicals were analytical grade. All water used was Milli-Q grade. Physiological carp saline (PCS) (contained 141.1 mM NaCl, 1.43 mM KCl, 0.99 mM CaCl₂, 2.64 mM NaHCO₃ and 6.16 mM glucose) modified to give a total osmolarity of 280 mosm L⁻¹ and pH 7.9 were prepared in our laboratory.

Erythrocyte isolation

The procedures of cell isolation were based on that described by Phillips et al. (2000) [2] with slight modifications. Individual healthy carp (*Cyprinus carpio* var. Jian) (100–110g) was anaesthetized, and the blood (approximately 1 ml) was drawn into a syringe via caudal puncture. The blood was placed in a chilled centrifuge tube containing heparinized (40 i.u.ml⁻¹) PCS. The blood from three different carp was pooled and centrifugated (Thermo, Waltham, MA, USA) at 900 × g and 4 °C for 10 min. Then, the plasma and buffy coat of pooled blood were removed in the centrifuge tube. After washing three times with PCS, the isolated erythrocytes were resuspended in PCS at a 1% hematocrit in following experiments. All procedures were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University in accordance with the Institutional Ethics Committee of the Chinese Institute of Chemical Biology guidelines.

Erythrocyte treatments

Erythrocyte treatments were based on the method described by Li et al. (2013) [3] with slight modifications. Alanine, Cit and Pro were combined in the molar ratio 10: 4: 1 (Ala₁₀Pro₄Cit₁) for investigating their mixture effects in the erythrocytes. Briefly, the isolated erythrocytes were suspended in PCS (control) and PCS containing 0.000, 0.175, 0.350, 0.700 or 1.400 mM of Ala, Cit, Pro or Ala₁₀Pro₄Cit₁ at a 1% hematocrit, respectively. All erythrocyte suspensions above were pre-incubated at 37 °C for 1.5 h. FeSO₄/H₂O₂ was then added at a final concentration of 40 μM/20 μM, except in the control. After incubation at 37 °C for 9 h, the erythrocytes were collected for measurement of the activities of caspase-3, caspase-8 and caspase-9 and the levels of

ROS and cytochrome c. The experiment was performed with 4 replicates per treatment and the control.

Measurement of caspases

The activities of caspase-3, caspase-8 and caspase-9 were determined by measuring cleavage of the Ac-DEVD-pNA (caspase-3 substrate), Ac-IETD-pNA (caspase-8 substrate) and Ac-LEHD-pNA (caspase-9 substrate) to pNA using the detection kits (Beyotime, Nantong, China) as described previously [4, 5]. The caspase activity in the samples was quantified with a Microplate reader (Thermo, Waltham, USA) at 405 nm.

Measurement of ROS

ROS levels in the erythrocytes were determined by measuring the oxidative conversion of 2', 7'-dichlorofluorescein diacetate (DCFH-DA) to fluorescent compound DCF using a detection kit (Beyotime, Nantong, China) as described previously [6-8]. DCF fluorescence in the erythrocytes was determined at 485 nm excitation and 520 nm emission using fluorescence spectrophotometer (Thermo, Waltham, USA).

Measurement of cytochrome c

The cytosolic proteins and mitochondria in fish erythrocytes were isolated by a commercial extraction kit (Beyotime, Nantong, China) as described previously [9]. The cytochrome c release from mitochondria in cytosol was evaluated by an enzyme-linked immunosorbent assay (ELISA) kit (ElabScience, Wuhan, China) [10]. Absorbance value in the samples was measured with the Microplate reader at 450 nm.

Protein measurements and statistical analysis

Protein concentration in the hemolysate was measured by Darbkin method [11]. All data are presented as means $\pm$ S.D. ($n = 4$). The data were subjected to one-way analysis of variance (ANOVA). If significant differences were found ($P < 0.05$), Duncan's multiple range tests were used to rank the groups [12]. The 50% and 5% inhibitory doses (ID_{50} and ID_5) were determined by the probit analysis [3]. All statistical analyses were performed using the SPSS 13.0 for Window software (SPSS, Chicago, IL, USA) [13].

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Figure and Table

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Fig. 1. Effects of inhibitor, Ala, Cit and Pro on the activities of caspase and the levels of cytochrome c and ROS in the erythrocyte after exposing to •OH for 7h. (A) Effects of Ac-IETD-fmk, Ala, Cit and Pro on caspase-8 activity. (B) Effects of Z-LEHD-fmk, Anti-Cyt C, Ala, Cit and Pro on caspase-9 activity. (C) Effects of Ac-IETD-fmk, Z-LEHD-fmk, Ala, Cit and Pro on caspase-3 activity. (D) Effects of Ac-IETD-fmk, Ala, Cit and Pro on the cytochrome c level. (E) Effects of Ac-IETD-fmk, Ala, Cit and Pro on the ROS level. The data represent the means $\pm$ S.D. of four replicates. Values with different superscripts are significant different ($P < 0.05$). Asterisks indicate significant differences from control (t -test, $P \leq 0.05$).

Fig. 2. Effects of zVAD-fmk, Ca^{2+} -free PCS, Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on the levels of ROS in $\bullet\text{OH}$ -induced carp erythrocyte as imaged by the fluorescent microscope. This experiment was repeated four times with similar results achieved.

Fig. 3. Effects of zVAD-fmk, Ca^{2+} -free PCS, Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on TUNEL in the $\bullet\text{OH}$ -induced carp erythrocyte. This experiment was repeated four times with similar results achieved.

Fig. 4 Effects of zVAD-fmk, Ca^{2+} -free PCS, Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ on annexin binding in the $\bullet\text{OH}$ -induced carp erythrocyte as imaged by the fluorescent microscope. This experiment was repeated four times with similar results achieved.

Fig. 5. Effects of Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on the modification of membrane proteins in the •OH-induced carp erythrocytes. In SDS-PAGE, lane M: marker, lane 1: the intact erythrocyte membrane proteins, lane 2: the erythrocytes were induced with •OH, lanes 3, 4, 5, 6 and 7: the •OH-induced erythrocytes were respectively incubated with Gln, Ala, Cit, Pro and Ala₁₀Pro₄Cit₁, HMWP: high-molecular-weight proteins. This experiment was repeated four times with similar results achieved.

Fig. 6. Effects of Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on Hb oxidation in the •OH-induced carp erythrocytes. (A) The absorption spectra of Hb. This experiment was repeated four times with similar results achieved. (B) The kinetics of Hb oxidation. This experiment was repeated four times with similar results achieved. (C) The delayed times of Hb oxidation (ΔT). These data represent the means $\pm$ S.D. of four replicates. Values with different superscripts are significant

1627 different ($P < 0.05$).

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Fig. 7. Effects of Ala, Cit, Pro and Ala₁₀Pro₄Cit₁ on the anti-hydroxyl radical (AHR) activity in the •OH-induced carp erythrocytes. The data represent the means $\pm$ S.D. of four replicates. Values with different superscripts are significant different ($P < 0.05$).

Table 1

Effects of zVAD-fmk, Ca^{2+} -free PCS, Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ on the activities of caspase-3, caspase-8 and caspase-9 and the levels of cytochrome c and ROS in the $\bullet\text{OH}$ -induced carp erythrocytes.

<i>Treatment</i>	<i>Caspase-3</i> (% Control)	<i>Caspase-8</i> (% Control)	<i>Caspase-9</i> (% Control)	<i>Cytochrome c</i> (% Control)	<i>ROS</i> (% Control)
Control	100.00±0.00 ^a	100.00±0.00 ^a	100.00±0.00 ^a	100.00±0.00 ^a	100.00±0.00 ^a
$\bullet\text{OH}$	212.19±10.03 ^d	354.27±21.57 ^f	325.12±16.09 ^e	348.68±15.74 ^f	815.86±58.21 ^f
$\bullet\text{OH}$ + zVAD-fmk	104.12±6.16 ^a	129.05±7.10 ^b	109.82±7.75 ^a	161.13±9.98 ^b	280.65±17.98 ^b
$\bullet\text{OH}$ + Ca^{2+} -free PCS	209.03±11.14 ^d	348.53±19.33 ^f	323.17±18.33 ^e	346.22±18.75 ^f	821.57±55.89 ^f
$\bullet\text{OH}$ + Ala	149.86±8.04 ^c	302.29±15.58 ^e	268.88±10.44 ^d	249.73±12.56 ^e	534.23±37.78 ^d
$\bullet\text{OH}$ + Cit	146.17±11.20 ^c	246.94±14.40 ^d	260.62±10.25 ^d	246.74±16.75 ^e	475.87±36.01 ^c
$\bullet\text{OH}$ + Pro	156.18±7.96 ^c	239.80±13.18 ^d	225.63±12.18 ^c	223.86±10.08 ^d	621.78±32.71 ^e
$\bullet\text{OH}$ + $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$	118.21±9.11 ^b	170.72±8.44 ^c	146.92±5.98 ^b	205.38±13.73 ^c	256.21±14.81 ^b

Data represent means $\pm$ S.D. of four replicates. Values within the same column with different superscripts are significantly different ($p < 0.05$).

1700 Table 2

1701 Effects of zVAD-fmk, Ca^{2+} -free PCS, Ala, Cit, Pro and $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$ on Ca^{2+} levels and calpain
1702 activity in the $\bullet\text{OH}$ -induced carp erythrocyte.

<i>Treatment</i>	<i>Ca^{2+} (% Control)</i>	<i>Calpain (% Control)</i>
Control	100.00±0.00 ^a	100.00±0.00 ^a
$\bullet\text{OH}$	226.76±14.59 ^d	225.49±12.25 ^c
$\bullet\text{OH}$ + zVAD-fmk	221.32±16.55 ^d	218.81±11.66 ^c
$\bullet\text{OH}$ + Ca^{2+} -free PCS	104.68±7.07 ^a	92.99±5.18 ^a
$\bullet\text{OH}$ + Ala	149.86±12.53 ^b	151.97±10.97 ^c
$\bullet\text{OH}$ + Cit	188.34±12.09 ^c	196.70±14.48 ^d
$\bullet\text{OH}$ + Pro	152.09±7.69 ^b	152.14±7.72 ^c
$\bullet\text{OH}$ + $\text{Ala}_{10}\text{Pro}_4\text{Cit}_1$	114.90±8.40 ^a	112.94±4.98 ^b

1703 Data represent means ± S.D. of four replicates. Values within the same column with different
1704 superscripts are significantly different ($p<0.05$).

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