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Evaluation of collagen mixture on promoting skin wound healing in zebrafish caused by acetic acid administration

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ABSTRACT

The aim of this study is to use zebrafish embryos as a quick platform for wound healing studies. At beginning, we optimized a protocol to induce skin lesion by acetic acid injection. The acetic acid injection induced regional inflammation wound hyperpigmentation by recruiting pigment cells to the wound area. Later, we applied established platform to evaluate the effect of tilapia's collagen peptide mixtures, including demonstration on promoting skin wound healing and eliminating inflammatory response. Results showed that after treating TY001, one of the above fish collagen peptide mixtures, not only repair and proliferation were induced, but also death and apoptosis cells were cleared within cutaneous lesion. Moreover, inflammatory response was suppressed along with collagen mixture treatment. Finally, the TY001-associated signaling was validated by real time-PCR, and numbers of gene associated with tissue repair and vessel proliferation were induced. To sum up, our findings provided a permissive model that may apply to generate a platform for further screening on repair and restoration technology. In addition, the tilapia fish collagen peptide mixture we applied on our model has great potential on developing clinical application on wound healing.

1. Introduction

It has been long considered that wound healing was one of the most complicated biological coordination during life [1]. Wound healing occurs faster in fish in a suitable environment compared to mammalian species, which myriad stages of repair and regeneration were involved in, such as phases of proliferation, migration, matrix synthesis, and contraction that aimed to reform the damaged tissue and prevent continuous infection [2]. Previous studies indicated that epidermal wound healing processes in mouse and mammalian embryos shared similar principles; however, a serial overlapping processes led it difficult to managing either direct or indirect effects of re-establishment of normal tissue after damage [3]. To date, zebrafish has become a well-described model to track the process of immune responses and signaling network during wound healing [3–5]. Compared to adult fish, tissue repair in embryonic fish is efficient and noting leaving a scar by actin-associated signaling instead of keratinocytes aggregation [3]. During tissue damage and repair, hyperpigmentation often occurred during healing process [6], which suggested transparency character of embryonic zebrafish is a promise model for cutaneous-related research.

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Previous studies demonstrated that several approaches subjected damage on fish skin to trigger downstream phenomenon and investigated the profound mechanism of wound healing process. While needle punctuation and dermatology laser-based damage were commonly applied on research [5,7,8], chemical reagent induced cutaneous damage were also a trend when investigating healing mechanism, including re-epithelialization and regeneration [3,9]. Low pH acids, such as acetic acid, citric acid, and succinic acid, were chosen to access behavioral or inflammatory responses [10–12]. Based on the past findings, we applied a novel technique by using 5% acetic acid to evaluate wound healing effect and mechanism in embryonic zebrafish.

Collagen, a main structural protein in animal skin, especially in fish [13]. Collagen played important role on skin structure and tissue repair which has potential on biomedical, pharmaceutical, and cosmetic applications [14–16]. Therefore, the mixtures of fish collagen peptide derived from Tilapia were applied onto zebrafish skin after cutaneous lesion in this study. The quick effects on death cell clearance and new cell proliferation were observed. Moreover, the inflammatory responds and genetic network were also elucidated during our examination and proved the provocative of our system.

2. Results

Due to the benefit on visualization of zebrafish melanocytes, we investigated the rate of skin regeneration by quantify the tissue transparency. For wounding, fish embryos were anaesthetized in MS222 and applied with 5% acetic acid injection to cause cutaneous lesions. Morphologically, tissue within zebrafish wounds was first visible with aggregation of pigments (Fig. S1B). After subjected with 200 μg/ml of 1822-1, 1822-2 and 1822-3 compounds which are containing different ratio tilapia collagen peptide mixtures, less pigment aggregation was shown within wounding regions (Fig. S1C-E). In addition, tissue morphology grew more smoothly compared to untreated group (Fig. S1A), which suggested the incidence of repair and regeneration after treatment. To quantify the healing activity, we measured the degree of tissue transparency and aggregation of pigment (Fig. S1F and G). Transparency helped determine the amount of newborn epithelial tissue that covered onto the healing region. Compare to untreated group with 11274 ± 293 pixels of transparency, 1822-1, 1822-2 and 1822-3 showed 9353 ± 238, 9961 ± 411 and 9986 ± 173 pixels, respectively (Fig. S1F). Moreover, the percentage of pigment number compare to untreated fish was decreased after compound treatment (17, 12, 11%, respectively, Fig. S1I) (also see Table 1 for detail). Furthermore, while regeneration happened around wounded region, angiogenesis occurred and newly formed blood vessels were observed (Fig. S2C-E) and we quantified the number of new vessels by taking the advantages of green fluorescent-labeled blood vessels in Tg(fli1:EGFP)^{y1} (Table 2). Among the three collagen mixtures we applied, 1822-1 showed the greatest efficiency on angiogenesis and tissue repair and will be named as TY001 and focused in the following examination (Fig. S2C).

To investigate the TY001 biological activity, we first observed the cell proliferation within wound area with subjecting different concentration of collagen mixtures. Serial concentration of TY001 in 100, 200, 300, 400 μg/ml were examined onto cutaneous lesion of zebrafish for 5 h and the BrdU incorporation experiment was performed to determine the cell proliferation status (Fig. 1). During wound healing process, epithelial cells proliferated to help cover the damage area. Compare to untreated group (Fig. 1A), we observed that BrdU-positive fluoresce intensity within wound region was significantly increased after treated with 200 and 300 μg/ml of TY001 (Fig. 1D, E, H). The percentage of cell proliferation of each treated group compared to untreated group was listed in Table 2. Interestingly, while treated with 400 μg/ml of TY001, proliferation was not occurred, which suggested that the high concentration of TY001 might cause cytotoxicity (Fig. 1F, H).

For healing process, it is important not only for induction of cell proliferation, but also death cell ablation. Therefore, we stained wound area with ethidium bromide (EtBr) to demonstrate the clean-up effect of TY001 on death cells after 5 h. We found that the amount of EtBr-positive death cells within wound areas was decreased after treating TY001 (Fig. 2 and Table 3). Low concentration of TY001 in 100 μg/ml showed promising clearance rate compared to other treated groups (Fig. 2D, I and Table 3). Furthermore, we demonstrated TUNEL assay to identify cells under apoptosis and quantify the ablation efficiency of TY001 on apoptotic cells after 5 h (Fig. S3). By detecting of relative fluorescence intensity in the region of interesting (red square), apoptotic cells were diminished after treated with TY001 (Fig. S3C-H). Quantitative analysis showed significant clean-up efficiency of embryos increased after treated with 100 μg/ml of TY001 (Fig. S3C) compared to positive group (Fig. S3B) (Table 4).

To validate the TY001 effect on inflammatory suppression, macrophage and neutrophil numbers were quantified in treated and untreated groups by using Tg(coro1a:EGFP)^{hkz04t} as a reporter line. We found that green fluorescence-labeled macrophages and neutrophils were induced after cutaneous damage, while decreased after serial concentration of TY001 were treated onto lesion after 18 h (Fig. 3). The inflammatory response was suppressed about 17% by TY001 (Table 5). In order to elucidate the molecular signaling that affected by TY001, we performed real time-PCR to evaluate the gene expression. Gene associated with cellular inflammatory responses, such as *Fan*, was elevated after tissue damage, which was known as a key factor of TNF-mediated leukocyte chemotaxis and activation (Fig. 4A). This suggested us that the TY001 induced clearance efficiency on death or apoptotic cells may be through *Fan* signaling. In addition, the increased expression of *Msxb*, *vegf-A*, *Wnt3a*, and *RARγ* after TY001 treatment (Fig. 4B–E), were considered consist with the tissue, cell, and vessel proliferation phenomenon we observed. Moreover, the upregulated *col1a1b* might play a role on speeded up the production of collagen (Fig. 4F).

Table 1
Cutaneous lesion repair after collagen mixture treating in zebrafish.

Group	Concentration (μg/ml)	Transparency (Mean ± SE, n = 10)	Inhibition of pigment aggregation (%)	Incidence of angiogenesis (%)
Normal tissue	–	7152 ± 34	–	–
Untreated lesion	–	11274 ± 293	–	50
1822–1	200	9353 ± 238***	17***	90
1822–2	200	9961 ± 411**	12**	70
1822–3	200	9986 ± 173**#	11**#	90

[1] Data compared to untreated lesion group, **, $p < 0.01$; ***, $p < 0.001$.
[2] Data compared to 1822-1 group, #, $p < 0.05$.

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Table 2
Effect of TY001 collagen mixture administration on cell proliferation in zebrafish.

Group	Concentration (μg/ml)	Fluorescence intensity (BrdU) (Mean ± SE, n = 10)	Induction of cell proliferation (%)
Untreated lesion	–	161273 ± 14797	–
TY001	100	204356 ± 20410	27
	200	304754 ± 17018***	89***
	300	294081 ± 29928**	82**
	400	203391 ± 26574	26

[1] Data compared to untreated lesion group, ***, $p < 0.001$.

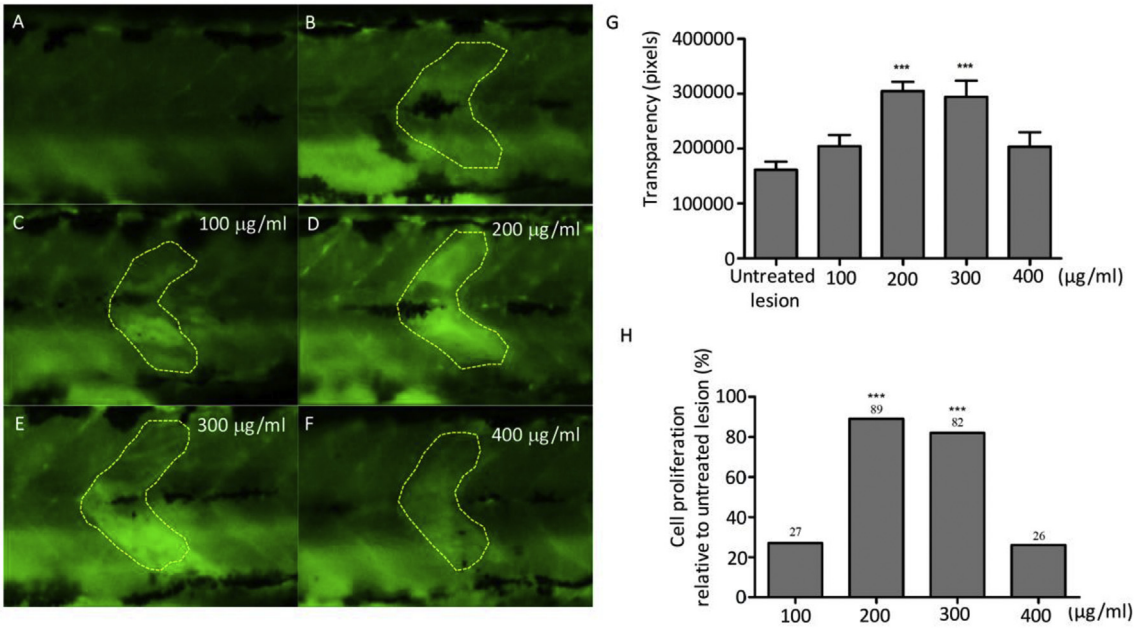

Fig. 1. Elevated cell proliferation was detected after treating TY001 collagen mixture.
Zebrafish larvae were applied with 5% acetic acid to cause cutaneous lesions at 2 dpf and treated with or without TY001 for treatment. Cell proliferation was demonstrated with BrdU staining. (A) Normal skin tissue on 2 dpf zebrafish larva from lateral view (B) Cutaneous lesion without TY001 treatment (C-F) Cutaneous lesion with different concentration of TY001 as indicated. (G) Quantitative of BrdU-positive area within lesion regions (yellow square) (H) Percentage of cell proliferation compared to untreated lesion after treating with different concentration of TY001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Discussion

During proliferation status after wound healing, interaction between keratinocyte and fibroblasts dominated cell migration and proliferation [17]. So far, numerous compounds, such as isoflavone, polyphenolic compounds in plant oils, and 18β-glycyrrhetinic acid (18β-GA) were identified as treatment for wound [17–19]. In this study, not only promotion on cell proliferation and angiogenesis, but also inhibition of pigment aggregation was demonstrated after TY001 treatment. Abnormal pigmentation, which involving four stages for melanosome maturation, was always unfavorable during healing process [20]. Currently, laser therapy has commonly used in clinical treatment for dyspigmentation; however, side effects, such as serial dermal and epidermal injury happened due to the heat when applied [21]. According to our results, after treating with TY001, the aggregation of pigment was significant dispersed, which suggested us TY001 effect on decreasing pigment deposition caused by damage within lesion may have potential on dermatological application.

Generally, angiogenesis was induced during healing process to maintain higher oxygen level to recruit cells to the wound spaces [22]. Factors that associated with angiogenesis have been identified and the related mechanism has also been elucidated [23]. Among these factors, vascular endothelial growth factor (VEGF) was one of the well-known mitogens that played initiation role during wound healing [24]. Tissue hypoxia caused by cutaneous damage induced expressions of VEGF and its receptor [25]. Therefore, consist with the new vessel formation phenomenon we observed after TY001 treatment, upregulated *vegf-A* was corresponding to the inducement of angiogenesis. According to our gene expressional analysis, *Fan*, a gene that associated with cellular inflammatory response and a key factor for leukocyte chemotaxis and activation, was elevated after tissue damage. At the meanwhile, we observed that macrophages and neutrophils were surged after damage, this suggested us that acetic acid-associated immune response was through *Fan* pathway. Although TY001 did not show suppression effect on *Fan* expression compared to untreated lesion group, it was indeed induced clearance efficiency on death cells which may be through inflammatory cells and *Fan* signaling activation. In addition, the increased expression of *Msxb*, *vegf-A*, *Wnt3a*, and *RARγ* after TY001 treatment which were associated with the tissue, cell, and vessel proliferation suggested that TY001 showed predominant therapeutic effect on wound healing by facilitate lesion recovery. Moreover, the upregulated *col1a1b* speeded up the production of collagen which is crucial for providing strength of cutaneous structure remodeling.

Another issue comes after cutaneous injuries was invocation of inflammatory responses that accompanied with recruitment of leucocytes to the lesion [18]. Therapeutic modulation of TY001 on cleaning death cells and diminish of macrophages and neutrophils

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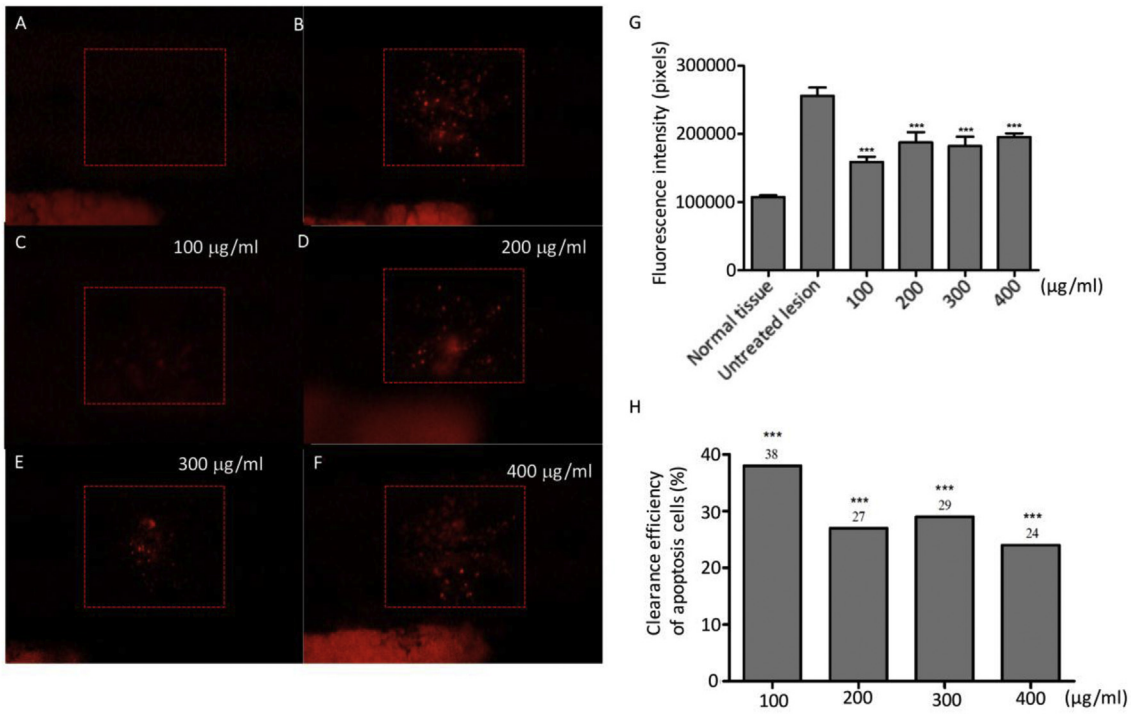

Fig. 2. Apoptotic cells were ablated after treating TY001 collagen mixture.
Cutaneous lesions were treated with or without TY001. Apoptotic cells were observed with TUNEL staining. Cutaneous lesion area was observed and quantified within red square region. (A) Normal skin tissue on 2 dpf zebrafish larva from lateral view (B) Cutaneous lesion without TY001 treatment (C-F) Cutaneous lesion with different concentration of TY001 as indicated. (G) Quantitative of TdT-stained cells within lesion regions (H) Percentage of apoptotic cell reduction compared to untreated lesion after treating with different concentration of TY001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Clearance efficiency of death cells on lesion in zebrafish.

Group	Concentration (μg/ml)	Cell death number (Mean ± SE, n = 10)	Clearance of death cells (%)
Normal tissue	–	4.4 ± 0.16	–
Untreated lesion	–	25.4 ± 1.44	–
TY001	100	10.7 ± 0.67***	58***
	200	15.5 ± 1.24***	39***
	300	15.4 ± 1.17***	39***
	400	14.4 ± 0.60***	43***

[1] Data compared to untreated lesion group, ***, $p < 0.001$.

Table 4
Clearance efficiency of apoptotic cells on lesion in zebrafish.

Group	Concentration (μg/ml)	Fluorescence intensity (TUNEL) (Mean ± SE, n = 10)	Clearance of apoptotic cells (%)
Normal tissue	–	107610 ± 2438	–
Untreated lesion	–	256020 ± 12085	–
TY001	100	158978 ± 7699***	38***
	200	187638 ± 15272***	27***
	300	182256 ± 13891***	29***
	400	195514 ± 5678***	24***

[1] Data compared to untreated lesion group, ***, $p < 0.001$.

facilitates healing repair and is important in maintaining skin homeostasis. While commercial readily lotions contain harmful compositions such as dihydroxyacetone (DCA), bleach, and heavy metals [20], the main component of TY001, collagen extract from tilapia, was comparably safe and efficient. Moreover, TY001 was the mixture of both animal and herbal peptides, which aimed to compensate the shortage of essential amino acids from different protein resources. While the wound healing was facilitated by great amount protein to perform repair process, TY001 showed high content of protein up to 80–90%. According to Liquid chromatography–mass spectrometry (LC/MS) analysis, two major type of peptides, Prolinly-Hydroproline (Pro-Hyp) and Hydroprolinly-Glycine (Hyp-Gly) were showed in TY001 with the ratio of 0.063–0.221. Moreover, among the peptides, TY001 was composed by 47% of peptide with size lower than 1 KDa, 24% of peptide within 1–3 KDa, 10% of peptide within 3–5 KDa, and 12% of peptide over 5 KDa. It has been proved that absorption of peptide was improved by smaller size and retention time in intestine [26].

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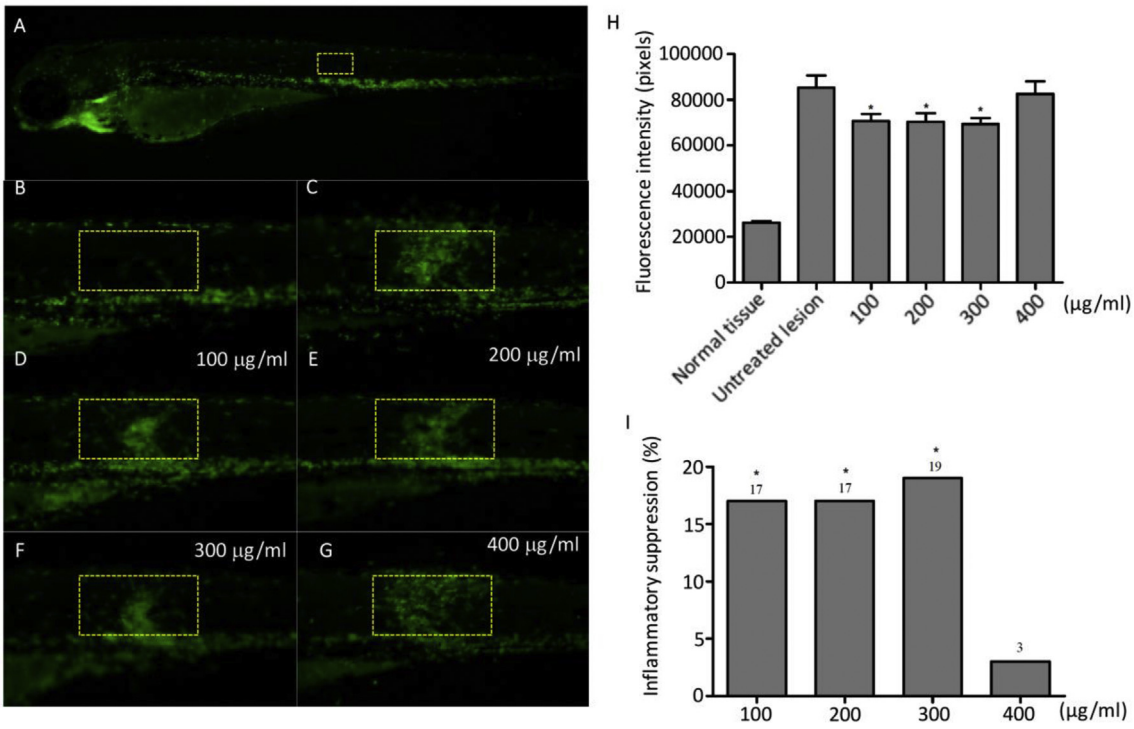

Fig. 3. Inflammatory cells were eliminated after treating TY001 collagen mixture.
Cutaneous lesions were treated with or without TY001. Macrophages and neutrophils were observed. (A) Cutaneous lesion area was observed and quantified within red square region. (B) Normal skin tissue on 2 dpf zebrafish larva from lateral view (C) Cutaneous lesion without TY001 treatment (D-G) Cutaneous lesion with different concentration of TY001 as indicated. (H) Quantitative cells within lesion regions (I) Percentage of suppression on inflammatory cells compared to untreated lesion after treating with different concentration of TY001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 5
Inflammatory suppression of TY001 collagen mixture in zebrafish.

Group	Concentration (μg/ml)	Fluorescence intensity (Mean ± SE, n = 10)	Suppression of inflammatory (%)
Normal tissue	–	26216 ± 674	–
Untreated lesion	–	85231 ± 5375	–
TY001	100	70631 ± 3099*	17*
	200	70370 ± 3724*	17*
	300	69339 ± 2652*	19*
	400	82516 ± 5534*	3

[1] Data compared to untreated lesion group, *, $p < 0.05$.

Therefore, the advantage of wound healing process by TY001 may also due to its small peptides that facilitate body absorption. To sum up, TY001 demonstrated significant benefits not only on cell proliferation and death cells clearance, but also inflammatory response suppression. It definitely showed promised wound healing activity and great potential on developing therapeutic application in the near future.

4. Material and methods

4.1. Animals

Zebrafish of wild type AB strain were applied in this study and all animal experiments were performed in accordance with the guidelines issued by the animal ethics committee (AAALAC Certificate NO.001458). Transgenic fish line of Tg(fli1:EGFP)^{y1} with EGFP expressed within blood vessel were used in angiogenesis assay. Transgenic fish line of Tg(coro1a:EGFP)^{hkz04t} with EGFP expressed within macrophage and neutrophils were used in inflammation assay. Both transgenic lines were obtained from China Zebrafish Resource Center (http://zfish.cn/). Two day-post-fertilization (dpf) embryonic zebrafish with the same batch of fertilization were used in this study. Embryos were collected and cultured in petri dishes containing fisher water (0.2% Instant Ocean Salt in deionized water, pH 6.9–7.2, conductivity 480–510 μS/cm, and hardness 53.7–71.6 mg/L $CaCO_3$) at 28 °C under 14 h on/10 h off light cycle.

4.2. Drug treatment and wound-healing assay

Two batches of fish collagen peptide derived from Tilapia, GBB 10SP (below 1000 Da) and GBB 50SP (3000–5000 Da), were purchased from Guangdong Baiwei Biotechnology Material Co., Ltd. GBB 10SP and GBB 50SP were used to prepare sample mixtures into different ratio (1822-1, 1:1; 1822-2, 4:1; 1822-3, 1:4). Granulated powder 1822-1, 1822-2, and 1822-3 were dissolved in water to 2 mg/ml stock and finally were diluted with culture water to working solution for later experiment. For wounding, zebrafish embryos aged at 2 dpf were anaesthetized in 0.13% Tricaine (w/v). A full thickness wound was introduced onto the intersection of anterior anal and dorsal fins by 10 nL of 5% acetic acid injection.

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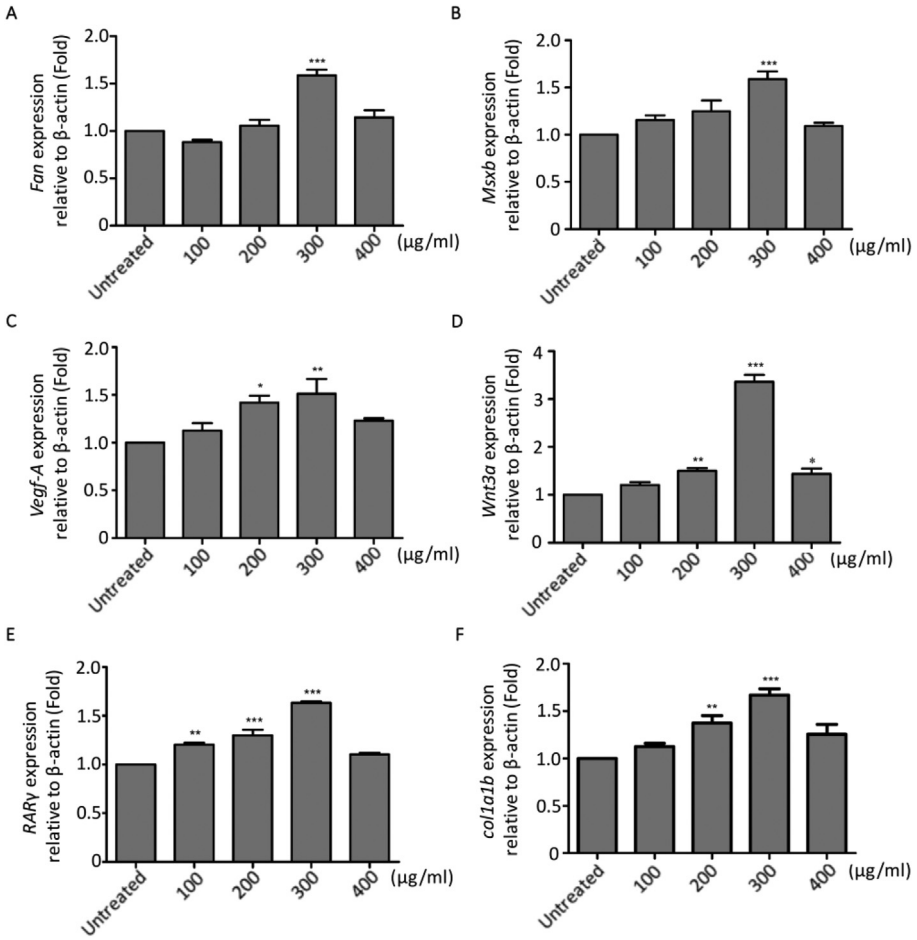

Fig. 4. Detection of marker gene expression by Real Time-PCR after TY001 collagen mixture treatment in zebrafish. Marker genes detected are (A) *Fan* (B) *Msxb* (C) *VegfA* (D) *Wnt3a* (E) *RARr* (F) *col1a1b*. β-actin gene is used as an house-keeping gene for expressional level normalization.

4.3. TY001 preparation and in vitro digestion

TY001 (patent granted by SIPO: 2017114143555) was composed by albumin concentrate, dehydrolyzed wheat protein, fish collagen, Calcium Caseinate, wheat oligopeptide, and casein hydrolyzed peptide, which blended with xanthan gum and silica. TY001 was dissolved in distilled water to prepare 10 mg/ml working concentration. 0.5 mol/L HCl was used to adjust pH value of TY001 solution to pH2.0. Protease (P110928, Aladdin) was added into TY001 solution with 1:40 ratio, and incubated in 37 °C for 120 min to mimic digestion in stomach. NaOH (0.5 mol/L) was used to adjust pH value to pH 7.5 to stop the reaction. The pancreatic protease (t10005532c protea) was added to mixture in 1:25 ratio and incubated in 37 °C for 120 min. The reaction was stopped by heating at 85 °C for 20 min.

4.4. BrdU assay

BrdU assay was performed in 10 mM solution with 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit I (11296736006, Roche). Fish was fixed overnight in 4%PFA overnight after BrdU and TY001 treated. The next day, fish was incubated for 30 min in 2 N HCl in PBST and 1 h prehybridization in PBST with 1% BSA and 5% FBS. The primary anti-BrdU antibody with nucleases (1:200) and secondary anti-mouse Ig-fluorescein antibody (1:2000) were used to detection.

4.5. Ethidium bromide staining

Zebrafish larvae were wounded and transferred into 6-well plate to treat with TY001 for 5 h. Larva then were incubated in 0.5 mg/ml ethidium bromide (V900319, Sigma) solution at dark for 30 min. Excess dyes were washed out from embryos three times.

4.6. TUNEL assay

TUNEL (Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling) was performed with In Situ Cell Death Detection Kit (C1089, Beyotime). Tissue was fixed with 4%PFA overnight, and dehydrated by methanol. The next day, tissue was rehydrated and then incubated in proteinase K (ST532, Beyotime) at 37 °C. An hour later, tissue was treated with 4%PFA to inactive proteinase K. Larvae were incubated overnight at 4 °C in labeling solution (TdT and fluorescein-conjugated deoxynucleotides). The next day, apoptotic cells were visualized under microscope in metal-enhanced DAB (Pierce).

5. Real Time-PCR

Thirty larvae after experiment were collected and homogenized in TRI reagent (1382739, Invitrogen, America) with homogenizer (TIANGEN, China) to isolate total RNA according to the manufacturer's instructions. Total RNA concentration was determined by spectrophotometry, and the RNA quality was checked by

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electrophoresis in denatured gels. For RT-PCR, 1 μg of total RNA was reverse-transcribed with FastQuant RT kit (KR106, TIANGEN, China) and then PCR was performed with iTaq Universal SYBR Green Supermix (1725121, Bio-rad, America) according to the manufacturer's instructions. PCR primers used to perform real time PCR are listed below: *β-actin* (5′TCGAGCAGGAGATGGGAACC3′ and 5′CTCGTGGATACCGCAAGATTC3′), *Fan* (5′GCAGCCATTAC TCTTCACCT3′ and 5′GCTATCACAGTCAACTCCTCC3′), *Msxb* (5′CTAAGGGACCCGTTGAAAC3′ and 5′CGCTTAGTTCTTCACTGTC3′), *vegfA* (5′CTCCTCCATCTGTCTGCT3′ and 5′GATACTCCTGGATGA TGTCTA3′), *wnt3a* (5′GAAACCGACCTCGTCTACTATGAAA3′ and 5′ACGTAGCAGCACCAGTGGAAA3′), *RARγ* (5′CCGACCCAACAAACCT CACA3′ and 5′TCCGTCCTCATCATCGTCATC3′) and *col1a1b* (5′GAAGCACGTCTGGTTCGG3′ and 5′CGTCCTCGCTGACTCTGTAT3′).

5.1. Image acquisition, quantification and statistics

Histological analysis of cutaneous of zebrafish embryo were performed to determine the effect of repair and regeneration. Transgenic fish were anesthetized in 0.13% Tricaine (w/v) and photographed for angiogenesis with microscope (AZ100, Nikon, Japan) equipped with a digital camera (TK-C1481EC) and a dissecting microscope (SZX7, Olympus, Japan). For quantifying, the original images captured at the wound position were processed using ImageJ software (http://rsbweb.nih.gov/ij/) and statistically compared using Dunnett's T test.

Author contribution

Conceptualization, J. L., Y-H L. and C-D H.; Methodology, X-y X and Y. L.; Validation, L-t S., Y-q X. and J. L.; Formal Analysis, X-y X. and Y. L.; Resources, J. L.; Data Curation, X-Y X and Y-q X; Writing, X-y X, Y-H L., and C-D H.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.09.148.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.09.148.

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