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The Effect of ACE Inhibitory Peptides with Aortic Endothelial Cells Function Factors of Rat Derived from Fermented Pork

Zhu-lian Zhao[1*]
Cai-qiong Zhou[1,2]
Rong Chang[1]
Yue-ying Xie[1]
Miao Ding[1]

[1]*Food Science College, Southwest University, Chongqing, 400716, China*
[2]*Chongqing Engineering Research Centre of Regional Food, Chongqing, 400716, China*

Abstract

ACE inhibitory peptides with less than 10 KDa were prepared by using natural lactic acid fermented pork as materials in Southwest China. The effects of ACE inhibitory peptide on vascular endothelial cell function were studied by primary culture and subculture of rat thoracic aorta endothelial cells. Result displayed that the content of peptide was 86.54%, and IC_{50} was 0.895 mg/mL. The hydrophobic amino acids, aromatic amino acids, and branched chain amino acids of the peptides were 39.35%, 10.69%, and 13.65%. The experimental group showed that the cell survival rate in each dose group was above 80%, which indicated that the concentration of each group was suitable. So ACE inhibitory peptides from fermented pork had no obvious toxic effect on vascular endothelial cells in a certain concentration range. These inhibitory peptides could increase the release of NO in endothelial cells and the high dose group was better than the low and medium dose group, but less than the positive control group. Besides, it also could reduce **endothelin-1** secretion with the high and medium dose group better than the low dose group but less than the positive control group. No significant in vitro antioxidant capacity and cell reactive oxygen species clearance was observed. The inhibition mechanism of ACE inhibitory peptides from fermented pork may promote vascular endothelial cells to release NO and reduce **endothelin-1** secretion.

Keywords: Fermented pork, ACE inhibitory peptides, Nitric oxide, **Endothelin-1**, Reactive oxygen species.

Abbreviations: ACE: angiotensin converting enzyme; $IC_{50:}$ half maximal inhibitory concentration; NO: nitric oxide; ET-1: **endothelin-1**; ROS: reactive oxygen species.

Introduction

Hypertension is a clinical syndrome that is characterized by increased artery systolic or diastolic blood pressure, which is often associated with diseases of the heart, blood vessels, brain, kidneys, and other organs. Hypertension is a serious hazard to human health, and about 45% of cardiovascular disease occurrence and mortality are associated with hypertension [1]. Vascular endothelial cell (VEC) is an endocrine and effector organ of the human body with important physiological function. The function of VEC is incomplete, and its disorder relates with cardiovascular disease, especially hypertension. Endothelial cells secrete endothelial-derived relaxing factors, such as nitric oxide. Endothelium-derived hyperpolarizing factor and prostacyclin can inhibit

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***Corresponding author:** Cai-qiong Zhou, College of Food Science, Southwest University, No.2 Tiansheng Road, Bei Bei District, Chongqing, 400715, PR China, Tel: +86-023-68251298; Email: zhoucaiqiong(at)swu.edu.cn

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the proliferation of vascular smooth muscle cells, reduce oxidative stress, and expand blood vessels. The secretion of endogenous shrinkage factors, such as endothelin, angiotensin II, thromboxane A2, and superoxide anion, can promote vasoconstriction and resist diastolic function [2]. The relaxation and contraction of vascular endothelial cells are balanced to maintain blood pressure stability. Instable blood pressure will lead to the emergence of hypertension and other diseases.

Angiotensin-converting enzyme (ACE) is the key enzyme that regulates renin-angiotensin system and Kallikrein-Kinin system. ACE can function in vascular tension element I and bradykinin, engender vasoconstrictor activity, and cause a rise in blood pressure [2]. Common synthetic antihypertensive drugs, such as captopril and enalapril, often accompany dry cough, taste disturbance, rash, and organ dysfunction when taken for a long time. The inhibition of ACE activity has a positive effect on reducing blood pressure [3]. Foodborne ACE inhibitory peptides are favored due to its organic contents, absence of side effects, and safety.

Different structures of ACE inhibitory peptides have been isolated from plant protein [4], animal protein [5], algae protein [6], dry fermented pickled ham [7], cheese [8], and fermented bean curd [9]. Pork is a widely consumed meat food of Chinese people. Many peptide fragments with different ACE inhibitory activities exist after the hydrolysis of porcine skeletal muscle protein [10]. Chinese traditional fermented pork is a meat product fermented with lactic acid. Fresh pork is added to rice flour, salt, and other ingredients and cultured via microbial anaerobic fermentation. Chinese traditional fermented pork is popular in Chongqing, Guizhou, Hunan, Guangxi, and other places [11]. Studies rarely reported on the function of fermented pork. Ye Chun [12] reported that the peptides from fermented pork had good scavenging capacity to 1,1- two phenyl-2- picryl hydrazine radical (DPPH) free radicals, hydroxyl free radicals, and nitrite. In this experiment, we intended to use the cells isolated from rat thoracic aorta endothelium and cultured in primary culture and subculture. We also studied the effect of ACE inhibitory peptides that were separated and purified from homemade fermented pork on the functional factors of NO, ET, and reactive oxygen species. We researched the mechanism of lowering the blood pressure with fermented pork ACE inhibitory peptides and provided relevant functional chemistry research data for the development of traditional lactic acid-fermented pork products.

Materials and Methods

Materials

Phosphoric acid, sulfanilic acid, ethanol, sodium nitrite, and potassium ferricyanide were analytically pure and were purchased from Chengdu Kelong Chemical Reagent Factory (Chengdu, China). N-1-naphthalene ethylene diamine hydrochloride was purchased from Tianjin Chemical Reagent Institute Co. Ltd (Tianjin, China). DMEM high-glucose medium and trypsin EDTA solution (0.25%) were purchased from HyClone (Logan, USA). DPPH was purchased from Ladder and Love (Chemical Industry Development Co. Ltd, Shanghai, China). 2',7'-double fluorescent yellow two chloro acetate were purchased from Sigma (San Francisco, USA). Captopril tablets were purchased from Shanxi Yun Peng Pharmaceutical Co. Ltd. (Shanxi, China). Fetal bovine serum was purchased from NQBB Australia (Australia). Penicillin streptomycin solution was purchased from Beyotime Biotechnology Research (Haimen, China). The 25 cm^2 culture bottle was purchased from Corning Company (Corning, USA). Endothelin-1 test kit was purchased from Nanjing Jincheng Biological Engineering Institute (Nanjing, China). SD rats: male, weight 180-200 gm, clean grade. Chongqing Teng Xin Bill animal Sales Co., experimental animal license No.: SCXK (Chongqing) 2013-0008.

Cells with CO_2 incubator were purchased from the Thermos Fisher Scientific Company. Clean bench was purchased from Sujing Group Suzhou Antai Air Technology Co. Ltd. 5702R type centrifuge was purchased from Germany Eppendorf. Full-wavelength enzyme standard instrument was purchased from American gene. Biological microscope was purchased from Olympus. 722 can see spectrophotometer was purchased from Shanghai Chaojing Science and Technology Co., Ltd.

Preparation of ACE inhibitory peptides from fermented pork

Fresh hind pork leg meat was washed and water drained. Meat cut into 3 cm×5 cm×0.4 cm pieces, salt added, stir fried until the rice ground was golden yellow (20 mesh sieve). Pork, rice, and salt were added according to the following ratio: 85:10:5. Fresh bamboo leaves were kneaded and used to plug the altar mouth, which was sealed with water. The sample was then fermented under the condition of 15 °C. ACE inhibitory peptides were prepared by ultrafiltration, macro porous resin, dextran gel, and reversed phase high performance liquid chromatography in 20 days.

Primary culture and subculture of vascular endothelial cells

Referring to Qu Hailong and Hu S [13,14] healthy male SD rats (200-250 gm) were killed, and their skin was cut along the midline of the abdomen. The thoracic aorta was placed in 4 degrees of sterile PBS solution. Extravascular connective tissue was removed. Fat was removed with microsurgical forceps, and vessels were washed with PBS solution repeatedly. Tiny arterial branches were removed with ophthalmic scissors and replaced with a new glass garden and 4 degrees of PBS solution in a sterile operation table. The vascular ring was cut wide open for 2-3 mm with ophthalmic scissors and then transferred to a 25 cm^2 culture flask. About 15-20 vascular rings were inoculated in each culture flask and in 2 mL of DMEM culture medium (containing 20% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin). The medium did not spill over the vascular rings, and the vascular rings were uniformly distributed on the bottom of the bottle. The bottle was positioned at 37 °C and 5% CO_2 (V/V) static culture box for 60 hrs. PBS was used to wash the vascular rings. The medium was replaced every 2-3 days.

When primary cultured cells grew to 80%-90% confluence, they were washed twice with sterile PBS, and 0.25% trypsin was added to digest the rings for 1 min. Other fluids containing fetal bovine serum were added to DMEM culture medium to stop the digestion immediately. The mixture was blown and mixed gently and then centrifuged for 5 min in 1100 r/min. The supernatant liquid was discarded, and 2 mL of new DMEM was added to the cell suspension. Cells were counted under the microscope and sub-cultured according to 1:3, in accordance with the amount of cells.

Experimental grouping and processing

Afterward, 1.0×10^5 cells/mL of cell suspension were confected with the third generation of cells and inoculated in 24 holes, with 1 mL of cells per plate. The old culture liquid was discarded and replaced with serum-free medium until the cell confluence grew to 80%. The ACE inhibitory peptides and captopril (50 μ L) liquid medium were replaced after 12 hrs of culture to synchronize cells. The experimental groups were cultured for 12, 24, and 48 h and counted with 0.4% Trypan blue. After incubation, cell viability was determined [15]. The supernatant was obtained to determine the content of each indicator. Experimental grouping was as follows:

A. Blank control group: without any drugs added;

B. Low-dose group: the final concentration of ACE inhibitor was 0.40 mg/mL;

C. Middle-dose group: the final concentration of ACE inhibitor was 0.80 mg/mL;

D. High-dose group: the final concentration of ACE inhibitor was 1.60 mg/mL;

E. Positive control group: the final concentration of captopril was 0.15 mg/mL.

Index analysis

A. Determination of NO in cell culture fluid: Griess reagent method [16] was used. About 100 μ L of supernatant was added to each experimental group in 96 plates. Then, 100 μ L of Griess reagent was prepared with 0.1% N-1-naphthalene ethylene diamine hydrochloride with ultrapure water and 1% sulfanilic acid with 5% phosphoric acid and then mixed with equal volume. Absorbance values were measured using an enzyme standard instrument at 540 nm after room temperature reaction for 10 min. Subsequently, 100 μ L of fresh culture medium was added to replace the experimental group as the experimental blank control. The $NaNO_2$ standard solution (100 μ L) concentrations were 100, 80, 60, 40, and 20 μ mol/L in 96-well plates. Then, 100 μ L of Griess reagent was added, and the absorbance values were measured using an enzyme standard instrument at 540 nm after room temperature reaction for 10 min. The $NaNO_2$ standard solution was replaced with 100 μ L of ultrapure water to be used as the blank control. $NaNO_2$ concentration was used as the horizontal coordinate and the absorption value was used as the vertical coordinate to make the standard curve. The regression equation was $y=0.0047x+0.0131$, $R^2=0.9971$. According to the standard curve, the content of NO_2 was calculated to reflect the content of NO in the cell culture medium.

B. Determination of ROS in cells [17] After 12 h of cell culture synchronization, the cells were treated with different experimental group drugs (50 μ L) and were cultured for 12, 24, and 48 h. The DCFH-DA fluorescent probe was diluted to 10 μ mol/L with no serum medium after the culture, and 100 μ L of probe was added to each cell. No serum medium was added to the probe after incubating in 37 °C for 20 min. The fluorescence intensity was detected by fluorescent enzyme. The excitation wavelength was 488 nm, and the emission wavelength was 525 nm. The relative intensity of each control group was 100%, and the relative fluorescence intensity of the experimental group was as follows: (fluorescence intensity of the experimental group/ fluorescence intensity of blank control group) × 100%.

C. Contents of ET in cell: determined by endothelin-1 test kit.

D. Determination of in vitro antioxidant capacity

DPPH free radical scavenging activity: This step was conducted according to Ma Haile's method [18] with slight modification. About 100 μ L of ACE inhibitory peptides was absorbed with different concentrations of up to 96 orifice meter. Afterward, 100 μ L of 0.1 mmol/L DPPH anhydrous ethanol solution was added. The mixture was mixed evenly and placed in the dark for 30 min. Absorbance A_1 was measured in 517 nm. The blank group was replaced by equal volume of ethanol instead of DPPH, and the absorbance value was recorded as A_0. The control group was replaced by equal volume of pure water instead of the sample solution, and the absorbance was recorded as A_2. The DPPH free radical scavenging rate was calculated by the following formula:

DPPH scavenging rate (%) = $[1-(A_1-A_0)/A_2] \times 100\%$

Determination of reducing power [19]: 1.0 mL of different concentrations of F3 group was absorbed. Subsequently, 1.0 mL of phosphate buffer solution with concentration of 0.2 mol/L, pH of 6.6, and 1.0 mL potassium ferricyanide solution (1% w/v) was added and mixed fully. Water bath at 50 °C was conducted for 20 min. Then, 1.0 mL of 10% tri-chloroacetic acid solution was added. The supernatant was obtained after centrifugation in 3000 r/min for 10 min. Afterward, 2.0 mL of ultrapure water and 1.0 mL of $FeCl_3$ solution (0.1%, w/v) were added. The absorption value was 700 nm after mixing the static set for 10 min. Subsequently, 1.0 mL of ultrapure water was used instead of the sample as the blank tube zero set. Ascorbic acid was used as control. The ability to absorb light at 700 nm was the size of the reduction ability. The greater the absorbance value, the stronger the ability of the antioxidant.

Amino acid composition and peptide content analysis

A. Peptide content analysis: By using the biuret method, the standard curve with Gly–Gly–Tyr–Arg peptides were drawn [20];

B. Amino acid composition analysis

Hydrolysis sample pretreatment: About 0.1200 g of the sample was added to the test tube, which was filled with 10

mL of 6 mol/L of HCl, and then oscillated. The sample was attenuated to 4-6 mm under 1/3 with alcohol burner tube. The test tube was sealed after vacuum pumping for 10 min. The treated test tube was placed in a constant temperature box in 110 ± 1 °C to sand hydrolysis for 22 h and cooled to room temperature. The test tube was shook and filtrated. Afterward, 1 mL of the filtrate was added into a 50 mL beaker. The filtrate was evaporated with a constant-temperature water bath in 60 °C. Then, 0.02 mol L HCl was added to dilute the filtrate four times. The sample was filtered with 0.22 um membrane for machine analysis.

Free sample pretreatment: About 0.2700 g of the samples was extracted and added to the 2 mL plastic centrifuge tube. Ultrasonic extraction was conducted for 30 min after adding 1 mL of 6% salicylic acid solution. Ultrasonic extraction was conducted for 30 min after static placement for 12 h. The sample was centrifuged in 16000 r/min for 5 min and filtered with 0.22 um membrane to machine analysis after diluting five times.

Analysis conditions: Amino acid composition was determined by automatic amino acid analyzer. (1) Separation column (4.6 mm×60 mm): The elution liquid flow rate was 0.4 mL/min; the column temperature was 70 °C, and the column pressure was 10.627 MPa; (2) Reaction column: the ninhydrin and indene ketone buffer flow rate was 0.35 mL/min; the column temperature was 135 °C; and the column pressure was 1.078 MPa.

Results and Discussion

Analysis of amino acid composition of ACE inhibitory peptides from natural lactic acid fermented pork

The content of laboratory homemade ACE inhibitory peptide was 86.54%, and the IC_{50} was 0.895 mg/mL. Table 1 shows the result of the amino acid composition analysis. The amino acids include glutamic acid, histidine, asparagic acid, phenylalanine, alanine, and glycine, which accounted for 15.3%, 11.3%, 8.1%, 7.5%, 6.9%, and 6.3% of the total composition, respectively (Figure 1). Glutamic acid can promote brain cell respiration, which is conducive to the elimination of ammonia in brain tissue, which will affect blood pressure [21]. The amounts of hydrophobic amino acids, aromatic amino acids, and branched chain amino acids were 39.35%, 10.69%, and 13.65%, respectively. This finding is consistent with the structure characteristic of ACE inhibitory peptides, which have high contents of hydrophobic amino acids, aromatic amino acids, and branched chain amino acids as well as high content of leucine and proline [22,23].

Effects of ACE inhibitory peptides on the morphology and cell viability of vascular endothelial cells

The cells were found to grow from blood vessel ring and stick to walls after being implanted in the cell culture flask for 60 h. However, the number of vessel rings was low, and the rings were triangular and short spindle in shape. The cells were covered by 2/3 of the cell culture flask after primary culture of about 10-12 d. The boundary between cells was clear and converged to the single layer in the relatively dense place, which shows a typical "paving stone" characteristic of the endothelial cells.

The cells were stained by using diamine blue. Dying cells were stained light blue, and live cells were rejected. Live cell rate was then calculated. Figure 2 shows the results of the experiment. The rate of the living cells in each group was more than 80%. This result indicated that the concentration of ACE inhibitory peptides in the experimental group was appropriate and that no obvious toxicity was made on the vascular endothelial cells in a certain concentration range.

Effect of ACE inhibitory peptides on the release of

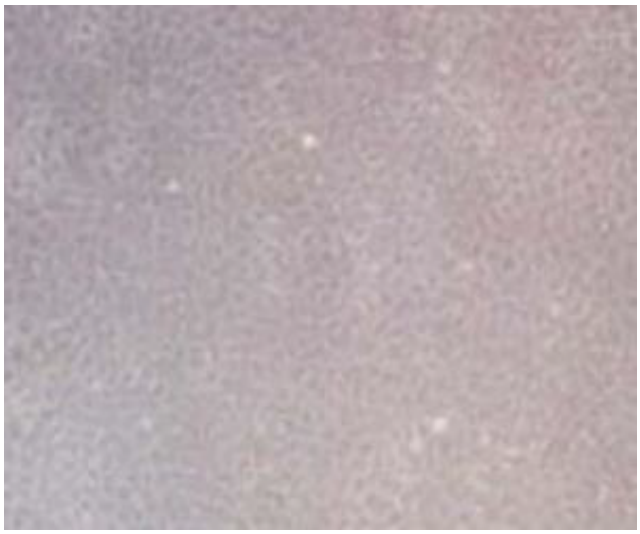

Figure 1: Endothelial cell morphology after cultured 10 ~ 12d (×100).

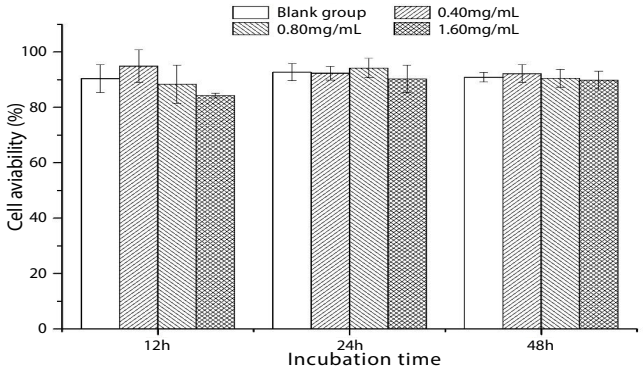

Figure 2: Viability of endothelial cell (%).

Table 1: Analysis of amino acid composition and content of ACE inhibitory peptides derived from fermented pork (mg/100g).

Amino acid	content	Amino acid	content	Amino acid	content
aspartic acid	3994.78	cysteine	1922.14	phenylalanine	3671.96
threonine	1799.64	valine	2626.16	lysine	2379.90
serine	1463.25	methionine	2007.43	histidine	5584.14
glusate	7527.77	isoleucine	1555.91	arginine	2070.51
glycine	3116.96	leucine	2537.67	proline	1988.95
alanine	3394.46	tyrosine	1592.09	total	49233.72

NO in vascular endothelial cells

NO has a strong ability to promote endogenous vascular dilatation and plays an important role in regulating the stability of human blood pressure and maintaining vascular tone [24]. Hypertension patients have vascular endothelial cell damage. The secretion of vascular endothelial cells increased with the increase of blood pressure, whereas the synthesis of vascular substance, such as NO, decreased. Blood vessels further shrink, the lumens narrowed, and the blood pressure increased [25]. ACE inhibitory peptides and captopril were used as positive control to deal with vascular endothelial cells in the experiment. Figure 3 shows the release amount of NO in the culture medium of each group. Both captopril and ACE inhibitory peptides could increase the release of NO in vascular endothelial cells. The release content of NO increased with the increase of culture time. After 12, 48, and 24 h, the content of NO in the medium-dose group is significantly different from that of the blank group ($p<0.05$) or extremely significantly different ($p<0.01$). In the high-dose group and the positive control group, the content of NO in the culture medium was significantly different than the control group ($p<0.01$). In the low-dose group, the content of NO increased, but not significantly ($p>0.05$). ACE inhibitory peptides with different concentrations had extremely significant difference ($p<0.01$) compared with captopril. These results indicated that the ACE inhibitory peptides could promote the release of NO in vascular endothelial cells, and the effect of high-dose ACE was better than that of low- and medium-dose ACE. However, the effect of high-dose ACE was less than that of the positive control group of captopril.

Effect of ACE inhibitory peptides on the content of ET-1 in vascular endothelial cells

ET-1 is known as the most potent and most enduring cytokine and plays a powerful role in promoting the proliferation of smooth muscle cells. ET-1 plays an important role in the formation and development of hypertension [26]. The ET-1 contents of the low-, medium-, and high-dose group of ACE inhibitory peptides and captopril positive control group were lower than the blank control group, as shown in Figure 4. Significant differences in ET-1 content were found in the medium-dose group compared with the control group ($p<0.05$). Significant difference ($p<0.05$) or extremely significant difference ($p<0.01$) was found in the high-dose group compared with the control group ($p<0.05$). No significant difference was found in the low-dose group and the control group ($p>0.05$) after culturing for 12, 24, and 48 h. The release of ET-1 in endothelial cells treated with different concentrations of ACE inhibitory peptides was significantly higher than that of the positive control group ($p<0.01$). The results showed that ACE inhibitory peptides inhibited the secretion of ET-1 in vascular endothelial cells, but the effect of low dose of ACE inhibitory peptides was less than that of middle dose and high dose. Some differences were found in the effect of the captopril positive control group.

Effect of ACE inhibitory peptides on ROS in endothelial cells and its antioxidant activity in vitro

ROS is a free radical produced by physiological and biochemical processes in the human body. ROS maintains the dynamic balance of production and removal. Once this balance is broken, it will lead to the generation of chronic diseases, such as aging, cancer, and hypertension. Figure 5 shows the effect of ACE inhibitory peptides on the ROS in endothelial cells. ACE inhibitory peptides were used to deal

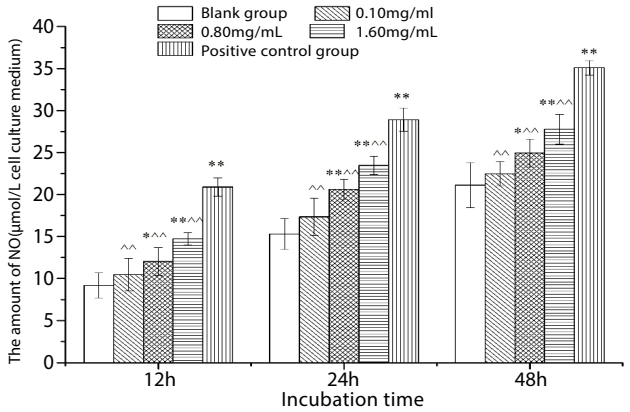

Figure 3: The amount of NO in each cell culture medium (μ mol/L broth).

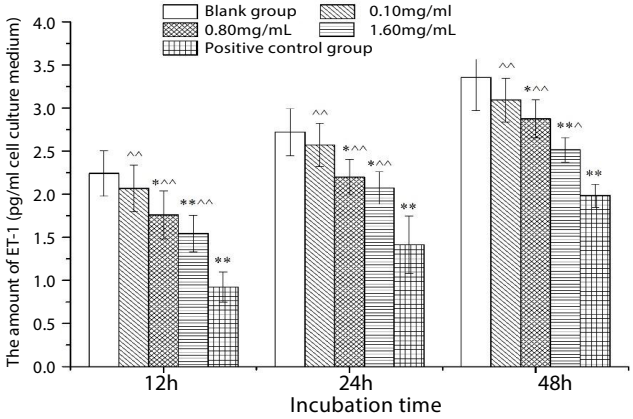

Figure 4: The amount of ET-1 in each cell culture medium (mg/mL broth).

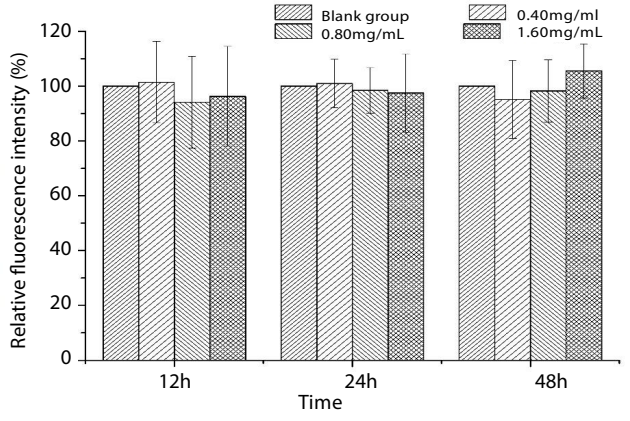

Figure 5: Effect of ACE inhibitory peptides on.

Table 2: Antioxidant capacity of ACE inhibitory peptide in vitro.

Antioxidant capacity In vitro	ACE inhibitory peptides(1mg/mL)	ACE inhibitorypeptides (5mg/mL)	ACE inhibitory peptides (10mg/mL)	Ascorbic acid (0.1mg/mL)
DPPH free radical scavenging rate (%)	3.33±2.23	14.43±1.58	20.41±3.58	—
Reducing power A_{700}	0.079±0.010	0.261±0.017	0.468±0.004	1.324±0.012

with vascular endothelial cells in different concentrations. No significant difference in relative fluorescence intensity was found between the groups compared with the blank group ($p>0.05$). The ACE inhibitory peptides from fermented pork had no obvious effect on the removal of ROS in endothelial cells. The results showed that the ACE inhibitory peptides from fermented pork had weak DPPH free radical scavenging according to in vitro antioxidant capacity test. The free radical scavenging rate of DPPH was only 20.41% when the concentration of ACE inhibitory peptides was 10 mg/mL. ACE inhibitory peptides did not show an apparent reducing power. The absorbance at 700 nm was 0.468 when the concentration was 10 mg/mL. A clear gap was found between ACE and ascorbic acid. The result is shown in Table 2.

Conclusion

In this study, the ACE inhibitory peptides with less than 10 KDa (the content of peptide was 86.54%, and the IC_{50} was 0.895mg/mL) were prepared by the traditional natural lactic acid fermented for 20 d in Southwest China, which is rich in glutamic acid and histidine. The contents of hydrophobic amino acids, aromatic amino acids, and branched chain amino acids were 39.35%, 10.69%, and 13.65%, respectively, which is in accordance with the characteristics of the ACE inhibitory peptides of amino acids.

The ACE inhibitory peptides were added after primary culture and subculture of rat vascular endothelial cells. The rate of living cells in each group was more than 80%, which indicated that the concentration of ACE inhibitory peptides in the experimental group was suitable for vascular endothelial cells. No obvious toxicity to the vascular endothelial cells in a certain concentration range was found.

The ACE inhibitory peptides of fermented pork were found to increase the release of NO in endothelial cells and reduce ET-1 secretion. The high-dose group was superior to the low- and medium-dose group, and some differences were found in the positive control group. ACE inhibitory peptides did not show significant in vitro antioxidant capacity and ROS clearance in cells. This finding indicated that the inhibition mechanism of ACE inhibitory peptides from fermented pork may be related to its ability to promote vascular endothelial cells to release NO and reduce ET-1 secretion.

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