Original Article

Efficacy of Topical Application of Chum Salmon (*Oncorhynchus keta*) Skin-derived Collagen Extracts in Improving Oral Traumatic Ulcer Healing

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Abstract

Background: Traumatic ulcer is a wound on the oral mucosa that often causes pain and impaired eating function. Healing of these wounds takes a long time and can interfere with an individual's daily activities. One therapeutic approach that is being developed is the use of topical application of chum salmon skin-derived collagen extract. Collagen is the main component of the extracellular matrix and plays a major role in wound healing. The skin of chum salmon (*Oncorhynchus keta*) contains collagen that is effective for the treatment of wounds. **Aim:** The aim of this study was to evaluate the effectiveness of topical applications of chum salmon (*O. keta*) skin-derived collagen extracts in improving the healing of traumatic ulcers through analysis of neutrophil and macrophage numbers and collagen density. **Materials and Methods:** Twenty-four male Wistar rats were randomly divided into four groups consisting of six rats each. The labial mucosa of the lower lips of the rats was injured with heated amalgam stoppers to create oral traumatic ulcers. Group 1 was a control group; in Groups 2, 3, and 4, 25%, 50%, and 75% of collagen extracts from chum salmon (*O. keta*) skin were applied topically once a day for 7 days, respectively. The neutrophil and macrophage numbers were observed by hematoxylin and eosin staining. Masson's Trichrome staining was used to analyze the collagen density. Data were analyzed using one-way analysis of variance and continued with *post hoc* least significant difference tests. Significance is considered if $P < 0.05$. **Results:** The oral traumatic ulcers gradually healed until day 7. The number of neutrophils and macrophages was significantly decreased in the treatment groups, and collagen density was increased, compared to the control group ($P < 0.05$). The decrease of neutrophil and macrophage numbers occurred significantly with the increased collagen extract concentrations ($P < 0.05$). Collagen density also increased significantly with the increased collagen extract concentrations ($P < 0.05$). **Conclusion:** Topical applications of chum salmon (*O. keta*) skin-derived collagen extracts accelerate the healing process of oral traumatic ulcers by decreasing neutrophil and macrophage numbers and increasing collagen density.

Keywords: *Chum salmon (Oncorhynchus keta) skin, collagen density, macrophages, neutrophils, oral traumatic ulcer*

Introduction

Traumatic ulcers are the most common lesions of the oral mucosa, with a high prevalence of nearly 25% of the world's population.[1] Oral traumatic ulcers are caused traumatically, chemically, mechanically/physically, thermally, and by radiation.[2] The healing process consists of four phases that include hemostasis, inflammatory, proliferative, and remodeling.[3,4] Fibroblast cells will appear in the injured area on the 3rd day of the healing process and then increase on the 7th day, which can be seen histologically. Fibroblasts play an important role in wound healing, especially during angiogenesis, epithelialization, and collagen formation.[5] Collagen is important for all phases of the wound healing process, including extracellular matrix (ECM) formation.[6]

Traumatic ulcers are self-limiting conditions. However, the pain makes the patient uncomfortable, the ulcers can trigger malnutrition and they can become more susceptible to infection.[7] The main management of traumatic ulcers are causative therapy to eliminate the etiologic factors, symptomatic therapy to reduce or eliminate the symptoms, and supportive therapy to accelerate the healing process. Treatments currently used are analgesics or topical anesthetics, antiseptic mouthwashes, anti-inflammatory steroids, and antibiotics,

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if needed, to prevent infections due to thermal trauma.[7] However, the use of that treatment can cause negative side effects.

Mammals have been widely used as sources of collagen, but their safety in therapeutic materials remains questionable. Fish, starfish, sponges, and jellyfish are alternatives as safer sources of collagen, compared to mammals. The skin of chum salmon (*Oncorhynchus keta*) contains collagen that is effective for the treatment of wounds.[8-10] Oral administration of collagen isolated from chum salmon (*O. keta*) skin enhances cutaneous healing and angiogenesis in rats.[10] However, little is known about chum salmon (*O. keta*) collagen's potential to enhance oral traumatic ulcer healing. Thus, this study aimed to evaluate the effectiveness of the topical application of collagen extracts isolated from chum salmon (*O. keta*) skin to enhance the healing of traumatic ulcers through analysis of the numbers of neutrophils and macrophages, as well as collagen density.

Materials and Methods

Ethical approval

This study is a *post test*-only control group design study which received approval from the Ethical Clearance for Health Research Committee, Faculty of Dentistry, Hang Tuah University (No. 149/KEPK/XII/2018).

Experimental animal

Twenty-four 3-month-old male Wistar rats weighing 220–250 g were used and randomly divided into four groups of six animals each. The experimental animals were kept in cages with good lighting cycles, a constant room temperature of 25°C, 60%–70% relative humidity, and access to food and water *ad libitum*. Group 1 was a control group; in Groups 2, 3, and 4, 25%, 50%, and 75% of collagen extracts from chum salmon (*O. keta*) skin were applied topically once a day for 7 days, respectively.

Extraction of collagen from chum salmon (*Oncorhynchus keta*) skin

The skin of chum salmon (*O. keta*) was homogenized and continued with an emulsion process in distilled water. A protease complex was added at 40°C, pH 8 for 3 h before the inactivation process and filtration. Collagen powder was produced by the spray dryer method. The collagen powder was then put into a solution consisting of sodium carboxymethyl cellulose (CMC-Na), sorbitol, aquadest, and methylparaben and stirred for 10 min at 700 rpm using a mixer to form chum salmon (*O. keta*) skin extract gels with concentrations of 25%, 50%, and 75%.

Induction of oral traumatic ulcer

Each Wistar rat was anesthetized until it was asleep using ketamine and xylazine in a combined injection into the rat's thickest muscle. As much as 0.1 ml/g/BW of both the ketamine and the xylazine were used. The lower lips of the rats were injured with an amalgam stopper that was heated in a spirit burner for 1 min and then used to touch the labial mucosa of each rat for 1 s, lightly and without pressure. The rats were kept in cages with adequate food after they were freed from the anesthesia.

Sample collection

The rats were terminated after 8 days by anesthetic overdoses with a ketamine 300 mg/kg and xylazine 30 mg/kg intraperitoneal injection. The labial mucosa of the lower lips was cut until the corners of the rats' mouths included the ulcers and the normal parts. The samples were put into the fixation solution and then immersed in a 10% formalin buffer for 24 h.

Histopathological examination

Histological examinations were performed to count the number of neutrophils and macrophages by means of hematoxylin and eosin staining (Sigma-Aldrich; St. Louis, MO), while the density of the collagen was determined using Masson's trichrome staining (Sigma-Aldrich; St. Louis, MO). The pathological changes were observed using an Olympus light microscope with a × 400 and five fields of view. The readings of the densities of the collagen fibers in the lamina propria layer were completed using a phase contrast microscope (Olympus B × 41, Olympus, Japan) equipped with a DP-70 digital camera and using OLYSIA Software with a × 400.

Statistical analysis

All data were expressed as mean ± standard deviations. Statistical analysis was performed using one-way analysis of variance and continued with *post hoc* least significant difference tests. Significance is considered if $P < 0.05$.

Results

The histopathological examination of the number of neutrophils and macrophages is shown in Figures 1 and 2, and the collagen density is shown in Figure 3. The results showed a significant difference in neutrophil numbers [Table 1], macrophage numbers [Table 2], and collagen density [Table 3] between treatment groups and the control group ($P < 0.05$).

A decrease in the numbers of neutrophils and macrophages occurred significantly as the collagen extract concentrations were increased ($P < 0.05$). Collagen density also increased significantly with increased collagen extract concentrations ($P < 0.05$).

Discussion

Traumatic ulcers are very common lesions that occur in the mouth due to trauma to the epithelium lining of the oral cavity. Traumatic ulcers are secondary lesions characterized when the epithelium layer exceeds the basement membrane or reaches a free nerve ending in the lamina propria, causing

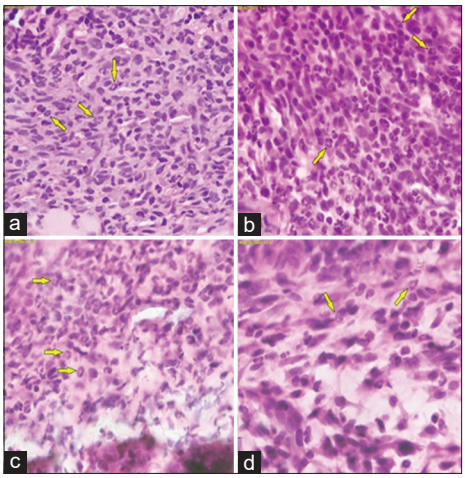

Figure 1: Histopathology analysis of the number of neutrophils in the lower lip mucosa of rats with hematoxylin and eosin staining, (a) Group 1 (Control): (b) Group 2 (c) Group 3 and (d) Group 4

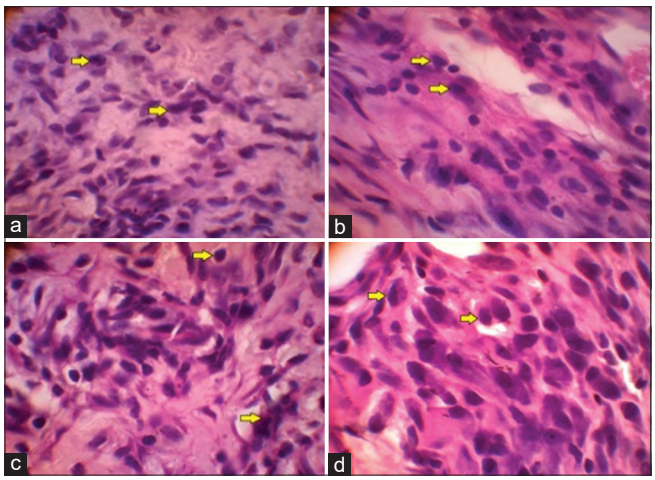

Figure 2: Histopathology analysis of the number of macrophages in the lower lip mucosa of rats with hematoxylin and eosin staining, (a) Group 1 (Control): (b) Group 2 (c) Group 3 and (d) Group 4

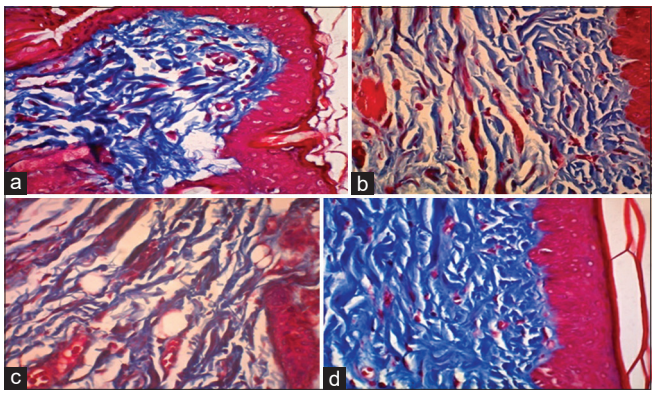

Figure 3: Histopathology analysis of collagen density in the lower lip mucosa of rats with Masson's Trichrome staining, (a) Group 1 (Control): (b) Group 2, (c) Group 3, and (d) Group 4

severe pain.[11] This process begins when the trauma injures the mucosa, and then inflammation follows as the body's first defense action to protect the mucosal tissue.[12-14]

Table 1: The mean and standard deviation of the calculation of the number of neutrophils in each treatment group

Group	Mean±SD
Group 1	88.8000±1.6721
Group 2	42.8400*±1.3933
Group 3	40.2000*±1.8201
Group 4	31.4400*±3.5844

*Significant versus control ($P \leq 0.05$). Data are mean±SD (n=6). SD: Standard deviation

Table 2: The mean and standard deviation of the calculation of the number of macrophages in each treatment group

Group	Mean±SD
Group 1	20.8000±1.0421
Group 2	12.4000*±1.2123
Group 3	10.2000*±1.3021
Group 4	8.4000*±3.0144

*Significant versus control ($P \leq 0.05$). Data are mean±SD (n=6). SD: Standard deviation

Table 3: The mean and standard deviation of the collagen density in each treatment group

Group	Mean±SD
Group 1	2061.3413±223.57772
Group 2	2419.3688*±298.06795
Group 3	3422.4875*±447.55264
Group 4	4542.7775*±182.40623

*Significant versus control ($P \leq 0.05$). Data are mean±SD (n=6). SD: Standard deviation

In the present study, topical applications of collagen extracts from the skin of chum salmon (*O. keta*) increased the healing of traumatic ulcers by decreasing neutrophil and macrophage numbers and by increasing collagen density. Wound healing is a series of processes with very high and precise dynamics consisting of stages that follow one another. It begins with the inflammation stage, then moves to cell proliferation, then to matrix deposition, and then ends with the remodeling phase. All phases of repair, including the inflammatory phase, wound regeneration by new epithelium, and granulation tissue formation, are part of a complex cycle influenced by growth factors and cytokines that move directly to the wound area.[15] The normal wound healing cascade begins with a sequential process of hemostasis and a deposition of fibrin, followed by a cascade of inflammatory cells specified by neutrophils, macrophages, and lymphocytes in the wound area. This process then ends, and a proliferation of fibroblasts is followed by the accumulation and remodeling by collagen until scar maturation occurs.[16]

Collagen plays an important role in the wound-healing process. Collagen has, therefore, been used as an adjunctive therapy to promote wound healing due to its low

biocompatibility and immunogenicity, its ability to recruit wound-healing response cells, and its ease of use. The use of animal collagen products is associated with serious drawbacks such as allergic reactions, transmissions of prion disease, and microbial contaminations.[17] Therefore, natural and engineered sources of collagen have been investigated.

Chum salmon (*O. keta*) is a marine animal that is rich in a protein that resembles a collagen protein group consisting of glycine, proline, and hydroxyproline.[18] Chum salmon (*O. keta*) was used to make fish extracts in the form of gelatines – protein derivatives developed by collagen hydrolysis. The gelatines have three-dimensional bonds between the collagen molecules that are broken into an assembly that is easier to rearrange.[19,20]

Collagen degradation in the wound healing processes is controlled by extracellular and intracellular pathways.[21] The extracellular pathways include membrane-bound and secreted proteolytic enzymes. The intracellular pathways include internalization of intact and fragmented collagen, followed by enzymatic breakdowns.[22-25] Throughout the inflammatory phase, soluble fragments of collagen degradation induct macrophages into the wound to phagocyte microbes and eliminate damaged tissue. This process initiates the transition from the inflammatory phase to the proliferative phase. During the proliferative phase, collagen fragments act as strong angiogenic signals to support the new blood vessel formation and keratinocyte migration that assist the reepithelialization.[26-28]

Collagen exposure due to injury initiates a clotting cascade and the creation of a fibrin clot that stops the initial bleeding. Collagen I and IV fragments are inflammation mediators that act as potent neutrophil chemoattractants, increase phagocytosis and immune responses, and act as gene expression modulators.[29,30] Inflammation is an important process in wound healing because it promotes the fibroblast proliferation that synthesizes collagen and ECM.[31] Healing the inflammation at the right time determines the process of wound healing. Resolution of inflammation is a process navigated by a balance of pro- and anti-inflammatory responses. Collagen promotes a temporary strong and sharp inflammatory response that quickly heals wounds.[32] In addition, collagen has a crucial role in promoting the anti-inflammatory phase by supporting the chemotaxis of neutrophils and macrophages.[33,34]

The skin of chum salmon (*O. keta*) can serve as an alternative source of protein, mainly as gelatine. The uniqueness of chum salmon (*O. keta*) skin-derived gelatine is in its amino acid content.[35] Gelatine obtained from salmon skin extracts with acetic acid will reduce the inflammatory response.[19,20] The inflammatory process occurs very quickly and lasts for 3 days. In the chronic inflammatory phase, macrophages clean the wound edges of the damaged cells. Activated macrophages will induce the growth factor and cytokines at the wound site, further amplifying and maintaining the activity of chemical and cellular mediators previously expressed by the degranulation of platelets and neutrophils.[18]

Wound closure requires a complex and extraordinary communication of cells, ECM, and cytokines. In this study, increased wound closure rates in wounds treated with salmon skin gelatine were associated with an increased proliferative phase and the conversion of fibroblasts to myofibroblasts. Fibroblasts are important for ECM synthesis, deposition, and remodeling. After migrating to the wound, fibroblasts begin synthesizing ECM.[24]

Collagen is a major structural component of ECM protein and is the component that has a role in wound strength. Increased hydroxyproline levels of gelatines can impart strength to regenerating tissue, thereby speeding up the wound closure process.[18] We have limitations in our study. In this study, we only observed the expression of neutrophils, macrophages, and collagen density on day 7. This is why future research should be conducted on more observation days to provide more in-depth insights on traumatic ulcer healing mechanisms by the topical applications of collagen extracts from the skin of chum salmon (*O. keta*).

Conclusion

Topical applications of 25%, 50%, and 75% chum salmon (*O. keta*) skin-derived collagen extracts accelerate the healing process of traumatic ulcers by decreasing neutrophil and macrophage numbers and increasing collagen density.

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Conflicts of interest

There are no conflicts of interest.

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