Potentiating cutaneous wound healing in young and aged skin with nutraceutical collagen peptides

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doi:10.1111/ced.14392

Summary

Background. Chronic wounds continue to be a burden to healthcare systems, with ageing linked to increased prevalence of chronic wound development. Nutraceutical collagen peptides have been shown to reduce signs of skin ageing, but their therapeutic potential for cutaneous wound healing remains undefined.

Aim. To determine the potential for nutraceutical collagen peptides to promote cutaneous wound healing in vitro in the context of age.

Methods. The potential for bovine- or porcine-derived nutraceutical collagen peptides to promote wound healing of primary cutaneous fibroblasts and keratinocytes derived from young and aged individuals in vitro was assessed by two-dimensional scratch and cell-viability assays and by immunofluorescence for the cell proliferation marker, Ki67. The achievement of peptide concentrations in vivo, equivalent to those exerting a beneficial effect on wound healing in vitro, was confirmed by pharmaco-kinetic studies of hydroxyproline, a biomarker for collagen peptide absorption, following peptide ingestion by healthy individuals over a wide age range.

Results. Results demonstrated significant nutraceutical collagen peptide-induced wound closure of both young and aged fibroblasts and keratinocytes, mediated by enhanced cellular proliferation and migration. Analysis of blood levels of hydroxyproline in young and aged individuals following porcine collagen peptide ingestion revealed peak serum/plasma levels after 2 h at similar concentrations to those exerting beneficial effects on wound healing in vitro.

Conclusion. These data demonstrate the capacity for nutraceutical collagen peptides to promote cutaneous wound closure in vitro, at pharmacologically achievable concentrations in vivo, thereby offering a potential novel therapeutic strategy for the management of cutaneous wounds in young and aged individuals.

Introduction

Chronic wounds are a major clinical and financial burden to worldwide healthcare systems, accounting for 5.5% of all expenditure in the UK National Health Service alone.1,2 Increased prevalence is further linked to ageing, with the socioeconomic burdens of wound care being ever-increasing within an ageing global population, emphasizing the acute unmet need for novel efficacious therapeutic approaches for cutaneous wound healing.2,3

Ageing has a negative impact on normal wound-healing responses, with age-related changes occurring in the structure and function of the extracellular matrix (ECM) as well as changes in growth factor secretion, contributing to chronic wound...
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development. Aged skin displays reduced collagen synthesis and increased matrix metalloproteinase (MMP) secretion, leading to increased collagen degradation. Consequently, while young skin mounts a robust and timely wound-healing response, aged skin displays extended inflammatory and remodelling phases, leading to delayed wound closure, decreased wound strength and the increased incidence of medical complications associated with chronic wounds in the elderly.

The structural and mechanical properties of collagen within the ECM are key to tissue integrity. Addressing the innate collagen deficit within aged skin through collagen supplementation therefore offers a promising strategy to restore ECM homeostasis and aid cutaneous wound healing. To this aim, there has been increased interest in the use of orally administered nutraceutical collagen peptides derived from animal skin collagen, constituting a mixture of low-molecular-weight, water-soluble peptides that are rich in amino acids such as hydroxyproline (Hyp), glycine and proline. Bioavailability studies indicate that Hyp-containing peptides are detected at concentrations of approximately 20 μmol/L 1 h post-ingestion, with distribution to the skin retained for up to 14 days. Furthermore, recent clinical trials demonstrated that daily collagen peptide ingestion improves skin hydration and elasticity while reducing signs of ageing. In addition, in vitro studies have illustrated chemotactic effects of collagen peptides on stimulating fibroblast migration and proliferation, and the synthesis of collagen and hyaluronic acid. Supporting the beneficial effects for cutaneous wound healing, studies in rat models demonstrate significantly increased wound-closure rates and enhanced collagen deposition following oral administration of collagen peptides. However, the potential for nutraceutical collagen peptides to promote wound healing in humans remains undefined. We therefore carried out this study to determine the potential for nutraceutical bovine or porcine collagen peptides to promote cutaneous wound healing of fibroblasts and keratinocytes derived from both young and aged individuals.

Methods

The study was approved by Newcastle University ethics committee (no. 1681/11689/2019), and all participants provided written informed consent.

Growth of primary human keratinocytes and fibroblasts

Primary keratinocytes and fibroblasts were isolated from surplus human foreskin (REC reference 19/NE/004_Lovat) maintained as previously described (Data S1), with donors grouped into young (18–35 years old), middle-aged (40–55 years old) or aged (≥ 60 years old).

Western blotting

Western blotting was used to detect collagen I, collagen III or hyaluronic acid expression in primary fibroblasts derived from young, middle-aged or aged individuals as previously described (Data S1).

Two-dimensional scratch-wound assays

For these assays, 15 000 fibroblasts or 30 000 keratinocytes derived from young or aged individuals were seeded on to 96-well plates (Incucyte ImageLock; Essen Bioscience, Germany), the wells of which were either uncoated or were coated with 1 mg/mL bovine or porcine collagen peptide (Rousselot, Ghent, Belgium). Cells were treated for 2 h in the presence/absence of 7.5 μg/mL mitomycin C (Sigma-Aldrich, Poole, Dorset, UK) to inhibit cell proliferation prior to scratch induction using a wound-making device (96-well Wound Maker; Essen Bioscience, Royston, Hertfordshire, UK). Treatment with 10 nmol/L CXCL12 (Almac Sciences, Craigavon, Armagh, UK) to uncoated wells was used as a positive control (Fig. S2, Data S2). Live-cell images were captured every 2 h for 72 h using analysis equipment and software (Incucyte ZOOM; Essen Bioscience) to assess wound confluence and calculate wound closure rate.

Cell viability/proliferation assays

Colorimetric MTS cell viability assays (Cell Titre 96; Promega UK, Southampton, Hampshire, UK) and immunofluorescent expression of Ki67 were used to determine the effect of nutraceutical collagen peptides on fibroblast and keratinocyte proliferation as previously described (Data S1). Cells were seeded on to 96-well plates or coverslips, which were either uncoated or coated with 1 mg/mL bovine or porcine collagen peptide prior to treatment for 2 h in the presence/absence of 7.5 μg/mL mitomycin C (Sigma-Aldrich) and incubated at 37 °C for 72 h.

Pharmacokinetics studies of porcine collagen peptide absorption

Six healthy volunteers aged between 20 and 66 years old ingested 10 g of porcine collagen peptides with
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5 mL fruit juice (Ribena; Suntory, Osaka, Japan) diluted in 250 mL water following an overnight fast. Venepuncture blood samples were obtained at 0, 2, 8 and 24 h post-ingestion, with serum and plasma samples subsequently stored at −80 °C prior to quantification of total Hyp by ultra-performance liquid chromatography tandem mass spectrometry, as previously described21 (Data S1).

Statistics

All data were analysed using statistical software (GraphPad Prism V8; GraphPad, La Jolla, CA, USA) and presented as mean ± SD. One-way ANOVA with Tukey multiple comparisons test was used to analyse wound closure rate, relative cell viability, collagen I and III expression. Paired t-test was used to compare total Hyp concentrations at baseline and 2 h post-ingestion, while unpaired t-test was used to compare Ki67 expression in the presence/absence of collagen peptides and to compare Hyp concentrations between young and aged individuals at each timepoint post-ingestion. Pearson correlation coefficient was used to determine the correlation between basal collagen expression and rate of wound closure. \( P < 0.05 \) was considered statistically significant.

Results

Collagen I but not collagen III expression decreases with age

Analysis of collagen I and III expression by young, middle-aged or aged fibroblasts revealed a significant \( (*P < 0.05) \) decrease in collagen I expression in aged compared with young fibroblasts (Fig. 1a,b), but no significant difference in collagen III expression was observed between aged fibroblasts from different aged individuals (Fig. 1c,d). Additionally, there was no significant correlation between collagen I or III expression by fibroblasts and rate of wound closure (Fig. S1, Data S1).

Bovine and porcine collagen peptides enhance wound closure of young and aged fibroblasts

Ageing has a negative effect on cellular responses to injury, causing delayed wound closure.22 To determine the potential for bovine or porcine nutraceutical collagen peptides to promote cutaneous wound closure in vitro, wound closure of primary fibroblasts or keratinocytes derived from young or aged individuals seeded on collagen peptide-coated plates was monitored using two-dimensional scratch assays, with 10 nmol/L CXCL12 treatment used as a positive control \( (*P < 0.05, **P < 0.01; \text{Fig. S2, Data S2}) \). Bovine and porcine collagen peptides significantly promoted wound closure of young and aged fibroblasts \( (*P < 0.05, **P < 0.01, ***P < 0.001; \text{Fig. 2}) \), although porcine collagen peptides were able to significantly enhance wound closure only in young keratinocytes \( (*P < 0.05, \text{Fig. S3, Data S1}) \).

To determine whether collagen peptide-induced wound closure was mediated through enhanced migration or proliferation, fibroblasts or keratinocytes (< 45 years old group) were cultured in the presence of mitomycin C for 2 h prior to wound induction. Collagen peptide-induced wound closure was significantly inhibited by mitomycin C in both fibroblasts and keratinocytes \( (*P < 0.01; \text{Figs S4 and S5a, Data S1}) \), suggesting that collagen peptides promote wound closure through enhanced cellular proliferation.

Bovine and porcine collagen peptides promote fibroblast proliferation

To confirm nutraceutical collagen peptide-induced fibroblast and keratinocyte proliferation, MTS cell viability assays and their effect on Ki67 expression were assessed. The results showed that bovine and porcine collagen peptides significantly increased both cell viability \( (**P < 0.01; \text{Fig. 3a}) \) and Ki67 expression \( (*P < 0.05; \text{Fig. 3b,c}) \) by primary fibroblasts. However, bovine or porcine collagen peptides were unable to enhance keratinocyte viability (Fig. S5b, Data S2).

Ageing does not affect nutraceutical collagen peptide absorption

To determine the bioavailability of porcine collagen peptides in vivo, total Hyp concentrations were analysed in serum and plasma samples derived from healthy volunteers before (0 h) and 2, 8 and 24 h after ingestion of 10 g of porcine collagen peptides. There was a significant increase in total Hyp concentration 2 h post-ingestion compared to total Hyp concentrations at baseline in both serum \( (**P < 0.01) \) and plasma \( (**P < 0.01) \) for each individual (Fig. S4 and Table S2, Data S2), with peak concentrations reached at 2 h post-ingestion (Fig. 4: serum 20 ± 4 μg/mL, plasma 23 ± 3 μg/mL), at equivalent concentrations to those achieved in vitro post-coating of tissue culture plates (Table S1, Data S2). Furthermore, there was no significant difference in total Hyp concentration at each timepoint between young and...
aged individuals (Fig. 4; Table S2, Data S2). Collectively, these data demonstrate that ingestion of 10 g of porcine collagen peptides is sufficient to achieve blood concentrations in vivo that are equivalent to concentrations shown to exert a positive effect on wound healing in vitro, using cells derived from donors who had not ingested nutraceutical collagen peptides.

Discussion

With an increasingly ageing population, chronic wounds are an increasing burden on healthcare systems.1,3 Ageing has a negative impact on wound healing through decreased collagen synthesis and diminished ability of aged skin to heal at rates comparable to those of young skin.4,7 The use of nutraceutical collagen peptides may therefore offer a viable strategy to promote cutaneous wound healing in both young and aged individuals.

Results from the present study, albeit from a small number of donors, demonstrated a significant reduction in collagen I expression by aged compared with young fibroblasts (Fig. 1a,b), consistent with the reported diminished ability of fibroblasts to produce collagen I and replenish the ECM, which decreases by 1% for each life year.7 However, we did not observe any significant difference in basal wound closure rates of young and aged fibroblasts, or any correlation with collagen expression, suggesting that an age-related decline in collagen expression by fibroblasts does not significantly affect basal wound closure in vitro (Fig. 2; Fig. S1, Data S2). Although similar age-related declines in collagen III have been reported, our results revealed no significant difference in collagen III expression of differently aged fibroblasts (Fig. 1c,d), which is likely to be reflective of the low collagen III content naturally present within unwounded skin, as collagen III is largely produced during the early phases of wound healing.7

While increasing evidence supports the potential for collagen peptides to promote wound healing, there is limited evidence surrounding their wound-healing activity in the context of age.16,17 The present study demonstrated that both bovine and porcine collagen peptides significantly increased wound closure rates in both young and aged fibroblasts (Fig. 2a–e), with subsequent studies suggesting that collagen peptide-induced wound closure results from enhanced cellular...
Figure 2 (a) Representative photomicrographs of wound closure of dermal fibroblasts seeded on to either uncoated control wells or wells coated with 1 mg/mL of bovine or porcine collagen peptides, at 0, 24, 48 and 72 h post-scratch. Scale bar = 100 µm. Original magnification × 10. (b,d) Wound confluence (%) of young (18–35 years old) or aged (≥ 60 years old) dermal fibroblasts seeded on to either uncoated control wells or wells coated with (b) bovine or (d) porcine collagen peptides, measured every 2 h over 72 h (mean ± SD, n = 3 independent experiments). (c,e) Rate of wound closure (µm/h) of young and aged dermal fibroblasts on to either uncoated control wells or wells coated with (c) bovine or (e) porcine collagen peptides (mean ± SD, n = 3 independent experiments, *P < 0.05, **P < 0.01, ***P < 0.001).
proliferation, as confirmed by the increase in peptide-induced Ki67 expression and cell viability (Fig. 3a–c). These results corroborate previous studies revealing enhanced fibroblast proliferation through the addition of prolyl-hydroxyproline-containing collagen peptides at similar concentrations used in the present study.\textsuperscript{14,15} The mechanism by which collagen peptides stimulate cell proliferation remains enigmatic.
however. It has been suggested that unlike native helical collagen, collagen peptides have specific exposed amino acids that are able to activate cell surface receptors on fibroblasts to directly or indirectly induce their proliferation.14 Alternatively, collagen peptides may be transported into fibroblasts via proton-coupled oligopeptide transports, including peptide transporter 2, and peptide histidine transporter (PHT) 1 and PHT2, hence further studies are warranted to ascertain the mechanisms by which collagen peptides exert their beneficial effects on cutaneous wound healing.23

While porcine collagen peptides were able to promote a significant increase in the wound closure rates of primary keratinocytes by promoting cell proliferation (Figs S3 and S5a, Data S1), interestingly, MTS proliferation assays performed on unwounded keratinocytes did not reveal any significant enhancement of keratinocyte proliferation in the presence of collagen peptides (Fig. S5b, Data S1). These data reflect the previously described incapacity of inactivated keratinocytes to proliferate in response to collagen, and highlight the need for the release of cytokines such as interleukin-1 from wounded keratinocytes to allow cell activation and subsequent induction of proliferation by collagen peptides.24,25

The ability of collagen peptides to exert a physiological effect on cutaneous wound healing is largely determined by their absorption and distribution to the skin.10-13 Pharmacokinetic analysis of Hyp revealed peak total Hyp concentrations of 20 µg/mL (150 nmol/mL) in serum and 23 µg/mL (177 nmol/mL) in plasma 2 h after ingestion of porcine collagen peptides (Fig. 4), with the concentrations achieved being comparable to those in other clinical pharmacokinetic studies and equivalent to micromolar concentrations shown in the present study to promote wound closure in vitro, using cells derived from donors who had not ingested nutraceutical collagen peptides (Table S1, Data S1).10,12 Importantly, no significant difference in porcine collagen peptide absorption was observed between young and aged individuals (Fig. 4), suggesting that age does not affect absorption and thereby bioavailability of these peptides.

Conclusion

The present study highlights the ability of nutraceutical collagen peptides used at clinically achievable concentrations to promote cutaneous wound closure in vitro by stimulating fibroblast and keratinocyte proliferation, thus suggesting their potential role as a novel therapeutic strategy for the treatment of cutaneous wounds in both young and elderly individuals.

What’s already known about this topic?
- Nutraceutical collagen peptides reduce signs of skin ageing.
- Nutraceutical collagen peptides promote wound healing in animal models.

What does this study add?
- Nutraceutical collagen peptides promote cutaneous wound healing by enhancing fibroblast and keratinocyte proliferation in cells derived from young and aged individuals.
• Consumption of porcine nutraceutical collagen peptides in vivo results in clinically achievable concentrations equivalent to those exerting a beneficial effect on cutaneous wound healing in vitro.

Acknowledgement

We thank the Newcastle University Dermatology Biobank and associated patients for donated tissue used for in vitro studies, and the volunteers for their participation in the pharmacokinetic studies of ingested collagen peptides. This study received financial support from Rousselot.

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Figure S3. (a) Representative photomicrographs of scratch-induced wound closure of keratinocytes seeded onto uncoated wells or wells pre-coated with 1mg/ml of bovine or porcine collagen peptides at 0, 24, 48 and 72 hours. Scale bar = 100µm. Magnification = x10. Wound confluence (%) of young keratinocytes (18-35 years old) or aged keratinocytes (60+ years old) seeded on wells pre-coated with 1mg/ml (b) porcine or (d) bovine collagen peptides or uncoated control wells (mean ± SD, N=3 independent experiments). Rate of wound closure of young and aged keratinocytes seeded on uncoated control wells or wells pre-coated with 1mg/ml (c) porcine or (e) bovine collagen peptides (mean ± SD, N=3 independent experiments, *P<0.05).

Figure S4. Wound confluence (%) of dermal fibroblasts (<45 years old) seeded onto uncoated control wells or 1mg/ml (a) bovine or (b) porcine collagen peptides coated wells in the presence or absence of 7.5µg/ml mitomycin C (mito c) measured every 2 hours for 72 hours (mean ± SD, N=3 independent experiments). (c) Rate of wound closure (µm/hr) of dermal fibroblasts seeded onto uncoated control wells or 1mg/ml bovine or porcine collagen peptide coated wells in the presence or absence of 7.5µg/ml mitomycin C (mito c) (mean ± SD, N=3 independent experiments, *P<0.05, **P<0.01).

Figure S5. (a) Rate of wound closure (µm/hr) of keratinocytes (<45 years old) seeded onto uncoated control wells or 1mg/ml bovine or porcine collagen peptide coated wells in the presence or absence of 7.5µg/ml mitomycin C (mito c) (mean ± SD, N=3 independent experiments, *P<0.05, **P<0.01). (a) Relative cell viability of primary keratinocytes seeded on uncoated control wells or 1mg/ml bovine or porcine collagen peptides coated wells in the presence or absence of 7.5µg/ml mitomycin C (mito c) for 72 hours (mean ± SD, N=3 independent experiments, *P<0.05, **P<0.01).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplementary Methods.

Table S1. Mean concentration of collagen peptides adhered to plastic after coating.

Table S2. Total hydroxyproline (Hyp) concentration in serum and plasma.

Figure S1. Correlation of relative (a) collagen I or (b) collagen III expression with rate of wound closure of dermal fibroblasts. (a) R² = 0.0001, (b) R² = 0.008.

Figure S2. (a) Representative photomicrographs of scratch-induced wound closure of dermal fibroblasts seeded on wells coated with 1mg/ml porcine collagen peptide or uncoated control wells treated in the presence or absence of 10nM CXCL12 at 0, 24, 48 and 72 hours. Scale bar = 100µm. Magnification = 10x. (b) Rate of wound closure (µm/hr) of dermal fibroblasts seeded on wells coated with 1mg/ml porcine collagen peptide or uncoated control wells treated in the presence or absence of 10 nM CXCL12 (mean ± SD, N=3 independent experiments, *P<0.05). (c) Representative photomicrographs of scratch-induced wound closure of keratinocytes seeded on wells coated with 1mg/ml porcine collagen peptide or uncoated control wells treated in the presence or absence of 10nM CXCL12 (mean ± SD, N=3 independent experiments, *P<0.05). (d) Rate of wound closure (µm/hr) of keratinocytes seeded on wells coated with 1mg/ml porcine collagen peptide or uncoated control wells treated in the presence or absence of 10nM CXCL12 (mean ± SD, N=3 independent experiments, *P<0.05, **P<0.01).