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Protein & Peptide Letters, **2019**, *26*, 1-10

RESEARCH ARTICLE

Accelerated Wound Healing Induced by a Novel Amphibian Peptide (OA-FF10)

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ARTICLE HISTORY

Received: March 19, 2018
Revised: December 4, 2018
Accepted: January 15, 2019

DOI:
10.2174/0929866526666190124144027

Abstract: ***Background:*** Despite the continued development of modern medicine, chronic wounds are still a critical issue in clinical treatment, placing a great physiological, psychological, and financial burden on patients. Researchers have investigated many methods to solve this problem, with bioactive peptides gaining increasing attention due to their considerable advantages and diverse functions, as well as low cost, simple storage, and easy transportation.

Methods: In this research, a novel peptide (named OA-FF10) was identified from the skin secretions of the odorous frog species *Odorrana andersonii*. The sequence of mature OA-FF10 was "FFTTSCRSGC", which was produced by the post-translational processing of a 61-residue prepropeptide.

Results: Similar to most frog peptides, OA-FF10 showed an intramolecular disulfide bridge at the C-terminus. OA-FF10 demonstrated no antibacterial, antioxidant, hemolytic, or acute toxic activity, but promoted wound healing and proliferation of human keratinocytes (HaCaT) both time- and dose-dependently. Furthermore, while OA-FF10 had no effect on wound healing of human skin fibroblasts (HSF), it did accelerate healing in a full-thickness skin-wound mouse model.

Conclusion: Our research revealed the strong wound-healing activity of OA-FF10 *in vivo* and *in vitro*, thus providing a new candidate for the development of novel wound-healing drugs.

Keywords: *Odorrana andersonii*, wound healing, skin secretions, bioactive peptide, prepropeptide, human keratinocytes.

1. INTRODUCTION

As the largest human organ, skin functions to regulate body temperature and sense the surrounding environment [1]. It protects internal tissues and organs from physical and chemical damage and from pathogenic microorganisms [2, 3]. Human skin contains epidermis, dermis, and subcutaneous tissue, and several accessory organs such as blood vessels, lymphatic vessels, nerves, and muscles [3]. The wound repairing process of skin is complicated and time-consuming, involving inflammation, granulation tissue hyperplasia, and epidermal tissue regeneration [4-6]. With so many complicated steps, any disturbance of the repair process may aggravate illness and delay wound healing of skin ruptures. Refractory wounds can also lead to the invasion of pathogens, resulting in secondary infections, water and electrolyte disturbance, infectious shock, organ failure, and even death [5, 7]. To improve the recovery process and reduce mortality from complications during the wound-healing process, an effective and rapid way in which to promote wound healing is critical.

With the increase in our standard of living and poor dietary choices, chronic diseases like diabetes and uremia, as well as associated complications such as gangrene and chronic ulcers, have become commonplace today. These complications make the healing and treatment of the initial illness more difficult and augment financial burdens on the patient and medical system. In the United Kingdom, for example, the cost of chronic wound treatment has reached £1 billion per year [8]. Currently, several different drugs are used in wound treatment, including Epidermal Growth Factors (EGFs), small molecular compounds derived from plants, and Heat Shock Proteins (HSPs) [9]. However, these drugs can exhibit substantial shortcomings, including poor

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0929-8665/19 $58.00+.00

stability and activity, difficulty and cost of storage, and scar hyperplasia [10-11]. As such, bioactive peptides have attracted growing interest due to their diverse functions and high activity, stability, and specificity [12-14]. Therefore, exploring novel drugs that can accelerate wound healing is vital for the development of modern medicine [15].

As species with bare skin that live in complex habitats, amphibians can easily suffer external damage and have therefore developed a protective skin peptide system. Previous studies on amphibian skin secretions have identified multiple bioactive peptides with diverse functions, including antiseptic peptides, antioxidant peptides, wound healing peptides, cytokines, bradykinins, neuromodulating peptides, and neurotoxins [12, 13, 16-18]. Due to their diverse functions, these bioactive peptides have the potential to be developed into new drugs, including those that can treat chronic wounds [19]. Of note, damaged amphibian skin has been found to heal quickly and often without scaring [20, 21], indicating the existence of molecules in amphibian skin secretions that can shorten the process of wound healing [2, 5, 22-24].

As an odorous frog species, *Odorrana andersonii* possesses various bioactive peptides in its skin secretions [25]. We isolated and identified a new peptide, OA-FF10, from the skin secretions of *O. andersonii*, which was found to be highly effective in promoting wound healing both *in vitro* and *in vivo*. This current research expands our knowledge of amphibian peptides and will hopefully contribute to the development of novel wound-healing drugs.

2. MATERIALS AND METHODS

2.1. Sample Collection and Animal Care

We collected the adult *O. andersonii* from Yunnan Province, China. This amphibian is not currently listed as being endangered, currently under any protective measures, or are distributed in protected areas. The frogs were transferred to the laboratory safely, where they were housed together in a 50 cm × 60 cm box and provided with daily food (*Tenebrio molitor*). The frogs were acclimated for two days before the start of the experiment. To obtain skin secretions, the frogs were first washed with deionized water and then placed in a beaker (10 cm × 20 cm) with deionized water containing 0.01% NaCl and stimulated by alternating current (6 V) using an electronic massager for 6 s. The skin secretions were obtained by washing the frogs with 25 mM Tris-HCl buffer (pH 7.8). The collected solutions were centrifuged at 4000 rpm for 15 min at 4°C, with the supernatants then collected, lyophilized, and stored at -80°C until use.

All animal care and handling procedures were conducted in accordance with the requirements of the Ethics Committee of Kunming Medical University.

2.2. Purification Procedure

The purification procedure was performed as per previous research [2]. The lyophilized skin secretions were applied to a handmade Sephadex G-75 (1.5 × 31 cm, superfine, GE Healthcare, Sweden) gel filtration column pre-equilibrated with a 25 mM Tris-HCl buffer (pH 7.8) containing 0.1 M NaCl. Elution was performed with the same buffer at a flow rate of 0.1 ml/min, and fractions were collected every 10 min by an automatic fraction collector (BSA-30A, HuXi Company, Shanghai, China). Fractions with wound healing activity were applied to a C18 RP-HPLC column (Hypersil BDS C18, 4.0 × 300 mm, Elite, China) pre-equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in water. Elution was conducted using a linear gradient of 0.1% (v/v) TFA in acetonitrile at a flow rate of 0.7 ml/min and monitored at 215 nm. Peaks with wound healing activity were collected and lyophilized for a second HPLC purification procedure using the same conditions as above. Cellular-level wound healing activity was tested on HaCaT cells *in vitro*.

2.3. Determination of Primary Structure of the Peptide

The average molecular mass and the purity of native OA-FF10 were determined using an AXIMA-CFRTM plus MALDI-TOF mass spectrometer (Shimadzu/Kratos, Manchester, UK) in linear mode with α-cyano-4-hydrorycinnamic acid matrix. All procedures were carried out as per the manufacturer's standard protocols, and data were analyzed using the provided software package. The complete amino acid sequence of the peptide was determined by automatic Edman degradation on a PPSQ-31A protein sequencer (Shimadzu, Japan) according to the manufacturer's standard GFD protocols, and recognition of the cysteine residues was conducted according to the standard protocols provided by the manufacturer.

2.4. Cloning of cDNA Encoding OA-FF10

An *O. andersonii* skin cDNA library was constructed as per previous research [2]. The cDNA synthesized by SMART techniques was used as a template for polymerase chain reaction (PCR) to screen the cDNAs encoding the mature peptide. Two primers, *i.e.*, 5' PCR primer (5'-CCAAA(G/C) ATGTTCACC(T/A)TGAAGAAA-3') and 3' PCR primer (5'-ATTCTAGAGGCCGAGGCGGCCGACA TG-3'), were commercially synthesized by the BGI Company (China) and used for PCR. The PCR procedure was conducted using PrimeSTAR® HS DNA polymerase (TaKaRa Biotechnology Co., Ltd., Dalian, China) and initiated with 2 min at 94°C and 25 cycles of 10 s at 92°C, 30 s at 50°C, and 40 s at 72°C. The PCR products were recovered using a DNA gel extraction kit (BioTeke, Beijing, China) and ligated into a pMD19-T vector (TaKaRa Biotechnology Co., Ltd., Dalian, China). The PCR products were then cloned into *Escherichia coli* DH5a competent cells and used for DNA sequencing with an Applied Biosystems DNA sequencer (ABI 3730XL, Foster City, CA, USA).

2.5. Peptide Synthesis

The OA-FF10 peptide ("FFTTSCRSGC"), which possessed an intramolecular disulfide bridge, was commercially synthesized by Wuhan Bioyeargene Biotechnology Co., Ltd. (Wuhan, China). The purity of the peptide was more than 95%.

2.6. Biological Activity Assays

2.6.1. Cellular Wound Healing Activity Assay

Cellular wound healing was determined as per previous research [22]. In summary, immortalized HaCaT and HSF cells were cultured in DMEM/F12 medium (BI, Israel) with 10% fetal bovine serum (FBS, BI, Israel), 100 units/ml of streptomycin, and 100 units/ml of penicillin in a humidified atmosphere of 5% CO_2 at 37°C. Monolayer cell formation was achieved by culturing the HaCaT and HSF (2.5 × 105 cells/well) cells in 24-well plates for 12–24 h. Serum starvation was performed for 24 h to increase peptide sensitivity. The cell monolayers were then wounded using a yellow 200-µl pipette tip (Axygen, USA), and twice washed with PBS to remove any detached cells. Subsequently, 500 µl of DMEM/F12 medium (serum free) containing various concentrations of OA-FF10 (12.5, 25, 50, and 100 µM) was respectively added to each well. Images of the wounded monolayers were acquired using a Primovert microscope (Zeiss, Germany) at time intervals of 0, 12, and 24 h. Cell migration activity was expressed as the percentage of the gap relative to the total area of the cell-free region immediately after the scratch, named the repair rate of scarification, using Image J software (National Institutes of Health, Bethesda, MD, USA). For each plate, six randomly selected images were acquired. All experiments were independently carried out in triplicate.

2.6.2. Hacat Cell Proliferation Assays

Cells were cultured in DMEM/F12 medium with 10% FBS, 100 units/ml of streptomycin, and 100 units/ml of penicillin in a humidified atmosphere of 5% CO_2 at 37°C. The cells (5,000 HaCaT cells/well, 100 µl, respectively) were then plated in 96-well plates and incubated for 3 h to allow the cells to adhere to the well walls. Afterwards, 20 µl of OA-FF10 dissolved in DMEM medium (serum free) at various concentrations (12.5, 25, 50, and 100 µM) was added to each well, followed by further incubation for 24 h. The same volume of DMEM medium (serum free) was used as a blank control. After incubation, the CellTiter 96® AQueous One Solution Assay (Promega, Madison, WI, USA) was used in accordance with the manufacturer's instructions to test the effect of OA-FF10 on HaCaT cell proliferation [25].

2.6.3. Animal Wound Healing Assay

Male adult mice (n = 12, 20–25 g) were obtained from the Experimental Animal Center of Kunming Medical University. The mice were housed individually in 50 cm × 65 cm cages and provided with free access to water and laboratory food. The mice were acclimated for two days, after which full-thickness skin wounds were surgically applied. In brief, the animals were anesthetized with an intraperitoneal injection of 100 ml of solution containing 1% pentobarbital sodium (0.1 ml/20 g body weight). The backs of the mice were shaved and disinfected with a 75% ethanol swab to prepare the skin for a standardized full-thickness cutaneous wound. Two 8 × 8 mm full-thickness excisional wounds were surgically made on the back of each mouse. After surgery, the cages were placed near a heating apparatus until mice fully recovered from anesthesia.

The mice with full-thickness skin wounds were randomly divided into three groups. The right-sided wounds of the first group were treated with 20 µl (100 µM) of OA-FF10, with the same volume of Kangfuxin (100 mg/L; KFX, Inner Mongolia Jingxin Pharmaceutical Co. Ltd., China, Z15020805; ethanol extract of *Periplaneta americana* (L.) used to treat common trauma, ulcers, and burns) used for wounds on the left side. For the second group, the wounds on the right side were treated with 20 µl (10 µM) of OA-FF10, with the same volume of saline used on the left side. The right-sided wounds of the third group were treated with 20 µl (1 µM) of OA-FF10, with the same volume of saline used on the left-sided wounds. All wounds were treated twice daily, and wound images were collected once every two days [26].

2.6.4. Wound Healing Rate Measurement

Mouse wound repair was determined with a D3000 digital camera (Nikon, Japan). Wound areas (percentage of residual wound area to original wound area) were estimated from the photographs by Image J software (NIH, USA), in which the edges of the wounds were traced, and the area of pixels were calculated. Mean values of successive tracings were computed as percentages of closure from the initial wound based on triplicate images using the following equation: Residual Wound Area (%) = (R (2, 4, 6, 8) / R (0)) ×100, where R (0) and R (2, 4, 6, 8) denote the remaining wound area on the day of operation and postoperative days 2, 4, 6, and 8, respectively. Wound-healing curves were constructed using GraphPad Prism software (v. 5).

2.6.5. Hemolytic Activity Assay

The hemolytic activity of the peptide was tested using erythrocytes according to previously reported methods [22, 27, 28]. Human erythrocytes were washed twice with Dulbecco's phosphate-buffered saline, and the pellets were resuspended and diluted (1%) with the same buffer. Various doses of OA-FF10 (10 nM, 100 nM, 1 µM, 10 µM, and 100 µM) were added, and the cells were incubated at 37°C in a water bath for 30 min. The mixtures were then centrifuged at 3000 rpm for 5 min at 4°C, and the absorbance of the supernatant was measured at 540 nm. Maximum hemolysis was determined by adding 0.1% Triton X-100 to the cell samples.

2.6.6. Acute Toxicity Assay

Toxicity was determined in two phases using previously described methods [29], with some modifications. Briefly, lethal range acute toxicity was determined by intraperitoneal injection of OA-FF10 in mice at concentrations of 12.5, 25, 50, and 100 µM/kg. We observed and recorded the mortalities, toxic effects, and changes in behavioral patterns of the mice over 24 h.

2.6.7. Antimicrobial Activity Assay

Highly sensitive radial diffusion was used to test the antimicrobial activity of the peptide [30]. Gram-positive bacterial strain *Staphylococcus aureus* (ATCC 25923), gram-negative bacterial strains *Escherichia coli* (ATCC 25922) and *Bacillus pyocyaneus* (CMCCB 10104), and fungal strain *Candida albicans* (ATCC 2002) were obtained from Kunming Medical University. The microbes were grown in *Luria Bertani* (LB) broth to OD_{600} = 0.8. A 10-µl aliquot of each

bacterium/fungus was then taken and added to 10 ml of fresh LB broth with 1% Type I agar (Sigma-Aldrich, St Louis, MO, USA) and poured over a 90-mm Petri dish. After the agar hardened, a small hole was made and a 7-μl aliquot of OA-FF10 (1 mM) was added to the hole and incubated overnight at 37°C. If a clear zone formed on the surface of the agar, we concluded that the sample inhibited microbial growth. Minimal inhibitory concentration (MIC) was determined in liquid LB medium at pH 7.0 by conventional serial dilution in 96-well microtiter plates. In these experiments, ampicillin (AMP, 1 mg/ml) was used as the positive control.

2.6.8. Antioxidant Activity Assay

The 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging test was performed as described previously [16], with some modifications. A stock solution of ABTS radical (Sigma-Aldrich, St Louis, MO, USA) was prepared by incubating 2.8 mM potassium persulfate (Sigma-Aldrich, St Louis, MO, USA) with 7 mM ABTS in water for at least 6 h in the dark, after which it was used immediately. The stock solution was diluted 50-fold with deionized water. The same volume of solvent was used as the negative control, with vitamin C dissolved in H_2O used as the positive control. The reaction occurred in the dark for 30 min. The activities of the samples were manifested by a decrease in absorbance at 415 nm. The rate of free radical scavenging (%) was calculated by $(A_{blank} - A_{sample}) \times 100 / A_{blank}$ [16].

Free radical scavenging activity was also determined using 2, 2^0-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, St Louis, MO, USA) as per previous research [31], with some modifications. Briefly, 190 ml of 5×10^{-4} M DPPH radical (Sigma-Aldrich, St Louis, MO, USA) was dissolved in methanol. The sample solution (10 μl) was then incubated in a 96-well microtiter plate. The plate was kept from light for 30 min at room temperature, and absorbance was read against a blank at 517 nm. The inhibition of free radicals by DPPH was calculated according to the formula: $(A_{blank} - A_{sample}) \times 100 / A_{blank}$. Vitamin C (10 mM) was used as the positive control.

3. RESULTS

3.1. Peptide Purification

The skin secretions of *O. andersonii* were separated into three main peaks using gel filtration. Samples were collected every 10 min and tested for wound-healing promotion activities on HaCaT cell scratches, as per our previous report (indicated by an arrow in Figure **1A** in our previous report [2]). The fractions showing the intended activities were combined and further purified by HPLC.

As shown in Figure **1A** (Figure **1A** in this manuscript is consist with Figure **1B** in our previous research [2] but with different indicating arrow), following further purification, more than 30 peaks were obtained, one of which showed cellular wound-healing activity (indicated by an arrow in Figure **1A**). This sample was collected and further purified by HPLC, from which we observed a peak with ideal shape and similar elution time (about 28.2 min) (Figure **1B**). For the activities tested, this peak showed a strong positive effect on HaCaT cell wound healing.

3.2. Primary Structure of OA-FF10

The sample purified by the second round of HPLC (shown by arrow in Figure **1B**) was analyzed by mass spectrometry. Results showed that the molecular mass of OA-FF10 was 1105.25 Da (Figure **2B**). Based on this result and the second RP-HPLC program (Figure **1B**), the purity of the sample was found to be more than 95%. The amino acid sequence of this peptide was validated by the combinational methods of peptidomics and genomics. Firstly, the RP-HPLC and activity tracking were used to screened and purified the natural OA-FF10, and then the mature peptide sequence was obtained and determined through an Edman sequencer (FFTTSCRSGC). Secondly, an *O. andersonii* skin cDNA library was constructed and the mature peptide of OA-FF10 was validated in the cDNA library to find the cDNA which was encoding it. As shown in Figure **2A** and **C**, the cDNA encodes the signal peptide, acidic segment, restriction site and mature peptide of OA-FF10. As shown in Figure **2C**, the prepropeptide of OA-FF10 was similar to several peptides previously discovered from amphibian skin [5, 16, 25, 32]. However, BLASTp searching of the NCBI database found no identical or similar mature peptides, and thus this peptide was considered novel and named OA-FF10 (OA: species name abbreviation, FF: two initial amino acids, 10: peptide length; Figure **2A**).

Sequence and structural analysis showed OA-FF10 to have an intramolecular disulfide-bridge heptapeptide segment at the C-terminus, which was produced by the post-translational processing of a 61-residue prepropeptide. In general, most amphibian bioactive peptides from frogs of the family Ranidae contain pairwise cysteines that form disulfide bonds [2, 5, 22, 25, 33, 34]. As shown in Figure **2A**, OA-FF10 also contained pairwise cysteines. To determine whether OA-FF10 had an intramolecular disulfide bridge, we measured its peptide mass (https://web.expasy.org/peptide_mass/). The Theoretical Molecular Mass (TMM) was 1107.45 Da, but the Observed Molecular Mass (OMM) was 1105.8 Da (Figure **2B**). This difference of ~2 Da suggested that the pairwise cysteines in OA-FF10 combined and formed a disulfide bond. To verify this, linear (two cysteine residues without formation of a disulfide bond) and annular OA-FF10 were synthesized. Results showed that only annular and native OA-FF10 could elute together, confirming that OA-FF10 contained a disulfide bond (data not shown).

3.3. Bioactivity of OA-FF10

3.3.1. Wound-healing Promotion of OA-FF10 on HaCaT Cells

After trauma, various procedures are involved in the wound-healing process, with the migration of keratinocytes of particular importance [24]. In the present study, the migration of keratinocytes was observed in the cell scratch experiments *in vitro* after treatment with OA-FF10. The migration rate of keratinocytes was calculated by testing the healing efficiency of a single layer of cells over 24 h. Various

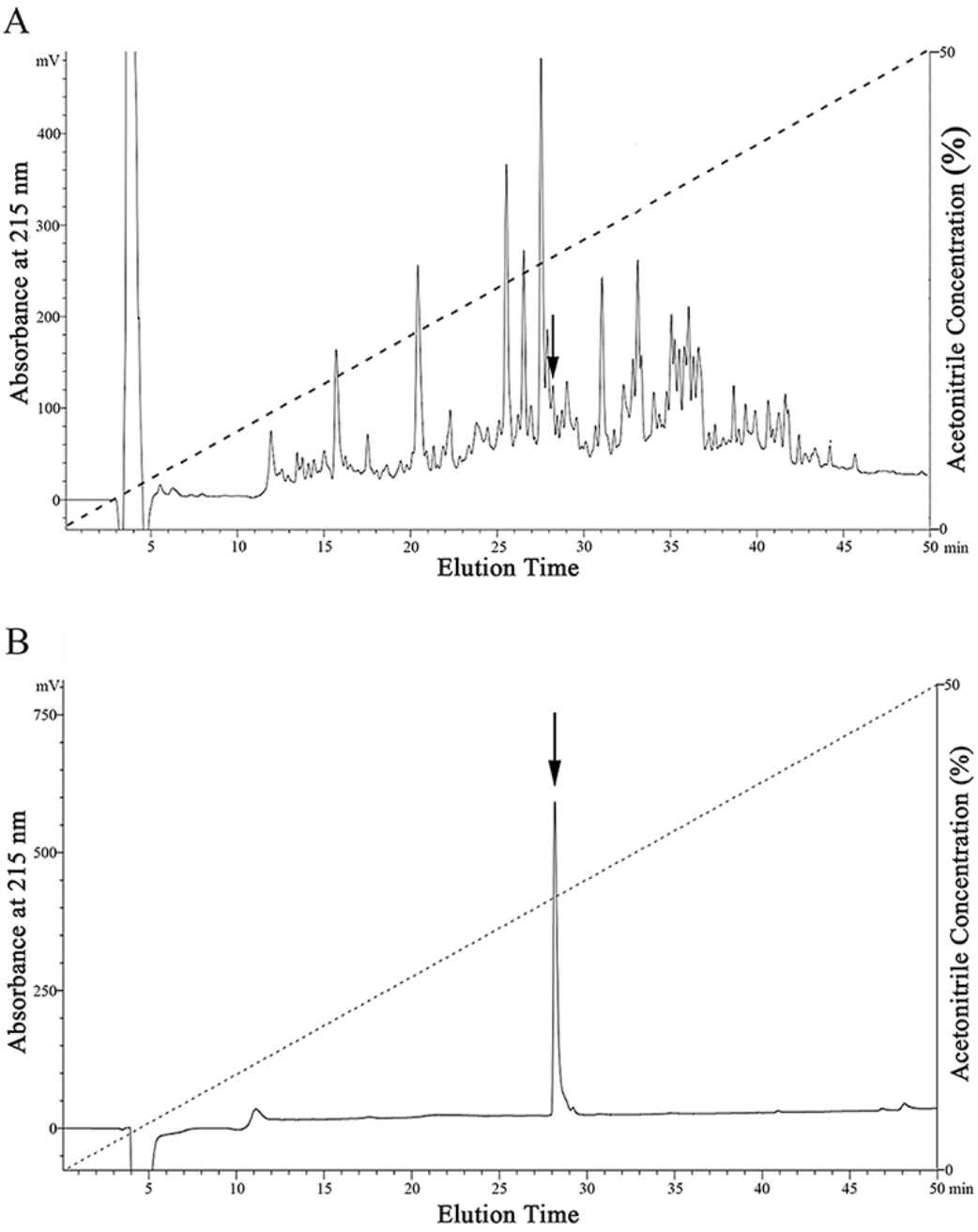

Figure 1. Peptide purification procedures from skin secretions of *Odorrana andersonii.* Skin secretions from *Odorrana andersonii* underwent Sephadex G-75 column gel filtration, with samples showing wound healing activity then collected and purified by RP-HPLC. The sample with wound healing activity (indicated by an arrow in Figure **1A**) was again purified by RP-HPLC. Finally, a peptide with wound-healing activity was obtained, as indicated by an arrow in Figure. **1B**, which was collected and used for further research.

concentrations of OA-FF10 (12.5, 25, 50, and 100 μM) were used to observe the wound healing of keratinocytes. In the negative control, the rates of HaCaT cell wound healing at 12 h and 24 h were 15% and 55%, respectively; at an OA-FF10 concentration of 12.5 μM, the healing rates at 12 h and 24 h were 22% and 70%, respectively (Figure **3B**); in the 25 μM-OA-FF10 group, the healing rates at 12 h and 24 h were 25% and 70%, respectively; in the 50 μM-OA-FF10 group, the healing rates at 12 h and 24 h were 30% and 75%, respectively. Interestingly, at 100 μM, the healing rates at 12 h and 24 h increased to 35% and 80%, respectively (Figure **3B**). As shown in Figure **3B**, the wound-healing promotion activity of OA-FF10 was both time and dose dependent.

3.3.2. No Wound-Healing Activity of OA-FF10 On HSF Cell Wound Repair

Fibroblasts play an important role in wound shrinkage and reconstruction [3]. We used the cell scratch test to explore the effect of OA-FF10 on HSF cells. The migration rate of fibroblasts was calculated after treatment with OA-FF10 at 12 h and 24 h. Same as HaCaT cells, various concentrations of OA-FF10 (12.5, 25, 50, and 100 μM) were

A

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atgtttacccttaaaaagtccatgcttcttctcctattcggtctgggtaccatt   54
 M   F   T   L   K   K   S   M   L   L   L   L   F   G   L   G   T   I    18
tccctgtcgctttgtgaacaaggtaaaaatgcagatgaagaagaggaagagcgg  108
 S   L   S   L   C   E   Q   G   K   N   A   D   E   E   E   E   E   R    36
cgagatgatgaagaagttgccaaaatggaagaagagattaaacggtttttacc   162
 R   D   D   E   E   V   A   K   M   E   E   E   I   K   R   F   F   T    54
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 T   S   C   R   S   G   C   *                            61
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aaaaaaaaaaaaaaa                                         341
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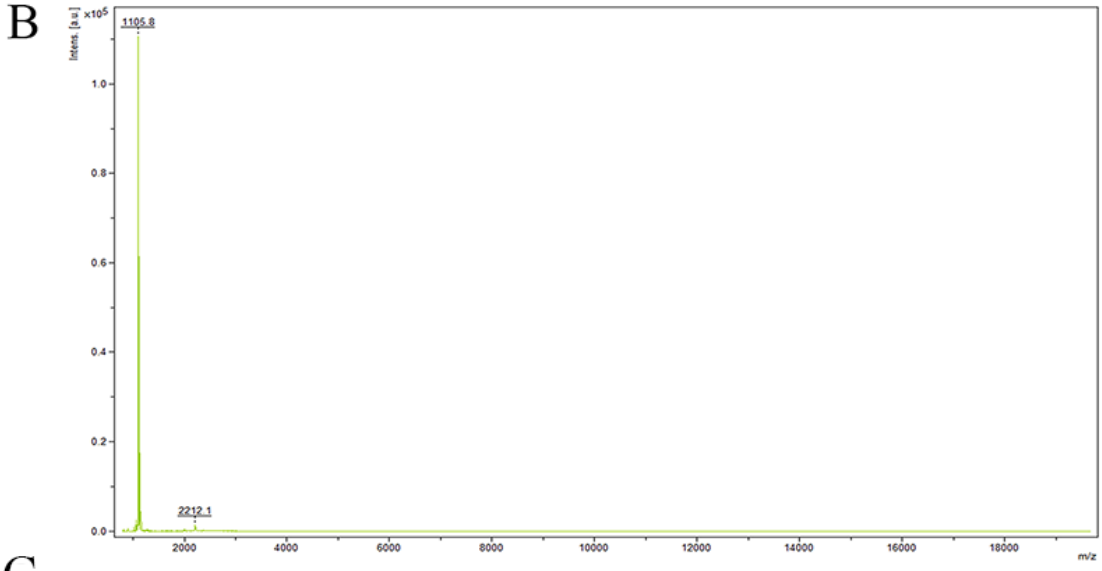
B



C

Name	Signal peptide	Acidic segment	Enzyme cleavage site	Mature peptide	References
Amolopin-N1	MFTLKKSLLLLFFLGTINLSLCEQERNAEEERRDDLGERQAEVE			KRFFPIVGKLLSG	37
Nigrocin-OA1	MFPLKKSLLLLFFIGTISLSFCQDETNAEEERRDEEVAKMEEI			KRGLLSGVLGVGKKIVCGLSGLC	25
Nigronain-H-OA1	MFTMKKSLLLLFFLGTISLSLCEEERDANDEEDGGEVTEEA			KRDLQRRCVIALPHKMRCRVTGRC	25
Andersonin-AOP1	MFTLKKSLLLLFFLGTINLSLCQEERRDDPNEMDAEGT			KRFLPGLECVW	16
Andersonin-AOP2	MFTLKKTLLLLFFLGIINLSLCEEEERNAEEEERRDDPDVRDVEVE			KRSLSCFLSFTR	16
Andersonin-AOP3	MFTLKKSLLFLFFIGIVSSSPYRGKRENGEEEAIEDI			KRQLFLQGSPQC	16
OM-LV20	MFTMKKSLLLYFFLGMVSLLLCKHERHAEERRHDPTEEEIIARNEENNEKRLVGKLLKGAVGDVCGLLPICL				5
OA-FF10	MFTLKKSMLLLLFGLGTISLSLCEQGKNADEEEEERRDDEEVAKMEEEIKRFFTTSCRSGC				

Figure 2. Primary structure of OA-FF10. **A**. The complete sequence of mature OA-FF10 ("FFTTSCRSGC") was 10 amino acid residues in length (shown in red) and produced by post-translational processing of a 61-residue prepropeptide. OA-FF10 contained an intramolecular disulfide bridge located at the C-terminus (indicated by blue lines). **B**. Molecular mass of native OA-FF10 and purity of the peak. **C**. Comparison of OA-FF10 with several other peptide sequences from amphibian skin secretions obtained from the NCBI database. (*The color version of the figure is available in the electronic copy of the article*).

used. OA-FF10 showed no effect on wound repair in HSF cells at 12 h and 24 h (shown in Supplementary Figure **1**).

3.3.3. OA-FF10 Induced Proliferation of HaCaT Cells

When tissue is damaged, the body rapidly initiates various procedures to participate in the healing process [3]. In the proliferation phase of wound healing, keratinocytes are extremely important. We conducted an *in vitro* experiment to test the effect of OA-FF10 on keratinocyte cell proliferation. As shown in Figure **4**, the proliferation of HaCaT cells was promoted (dose-dependently) by OA-FF10 at concentrations of 12.5 μM to 100 μM. Because OA-FF10 had no effect on HSF cell wound repair, we did not perform the proliferation assay on these cells.

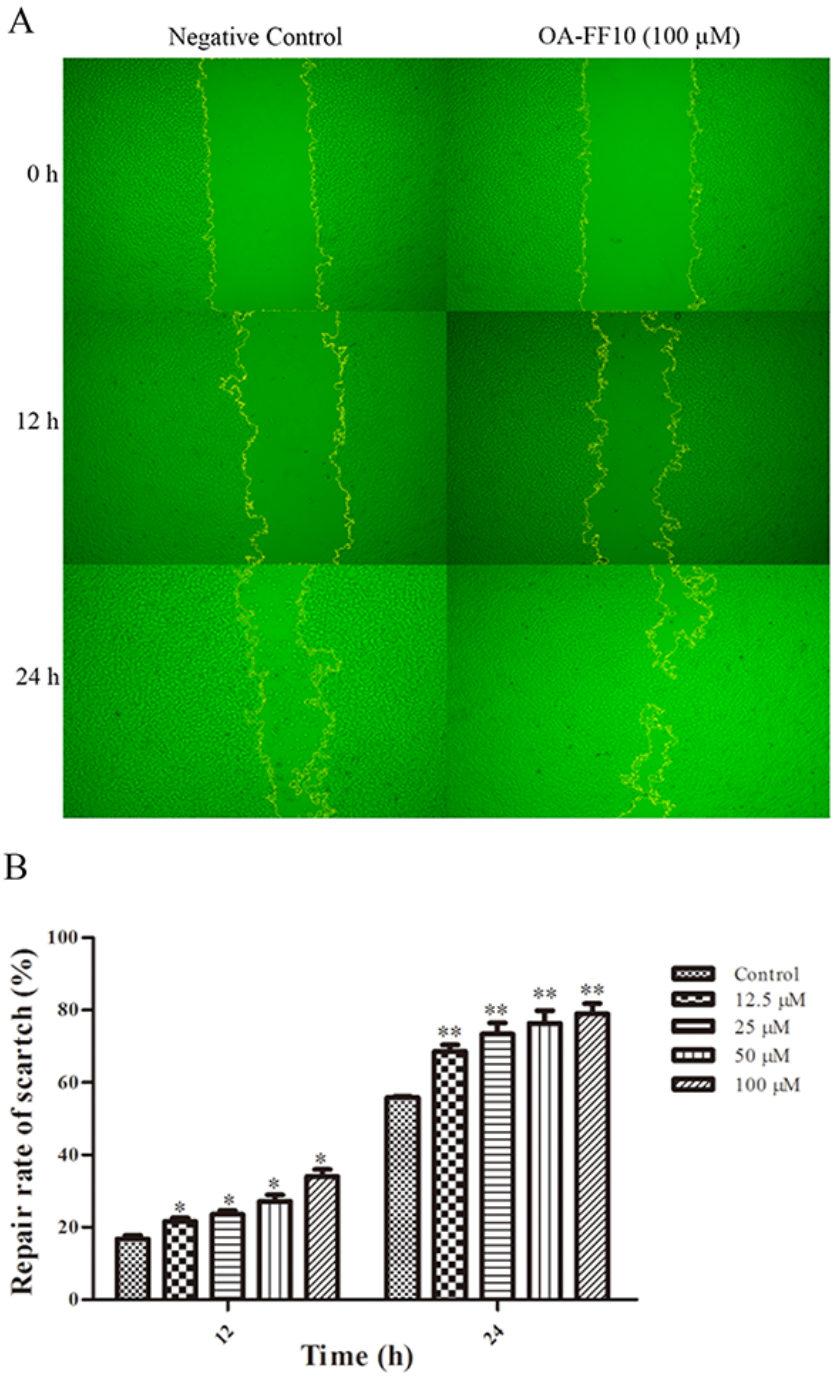


Figure 3. Effect of OA-FF10 on HaCaT cell wound healing. **A**. OA-FF10 (100 μM) showed obvious wound healing promotion activity on HaCaT cells. **B**. OA-FF10 accelerated HaCaT cell wound healing in both a time and dose dependent manner. Data are means ± SEM of six independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.0001$ indicate significantly different from the control (Student's *t*-tests).

3.3.4. OA-FF10 Shortened the Healing Process of Full-Thickness Wounds in Mice

As OA-FF10 showed a marked effect on promoting wound repair *in vitro*, we hypothesized that it may also exhibit *in vivo* effects. To test this, full-thickness skin wounds were established on mice to explore the role OA-FF10 in wound repair. We treated the wounds with OA-FF10 (1, 10, 100 μM, 20 μl), KFX (100 mg/L, 20 μl as a positive control), or saline solution (20 μl as a negative control) twice daily. Data were collected by taking images of the wounds on the day of surgery (0 day) and on days 2, 4, 6, and 8 after surgery. Compared with the negative control, the trauma area treated with OA-FF10 decreased significantly on day 2 (Figure **5**). It is worth noting that OA-FF10 showed greater wound healing activity than KFX (100 mg/L) and was time and dose dependent. In addition, there were no adverse changes in the general conditions of mice after exposure to OA-FF10 (data not shown).

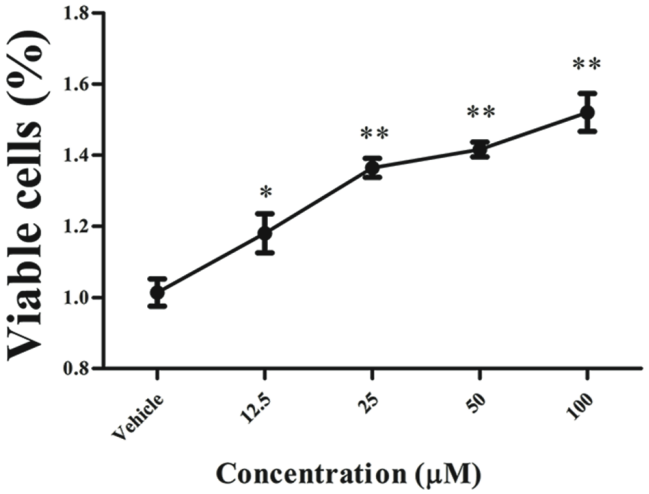


Figure 4. Effects of OA-FF10 on HaCaT cell proliferation.
OA-FF10 promoted HaCaT cell proliferation both time- and dose-dependently. Data are means ± SEM of six independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.0001$ indicate significantly different from the control (Student's *t*-tests).

3.3.5. OA-FF10 Showed No Hemolytic Activity in Human Red Blood Cells and No Acute Toxicity to Mice

No hemolytic activity was observed under the maximum concentration of OA-FF10 (100 μM; shown in Supplementary Figure **2**). In the acute toxicity test, after injection with OA-FF10 (12.5, 25, 50, and 100 μM/kg), mice were observed for 24 h and no lethal effects were observed (shown in Supplementary Table **1**).

3.3.6. OA-FF10 Showed No Obvious Antibacterial or Antioxidant Activity

Under the maximum concentration of 1 mM, OA-FF10 had no effect on gram-positive bacterial strain *S. aureus*, gram-negative bacterial strains *E. coli* and *B. pyocyaneus*, or fungal strain *C. albicans* (shown in Supplementary Table **2**). The antioxidant activity of OA-FF10 was also tested with the maximum concentration set to 100 μM. Results showed that OA-FF10 exhibited no ABTS+ or DPPH+ scavenging activity (shown in Supplementary Figure **3**).

4. DISCUSSION

Amphibian skin secretions contain rich and diverse bioactive peptides, including antimicrobial and antioxidant peptides [16, 25]. However, wound healing peptides from amphibian skin remain poorly reported [2, 5, 22-24]. Therefore, the purpose of this study was to isolate and identify a wound-healing promoting peptide from the skin secretions of *O. andersonii*.

The products collected from gel filtration were diluted to low concentration to test their effect on cell scratch wound repair. Samples that displayed activities were combined and further purified by RP-HPLC (Figure **1A** and **B**), with a peptide that promoted wound healing at the cellular level then obtained (Figure **1B**). Using Edman sequencing and cDNA cloning, the amino acid sequence of this peptide was

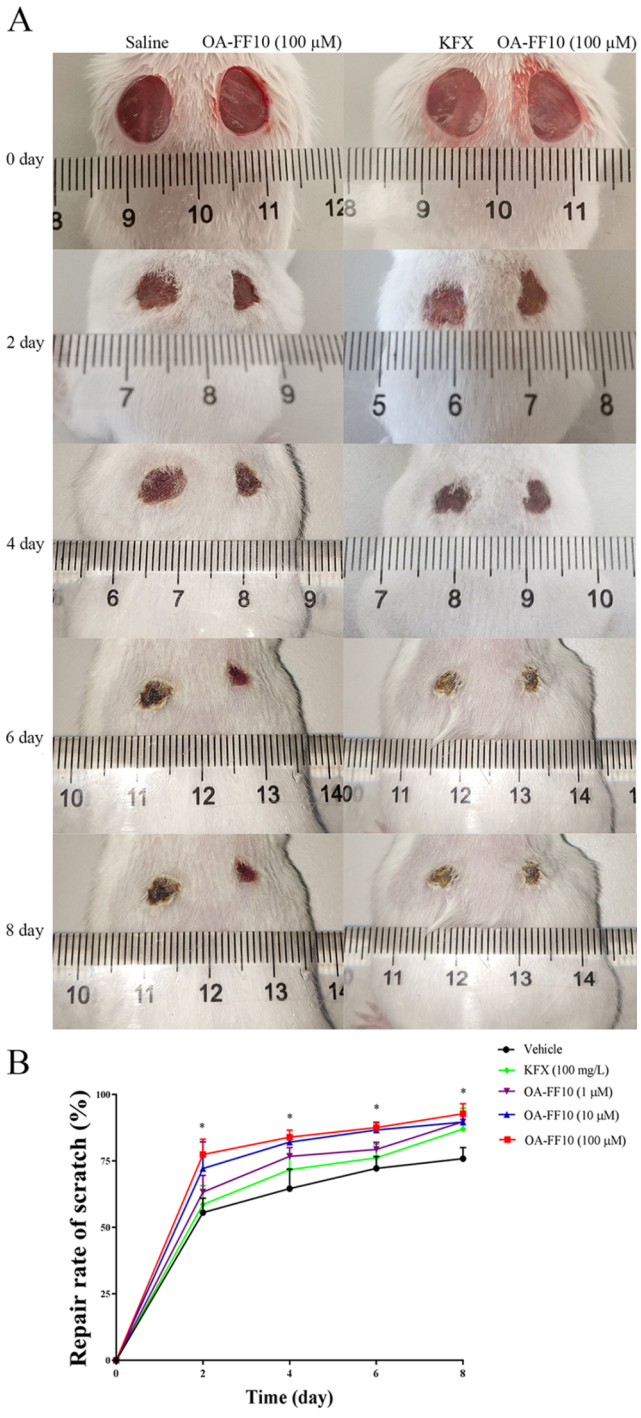


Figure 5. Increase in healing of full-thickness skin wounds in mice following topical application of OA-FF10. Negative control was saline solution, positive control was KFX (100 mg/L). **A**. OA-FF10 (100 μM) obviously promoted wound healing in mice. **B**. OA-FF10 accelerated full-thickness skin wound healing in mice in a time and dose dependent manner. Data are means ± SEM of six independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.0001$ indicate significantly different from the control (Student's *t*-tests).

determined to be "FFTTSCRSGC" (Figure **2A**). Furthermore, mass spectrometry revealed the peptide to have a molecular mass of 1105.8 Da. Searching the NCBI database, no

peptides were found with similar sequences, and thus the current peptide was considered novel and named OA-FF10. The OA-FF10 sequence also contained an intramolecular disulfide-bridge segment at the C-terminus, which is usually known as a Rana box and demonstrates various antimicrobial, cytotoxic, or hemolytic activities [31-32, 35-39]. For further confirmation, the primary structure of OA-FF10 was compared with that of other amphibian skin secretion peptides [5, 16, 25, 32]. A series of studies were also conducted to test the bioactivity of OA-FF10.

Various functions and uses have been determined for peptides obtained from the skin secretions of amphibians, with wound repair promotion gaining particular attention. We conducted several *in vivo* and *in vitro* experiments to explore the function of OA-FF10 in wound repair. After skin injury, keratinocytes and fibroblasts play important roles in the proliferative stage of wound healing. In this study, the effects of OA-FF10 on the migration and proliferation of HaCaT and HSF cells were tested *in vitro*. Interestingly, unlike other bioactive peptides from frogs [2, 5], OA-FF10 accelerated HaCaT cell scratch repair in both a time and dose dependent manner but had no effect on HSF cell wound healing under the same concentrations (12.5, 25, 50, and 100 μM; Figure **3** and Supplementary Figure **1**). These conflicting results may be due to the sensitivity of different cells to the concentrations of OA-FF10, or the different mechanisms they exhibit during wound healing. These findings highlight the uniqueness of OA-FF10. Furthermore, the *in vitro* experiments on HaCaT cells showed that OA-FF10 promoted the proliferation of these cells both time- and dose-dependently (Figure **4**). This may be an important mechanism by which OA-FF10 accelerates the process of wound repair in HaCaT cells. To confirm the wound-healing activity *in vivo*, a full-thickness skin-wound mouse model was established. Twice a day, skin wounds were treated with OA-FF10 (1 μM,10 μM, and 100 μM), KFX (100 mg/L), or normal saline. As shown in Figure **5**, OA-FF10 demonstrated strong time and dose dependent wound healing promotion. Compared with 100 mg/L of KFX, OA-FF10 showed stronger activity in promoting wound healing. As a peptide only 10 amino acids in length, OA-FF10 exhibits high wound healing activity, and thus its further development should be undertaken.

To confirm the practical operability of OA-FF10 in clinical drug use, its hemolytic activity against human erythrocytes and its acute toxicity against mice were determined. At the maximum concentration of 100 μM, no hemolytic activity or acute toxicity were observed (Supplementary Figure **2** and Table **1**). In previous studies on the skin secretions of amphibians, most bioactive peptides have shown antimicrobial and antioxidant functions [40]. Therefore, the antibacterial and antioxidant activities of OA-FF10 were tested. Unfortunately, OA-FF10 showed no antimicrobial activity at the maximum concentration of 1 mM, nor antioxidant activity at the concentration of 100 μM (Supplementary Table **2** and Figure **3**). It is worth noting, however, that OA-FF10 showed significant wound healing activity at concentrations as low as 12.5 μM. This study adds to our understanding of amphibian skin peptides that demonstrate considerable and important wound healing promotion and acceleration. Furthermore, this work offers a new direction for research on the diversity of amphibian skin peptides and provides a novel peptide template for the development of the wound-healing drugs.

CONCLUSION

In this study, a novel peptide, OA-FF10, was identified from the skin secretions of *O. andersonii*. OA-FF10 was found to contain an intramolecular disulfide bridge located at the C-terminus, with an amino acid sequence of "FFTTSCRSGC". This peptide showed no acute toxicity or antibacterial, antioxidant, or hemolytic activity, but did exhibit strong wound-healing activity *in vitro* and *in vivo*. Our results provide a new candidate for the development of novel and effective wound-healing drugs.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethics Committee of Kunming Medical University.

HUMAN AND ANIMAL RIGHTS

No humans were used for the studies that are basis of this research. The reported experiments on animals, were in accordance with the guidelines of Kunming Medical University, China.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICTS OF INTEREST

All authors declare no conflicts of interest in this work.

ACKNOWLEDGEMENTS

This work was supported by grants from the Chinese National Natural Science Foundation (81760648, 31460571, 31660244, 81500983, and 31670776) and Yunnan Applied Basic Research Project Foundation (2017FB035).

AUTHOR CONTRIBUTIONS

Xinwang Yang and Jing Tang designed the study. Naixin Liu, Zhe Li and Buliang Meng performed most of the research, including data collection, purification procedures, peptide structure determination, data analysis, and manuscript composition. Wenxin Bian, Siyuan Wang, and Xiaoqing Cao participated in the purification procedures. Ying Wang and Yongli Song participated in the antioxidant activity determination of peptides. Meifeng Yang and Xiaojie Li participated in the collection of animals. All authors contributed substantially to this research and reviewed this manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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