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Research Article

ANTIOXIDANT ACTIVITIES AND *IN VITRO* WOUND HEALING EFFECTS OF META COLLAGEN

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ABSTRACT. The imbalance of reactive oxygen species (ROS) may cause oxidative stress which is harmful to skin health. Moreover, oxidative stress could also impair the healing of a wound. Antioxidants have been demonstrated their benefits to skin health and wound healing. Previous studies have shown collagen tripeptide has beneficial effects on skins, such as alleviating photoaging, improving skin elasticity and hydration, improving type I collagen synthesis and more. In this study, anti-oxidant activities and *in vitro* wound healing effects of Meta collagen (Highly content dipeptide and tripeptide) were investigated. The skin irritation test of Meta collagen was also studied. In the DPPH scavenging assay, Meta collagen could scavenge DPPH dose-dependently and showed the anti-oxidant activity. Meta collagen could enhance the migration of NIH-3T3 fibroblasts, suggesting its *in vitro* wound healing effects. Meanwhile, Meta collagen (1000 mg/mL) is considered as non-irritant to skin by using a reconstructed human epidermis. According to these results, Meta collagen shows anti-oxidant activities and *in vitro* wound healing effects without skin irritation. Meta collagen might be a potential material to be developed as a skin care product in the future.

Keywords: *Collagen, antioxidant, wound healing, skin irritation.*

INTRODUCTION

Reactive oxygen species (ROS) are molecules derived from oxygen, including non-radical and free radical species. ROS include singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{-\bullet}$), hydroxyl radical ($^\bullet OH$), hydrogen peroxide (H_2O_2) and more [1, 2, 3]. ROS are byproducts generated from the respiratory chain in mitochondria of cells. ROS are generated in many physiological reactions, such as aerobic metabolism, inflammation, antimicrobial response, and more. Many factors (e.g. visible light, ultraviolet, visible light, infrared lights, ozone, pollutants and more) could induce the generation of ROS in skin [2, 4]. Excess ROS could induce cell death and damages [5]. The loss of balance between the production and the neutralization of ROS in the body would lead in oxidative stress [6]. Oxidative stress in the skin is harm to skin health and plays important roles in

the ageing of skin. Antioxidants which could scavenge ROS could be beneficial to skin health and alleviate the ageing process [7].

When injuries occur in skin, a wound would be generated. After a wound occurs, the wound healing processes would begin, including coagulation, inflammation, proliferation and tissue remodeling [8, 9]. Suitable level of ROS play important roles in the wound healing processes, e.g., inflammation and proliferation. However, oxidative stress could cause chronic inflammation and then results in chronic wounds[10]. Growth factors or substances that could improve proliferation and migration of fibroblasts, endothelial cells, and keratinocytes would be beneficial to the wound healing [10]. Antioxidants have been also shown to be beneficial to the healing of wounds [1, 11, 12].

Collagen and gelatin (a degraded form of collagen) have been used in many areas, such as the fabrication of hydrogel wound dressings and more [13]. Recent studies have shown that peptides obtained from protein hydrolysis of collagen are functional in health. For example, collagen tripeptide has been used as a functional food material and has been shown several beneficial effects on skins. Oral supplement of collagen tripeptide could alleviate photoaging and improve the functions of epidermal skin barrier using UVB-exposed hairless mice [14]. In a prospective, randomized, controlled clinical study, oral supplement of collagen tripeptide may improve skin elasticity and hydration [15]. Collagen tripeptide could improve Hs68 fibroblasts to produce type I collagen [16].

In this research, we firstly report the antioxidant activities and *in vitro* wound healing effects of Meta collagen, a novel material comprising a high content of dipeptides and tripeptides. We demonstrated the anti-oxidant activities of Meta collagen through showing its scavenging effects on reactive oxygen species. Moreover, we described the *in vitro* wound healing effects of Meta collagen through enhancing the migration of fibroblasts. On the other hand, Meta collagen is a non-irritant for the skin from the results using a 3D skin tissue model. According to the above results, we believe to offer a valuable information that Meta collagen may be a potential material for skin health.

MATERIALS AND METHODS

Chemicals and reagents

All Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise mentioned. Materials for cell culture were purchased from Corning (Corning, NY, USA). Transforming Growth Factor-β1 (TGF-β) was purchased from R&D Systems (Minneapolis, MN).

Sample preparation

Meta collagen was provided by Jellice Pioneer Private Limited Taiwan Branch (Singapore). Brief manufacturing method was shown as below: skins of fish or pigs were crushed and sieved. Further use of acid or alkali degradation and extraction to obtain gelatin. A further step using enzymatic hydrolysis to obtain Meta collagen (Highly content dipeptide and tripeptide, e.g. Gly-x-y).

DPPH scavenging assay

DPPH was a hydrogen acceptor and used for evaluating antioxidant ability of test substances [17, 18]. DPPH (Sigma, D9132) was dissolved in ethanol to the concentration of 0.2 mM. A serial concentration of Meta collagen, Vitamin C or vehicle control were

prepared and 100 μL of each sample were added in 96 well plates. The absorbance at 517 nm (OD517) was measured by a photo-spectrometer (FlexStation 3, Molecular Devices) as background. One hundred microliter of 0.2 mM DPPH were added to each sample to a final volume of 200 μL in 96 well plates. After reaction at room temperature for 30 minutes in the dark, the final OD517 was measured. After subtracting the background from the final OD517 to obtain $D517_{\text{-background}}$, the DPPH scavenging ability was calculated by the following equation: (1- [$OD517_{\text{-background}}$ of test article / $OD517_{\text{-background}}$ of vehicle control]) x 100%. All studies were performed in triplicate wells.

Cell culture

NIH-3T3 cells were purchased from the Bioresource Collection and Research Center (BCRC, Taiwan). NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium which contained 10% fetal bovine serum and 1% Penicillin-Streptomycin. The cells were cultured in a humidified incubator at 37 °C and 5% CO_2.

In vitro wound healing assay

NIH-3T3 cells were seeded onto 24-well cell culture plates with SPLScar™ Block (SPL Life Sciences). When the cell growth reached confluence, the SPLScar™ Block was moved to generate a wound area and then photographed. Meta collagen, TGF-β or vehicle control were then added the cell medium. After cultured at 5% CO_2 and 37 °C for 8 hours, the wound areas were photographed again. The initial wound area and final wound area after exposed to each treatment were determined by imageJ. The percentage of wound closure was calculated through subtracting the initial wound area to the final wound area as the following equation: Wound Closure = $Area_{t=0} - Area_{t=8h}$. The relative wound closure compared to vehicle control group was calculated as the following equation: (Wound Closure of test article - Wound Closure of vehicle control) / Wound Closure of vehicle control x 100% [19].

Skin irritation test

The assessment of skin irritation of Meta collagen was determined by using a reconstructed human epidermis (RHE). The 3D skin tissue model (EpiDerm™) provided from MatTek Corporation was used this study. The operating procedures were performed according to the manufacturer [20]. The operating procedures were based upon the OECD guideline, "*In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method" (OECD TG 439)[21]. Briefly, 30 μL DPBS (negative control), 5% SDS (positive control) or 1000 mg/mL Meta collagen were added onto three reconstructed human epidermis tissues, respectively. After exposing to each treatment for 60 minutes, each tissue was rinsed with DPBS to remove the samples on tissues. The tissues were incubated at 37°C and 5% CO_2 for 24 hours. The culture medium was refreshed and the tissues were incubated for 18 hours. Then, tissues were collected and MTT assay was performed. After exposing 1 mg/mL MTT for 3 hours, formazan was extracted using isopropanol. Two formazan extract aliquots were taken from each tissue extract. The absorbance at 570 nm (OD570) of tissues extract was measured by a photo-spectrometer (FlexStation 3, Molecular Devices). The mean tissue viability of three skin tissues after exposed to the Meta collagen compared to negative control was used to predict the irritancy potential. If the mean relative viability was more than 50% of the negative control, the test substance would be considered not to cause irritation to skin [21].

Statistics analysis

Graphical and data analysis were processed with Excel and GraphPad Prism. All data were expressed as the mean with the standard deviation (mean ± SD). Student t-test was conducted to examine significant differences between groups. If the *p*-value was less than 0.05, statistical significance will be determined.

RESULTS AND DISCUSSION

Anti-oxidant activity of Meta collagen

Substances with anti-oxidant activity of could inhibit oxidative stress and may be beneficial in skin health, skin aging, wound healing and more [7, 22]. In order to investigate the anti-oxidant activity of Meta collagen, the effect of Meta collagen on scavenging DPPH, a free radical with hydrogen acceptor capability, was studied. In the DPPH scavenging assay, the DPPH scavenging abilities of 6.25, 12.5, 25, 50 and 100 mg/mL Meta collagen were 13.98 ± 5.51%, 19.77 ± 0.44%, 35.14 ± 1.53%, 55.54 ± 2.79% and 78.21 ± 3.08%, respectively (Table 1, Fig 1). The results showed Meta collagen could scavenge DPPH dose-dependently, suggesting the anti-oxidant activity of Meta collagen. One the other hand, the positive control (10 mM Vitamin C) showed the DPPH scavenging ability of 93.07 ± 0.44% (Table 1, Fig 1).

Table 1. *Effects of Meta collagen on DPPH scavenging assay.*

Treatments / N		Background corrected OD517			DPPH scavenging activity (%)			
		1	2	3	1	2	3	Mean ± SD
Vehicle control		0.285	0.304	0.311	5.92	-1.64	-4.28	0 ± 5.29
10 mM Vitamin C		0.053	0.054	0.054	93.58	92.82	92.82	93.07 ± 0.44**
Meta collagen (mg/mL)	**6.25**	0.25	0.273	0.278	20.28	11.59	10.08	13.98 ± 5.51*
	12.5	0.251	0.252	0.254	20.28	19.52	19.52	19.77 ± 0.44*
	25	0.211	0.208	0.217	35.39	36.52	33.50	35.14 ± 1.53***
	50	0.169	0.156	0.158	52.39	57.68	56.55	55.54 ± 2.79****
	100	0.098	0.096	0.11	79.60	80.35	74.69	78.21 ± 3.08****

** p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 compared to vehicle control by Student's t-test.*

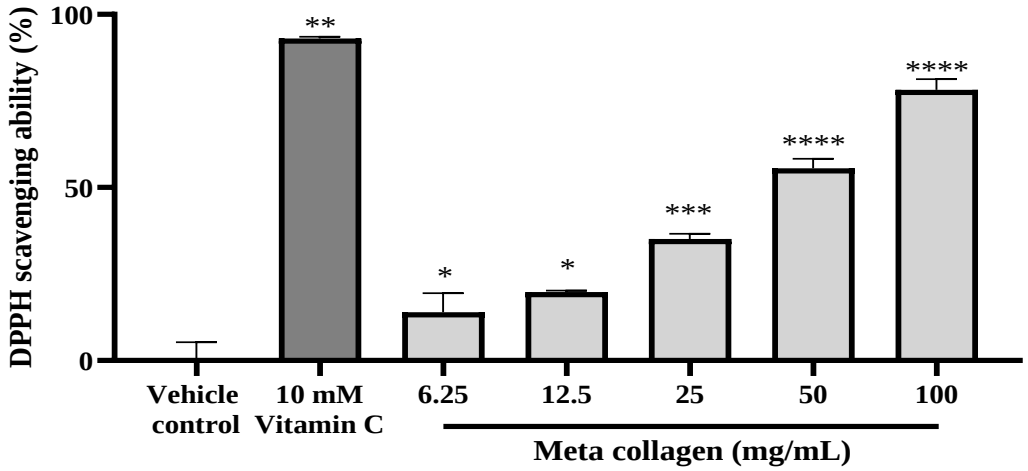

Fig. 1. *Effects of Meta collagen on DPPH scavenging assay. Values are demonstrated as mean ± SD. n = 3 in each group. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 compared to vehicle control by Student's t-test.*

In vitro wound healing activities of Meta collagen

The migration of fibroblasts toward a wound contributes the healing of a wound. A substance which could enhance the migration of fibroblasts may promote wound healing. In order to evaluating the effects of Meta collagen on the migration of fibroblasts, NIH-3T3 fibroblasts were used [23]. After creating a wound area on the NIH-3T3 cell monolayer through using SPLScar™ Block, the migration of NIH-3T3 fibroblasts were measured to calculate the relative wound closure compared to control group. After treated with the positive control (10 ng/mL TGF-β), the relative wound closure compared to control group was 163.0 ± 8.6% with statistical significance (Table 2, Fig 2). On the other hand, the relative wound closure of 1, 2, 10 and 20 mg/mL Meta collagen groups were 107.5 ± 14.6%,113.5 ± 12.9%, 115.4 ± 8% and 121.5 ± 11.7%, respectively (Table 2, Fig 2). Moreover, the relative wound closure of 10 and 20 mg/mL Meta collagen were significantly higher than the control group. These results suggest Meta collagen could enhance the migration of NIH-3T3 fibroblasts.

Table 2. *Effect of Meta collagen on NIH-3T3 fibroblasts migration in an in vitro wound healing assay.*

		Wound closure (μm²)			**Relative wound closure (%)**			
Treatments / N		1	2	3	1	2	3	**Mean ± SD**
Vehicle control		179947	180016	158597	104.1	104.1	91.8	100.0 ± 7.1
10 ng/mL TGF-β		289775	264658	290797	167.6	153.1	168.2	163.0 ± 8.6***
Meta collagen (mg/mL)	**1**	194743	205470	157269	112.7	118.9	91.0	107.5 ± 14.6
	2	209982	208285	170500	121.5	120.5	98.6	113.5 ± 12.9
	10	190639	192234	215361	110.3	111.2	124.6	115.4 ± 8.0*
	20	212187	228997	188645	122.8	132.5	109.1	121.5 ± 11.7*

*** *p<0.001, * p<0.05 compared to vehicle control by using Student's t-test.*

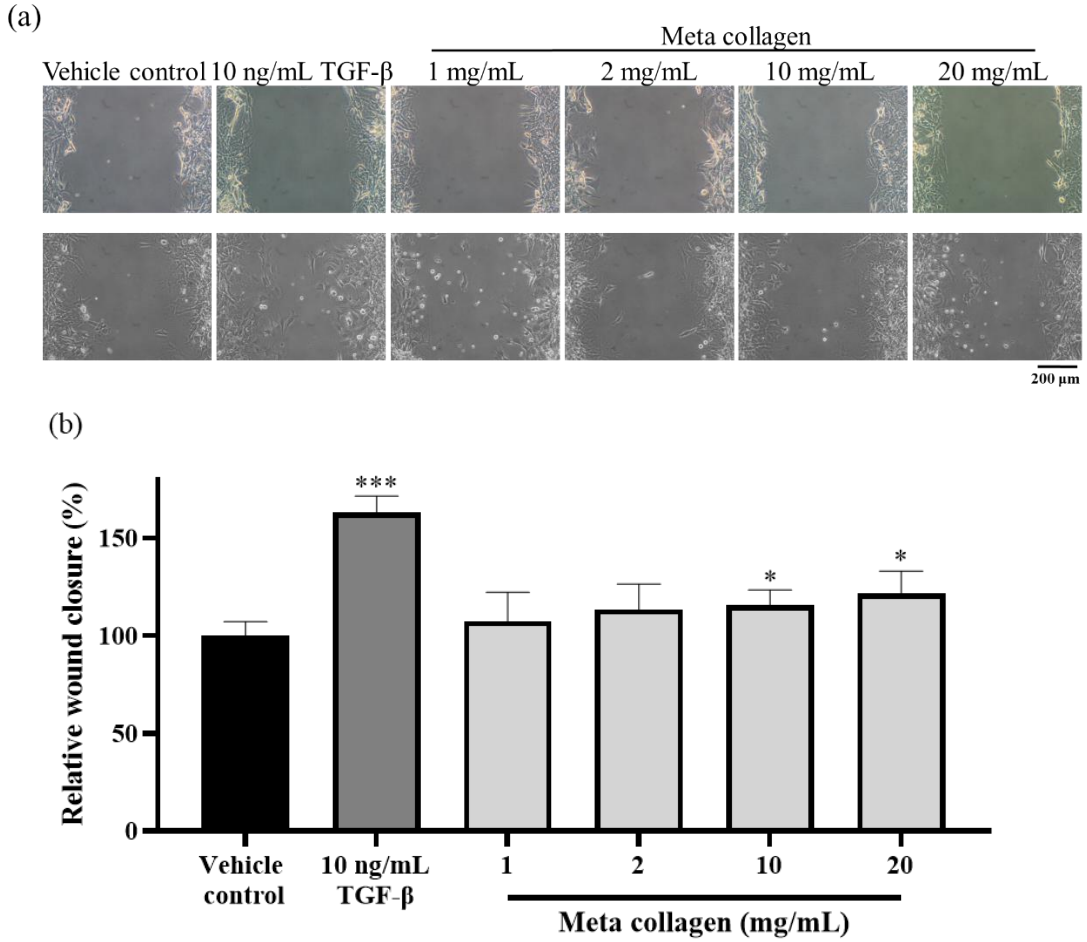

Fig. 2. *Effect of Meta collagen on NIH-3T3 fibroblasts migration in an in vitro wound healing assay. (a) The images of each group under 100 times magnification (100x). (b) The relative wound closure of each group. Positive control: 10 ng/mL TGF-β. Results are shown as mean ± SD, n = 3 in each group. *** p<0.001, * p<0.05 compared to vehicle control by using Student's t-test.*

Skin irritation test

Skin irritation means reversible damages to the skin caused by skin application of a substance or mixture [21]. A substance or mixture which would cause skin irritation should be avoid to be used as a product for skin applications. In order to investigate whether Meta collagen may cause skin irritation, *in vitro* skin irritation test (OECD TG 439) using the reconstructed human epidermis was used in the study. After exposing DPBS (negative control), 5% SDS (positive control) and 1000 mg/mL Meta collagen according to the operating procedures provided by the manufacturer of the reconstructed human epidermis, the relative viability of the reconstructed human epidermis exposed to positive control (5% SDS) was 2.36 ± 0.11% of the reconstructed human epidermis exposed to negative control (Table 3 and Fig. 3). On the other hand, the relative viability of the reconstructed human epidermis exposed to 1000 mg/mL Meta collagen was 94.12 ± 3.83% when compared to negative group (Table 3 and Fig. 3). If the mean relative viability less than 50% of the negative control, the test substance is considered to be an

irritant to skin, otherwise it will be considered to be non-irritant to skin according to OECD TG 439. Accordingly, 1000 mg/mL Meta collagen is considered not to cause irritation to skin but 5% SDS is an irritant to skin. The effective concentration of Meta collagen in scavenging DPPH and improving the migration of fibroblasts are lower than the tested concentration of Meta collagen in the skin irritation test. The results demonstrated Meta collagen might not cause irritation to skin when be used as a product for skin care.

Table. 3. *The data of EpiDerm™ tissues exposed to different controls and 1000 mg/mL Meta collagen.*

Treatments	Tissue N	Blank corrected OD570 Aliq. 1	Aliq. 2	Mean of aliquots (OD570)	Relative viability (%)	Mean	SD
DPBS (Negative control)	1	1.882	1.812	1.847	100.08	100.00	2.12
	2	1.906	1.861	1.884	102.08		
	3	1.817	1.794	1.805	97.84		
5% SDS (Positive control)	1	0.042	0.041	0.041	2.24	2.36***	0.11
	2	0.046	0.043	0.045	2.43		
	3	0.044	0.045	0.045	2.42		
1000 mg/mL Meta collagen	1	1.727	1.759	1.743	94.47	94.12	3.83
	2	1.803	1.804	1.804	97.76		
	3	1.696	1.630	1.663	90.12		

**** p<0.001 compared to negative control group by Student's t-test.*

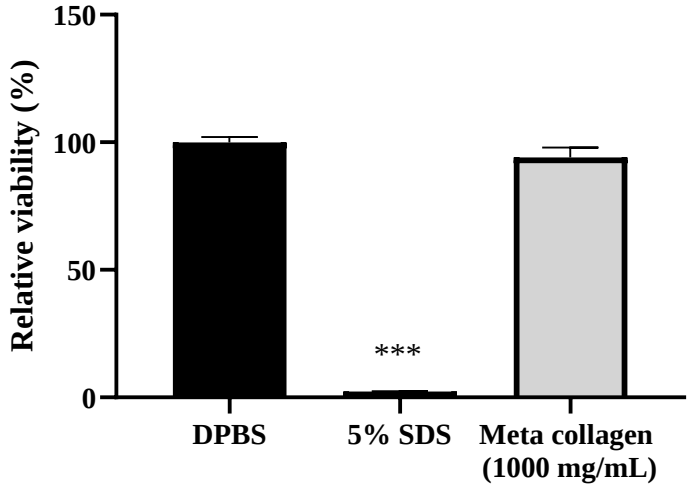

Fig. 3. *The relative viability of EpiDerm™ tissues after exposure to Meta collagen. DPBS (Negative control); 5% SDS (Positive control). Data are shown as mean ± SD. n = 3 in each group. *** p<0.001 compared to DPBS group by Student's t-test.*

Many natural materials have been utilized in the fabrication of hydrogel wound dressings, including chitosan, hyaluronic acid, alginate, gelatin, collagen, and more. These dressings have exhibited promising effects on wound healing [13]. Natural materials can also be employed in self-healing polymer matrix composites capable of spontaneously repairing microcracks, which could be beneficial in wound dressings [24, 25]. Meta collagen, a novel collagen-derived natural material rich in dipeptides and tripeptides, was the subject of studies in this research. These studies demonstrated its antioxidant properties and wound healing effects. The results support the assumption that Meta collagen could be a valuable material for skin care, including wound healing and more. For potential future applications in wound healing, Meta collagen could be considered for incorporation into wound dressings, potentially leading to innovative products. However, this study had limitations. For example, this was an exploratory study, and the possibility of future animal or clinical experiments depends on financial support.

CONCLUSION

In this study, antioxidant activities and *in vitro* wound healing effects of Meta collagen (Highly content dipeptide and tripeptide) were investigated. Meta collagen could scavenge DPPH dose-dependently in the DPPH scavenging assay. Meta collagen could enhance the migration of NIH-3T3 fibroblasts. These results showed the antioxidant activity and *in vitro* wound healing effects of Meta collagen. On the other hand, Meta collagen (1000 mg/mL) is considered as non-irritant to skin by using a reconstructed human epidermis. Accordingly, Meta collagen might be a potential material to be developed as a skin care product in the future.

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Conflict of Interest. The authors declared that there is no conflict of interest.

Authorship Contributions. Concept: H.K., S.L., Design: S.J., Y.K., Data Collection or Processing: S.J., Y.K., Analysis or Interpretation: S.J., Y.K., Z.K., Literature Search: S.J., Z.K, Writing: Z.K., S.L.

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