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Microstructure, rheological and wound healing properties of collagen-based gel from cuttlefish skin

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ABSTRACT

Collagen-based biomaterials are of the utmost importance for tissue engineering and regenerative medicine. The aims of the present investigation were to evaluate structural and rheological properties of collagen-based gel obtained from cuttlefish skin, and to investigate its ability to enhance wound healing. Scanning electron microscopy of resulted gel showed a dense fibrillar microstructure with high interconnection network with a smaller pore size. In addition, the rheological characterization of collagen gel showed an excellent reversibility, when subjected to a temperature variation. Moreover, in the wound-healing study, topical application of collagen based gel increased significantly the percentage of wound closure over a period of 12 days, when compared to the untreated and CICAFLORA®-treated groups. Wound-healing activity of collagen gel was confirmed by histopathology study. Thus, cuttlefish collagen based gel might be useful as a wound healing agent.

1. Introduction

Collagen is commonly used in medical and pharmaceutical industries as carrier molecules for drugs, proteins and genes [1]. Especially, microfibrous collagen sheets are used as promising drug carrier for the treatment of cancer [2]. In fact, collagen qualified as an excellent material for wound healing due to its biodegradable and biocompatible properties. Hence, implanted collagen will be degraded through native enzymatic pathways without any toxic response. In order to produce collagen biomaterials, different methods were developed to extract collagen from bio-sources. Modern extraction approaches are based on three basic principles of solubilization: acidic solution [3], neutral salt solution [4] and proteolytic solution [5].

The utilization of fish skin collagen and gelatin is expected to attract the interest of the industry as an alternative source. This may be due to comparative unpopularity of porcine skin collagen and gelatin in relation to some religious reasons. At the same time, use of bovine derived collagen and gelatin are also in active discussion due to the mad cow disease, bovine spongiform encephalopathy (BSE) and the risk they pose in human. In contrast, fish collagen and gelatin have relatively a low risk of possessing unknown pathogens such as BSE.

A wound is a break in the normal tissue continuum, resulting in a variety of cellular and molecular sequels. The wound may be created by physical, chemical, thermal, microbial, or immunological, abuse of the tissue [6]. Wound healing is a complex multifactorial process involving inflammation, proliferation, remodeling, which behave in a harmonious way in order to guarantee tissue reparation. The process of wound healing may be hampered by the presence of free radicals, which can damage wound surrounding cells [7]. Many biomaterials used as skin substitutes represented natural components existing in the wound that will be activated during the healing process. Recently, gelatin forming gel has also been investigated successfully for its use in wound healing alone [8]. Due to its intrinsic biological properties and biocompatibility, this material is advantageous.

Results of previous study investigating the production of gelatin from the skin of cuttlefish proved that this protein can serve as a potential biomaterial [9]. In addition, advanced researches are carrying out to find novel source of collagen, with a potential tissue repairing ability [10–13].

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The aims of this paper were to evaluate structural and rheological properties of collagen based gel, obtained from cuttlefish skin, and to investigate its ability to enhance wound healing.

2. Materials and methods

2.1. Cuttlefish skin preparation

Cuttlefish by-products were obtained from marine processing industry "IMPEX" located in Sfax City, Tunisia. The samples were packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w). They were washed twice with water to eliminate the dark ink. Finally, cuttlefish outer skin was collected and then stored in sealed plastic bags at −20 °C until used for gelatin extraction and analysis.

2.2. Collagen extraction

In order to remove non-collagenous proteins, washed skins were first soaked in 0.05 M NaOH with a skin/solution ratio of 1/10 (w/v) for 2 h at 4 °C and the solution was changed every 30 min. The alkaline treated skins were then washed with cold tap water until neutral pH wash water was obtained. The alkaline-treated skins cuttlefish were soaked in 0.1 M acetic acid with a solid/solvent ratio of 1:10 (w/v), as described by Jridi et al. [9]. The mixture was stirred for 48 h at 4 °C. Finally, the pH of the mixture was raised to 5 using 10 M NaOH and stirred gently for 1 h at 4 °C.

The extracted solution was filtered with a double layer of gauze and the filtrate was centrifuged at 10,000 × *g* for 30 min. The filtrate was then freeze dried (Bioblock Scientific Christ ALPHA 1-2, IllKrich-Cedex, France) and the resulting extract was used for further investigations.

2.3. Viscoelastic properties

Dynamic studies were performed on an AR1000 Rheometer (Physica MCR-301, Anton Paar, Germany) using a cone-plate geometry (cone angle 2°). Collagen solution at 6.67% (w/v) was prepared by dissolving in distilled water under continuous stirring at 45 °C for 20 min. The viscosity measurement was performed at a scan rate of 1 °C/min, frequency 1 Hz, oscillating applied stress of 3.0 Pa and gap 0.15 mm. During the testing, the gel was heated from 5 to 50 °C, cooled from 50 to 5 °C and then kept at 5 °C for 10 min. The tan δ was calculated from the ratio of G″ and G′ obtained from frequency sweep tests and result was represented as a function of temperature.

2.4. Scanning electron microscopy

Collagen based gel microstructure was visualized using a scanning electron microscope (Cambridge Scan-360 microscope) at an accelerating voltage of 3.0 kV. The sample was frozen under liquid nitrogen. Prior to visualization, sample was mounted on brass stub and sputtered with gold in order to make the sample conductive. Samples were photographed with an angle of 90° to the surface to allow observation of the films cross section.

2.5. In vivo *wound healing study*

2.5.1. Animals

Healthy young female Wistar rats weighing between 150 and 200 g were housed in individual clean polyethylene cages under controlled conditions (12 h high-dark cycle at 22–25 °C and 60–70% relative humidity). Animals were left for 2 weeks at room conditions for acclimatization. They were maintained on standard pellet diet and water throughout the experiment. Procedures and animals comfort were controlled by the International Guidelines for Animal Care.

2.5.2. Excision wound healing model

After total anesthesia with ketamine (100 mg/kg body weight) by intramuscular injection, a circular area of approximately 150 mm^2 wound was made on depilated thoracic region of rats. The wounding day was considered as day 0. The animals were housed individually and periodically weighted before and after the experiment. The ointment was topically applied every two days till the complete epithelization.

2.5.3. Wound healing activity

Eighteen rats in all were used in the study. They were divided into three groups consisting of six animals each. Group I was untreated and served as the control (just cleaning the wounds with a physiologic serum). Group II was treated with "CICAFLORA®" and served as a reference standard (positive control). Group III was treated with cuttlefish skin collagen gel (6.67%).

After rinsing wounds with physiologic serum, the collagen gel and the standard drug (CICAFLORA®) were applied, in a fine layer covering the surface of the wound, every two days till the wound was completely healed. On the last day, all the rats were anaesthetized with ether, sacrificed and the granulation tissues were excised from the sacrificed animals. A part of wet tissue was preserved for hydroxyproline estimation and another one was fixed in formalin 10% (v/v), embedded in paraffin and processed for histological observation.

2.6. Wound healing evaluation parameters

2.6.1. Chromatic study

This study consisted on attributing a chromatic code to each wound rate as the following: bright red = blood covering the wound; dark red coagulation of blood in the epidermis, red = granulation tissue and pink epithelialization step [14].

2.6.2. Wound contraction and epithelialization time

An excision wound margin was traced after wound creation by using transparent paper and area was measured [15]. Wound contraction was measured every 2 days interval, until complete wound healing and expressed in percentage of healed wound area. The percentage of wound closure was calculated using the following expression:

$$\text{Wound closure}\,(\%) = \frac{A_0 - A_d}{A_0} \times 100$$

where A_0 and A_d are the initial wound area (day 0) and the area of wound on day (d), respectively. The period of epithelization was calculated as the number of days required for falling of the dead tissue without any residual raw wound.

2.6.3. Hydroxyproline estimation

Hydroxyproline content of tissue samples was estimated according to the method of Lee and Tong [16]. Wound tissues were dried in a hot air oven at 60–70 °C to a constant weight and then hydrolyzed in 6 N HCl for 4 h at 130 °C in sealed glass tubes. The hydrolysates were neutralized to pH 7.0 and then were subjected to Chloramine-T oxidation for 20 min. The reactions were terminated by addition of 0.4 M perchloric acid and the color, developed with the help of Ehrlich reagent at 60 °C, was measured at 650 nm using a spectrophotometer. Hydroxyproline concentrations were calculated from the linear standard curve and presented as mg/100 mg of dry tissue weight.