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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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