The potential of a niacinamide dominated cosmeceutical formulation on fibroblast activity and wound healing in vitro

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Key words
L-Carnosine; Dermal fibroblasts; Hesperidin; HSP70 homologue; Niacinamide; Wound healing

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Abstract
Knowledge on the intrinsic mechanisms involved in wound healing provides opportunity for various therapeutic strategies. The manipulation of dermal fibroblast proliferation and differentiation might prove to beneficially augment wound healing. This study evaluated the combined effects of niacinamide, L-carnosine, hesperidin and Biofactor HSP® on fibroblast activity. The effects on fibroblast collagen production, cellular proliferation, migration and terminal differentiation were assessed. In addition, the authors determined the effects on in vitro wound healing. The optimal concentrations of actives were determined in vitro. Testing parameters included microscopic morphological cell analysis, cell viability and proliferation determination, calorimetric collagen detection and in vitro wound healing dynamics. Results show that 0·31 mg/ml niacinamide, 0·10 mg/ml L-carnosine, 0·05 mg/ml hesperidin and 5·18 μg/ml Biofactor HSP® proved optimal in vitro. The results show that fibroblast collagen synthesis was increased alongside with cellular migration and proliferation.

Introduction
The vast amount of data on the intrinsic mechanisms involved in wound healing provides opportunity for various therapeutic strategies. Current scientific literature serves as a primer with information that allows for the manipulation of wound healing mechanics. One such example is the benefit of the down-regulation transforming growth factor (TGF-β1) which has been implicated in the pathogenesis of keloid and hypertrophic scar formation (1–3). This leads to the question whether it is possible to manipulate dermal fibroblast proliferation and differentiation in a dose-dependant and localised manner towards accelerated wound healing. The activity of fibroblasts in the production and maintenance of the dermal framework during wound repair or regeneration is well researched. Morphological subsets of dermal fibroblasts have been extensively described in studies by Mollenhauer and Bayreuther (4,5). These subsets are defined as either mitotically active fibroblasts (MF) or postmitotic fibrocytes (PMF). The latter are irreversibly differentiated cells and are known to produce five to eight times more collagen than their mitotically active precursors (5). Additional sub-classifications have also been identified, based on the ability to synthesise specific cytokines and growth factors such as TGF-β and keratinocyte growth factor (KGF) (6–9). Endogenous and exogenous factors are thought to alter cellular homeostasis, leading to pathologic tissue remodelling and consequent fibrosis as observed in the skin, lungs and kidneys (7–13). Thus, in vivo strategies such as changing the dynamic wound environment, in order to accelerate or improve the clinical outcome, must therefore be considered.

Key Messages
• endogenous and exogenous factors are thought to alter cellular homeostasis, leading to pathologic tissue remodelling and consequent fibrosis as observed in the skin, lungs and kidneys
• in vivo strategies such as changing the dynamic wound environment, in order to accelerate or improve the clinical outcome, must therefore be considered
• in this study, the prospect of altering the cellular dynamics associated with dermal fibroblasts matrix
deposition and remodelling was explored through the testing of a formulation consisting of niacinamide, L-carnosine, hesperidin and an HSP70 homologue

• the rationale for the formulation used in this study is motivated by the efficacy evidence of niacinamide, L-carnosine, hesperidin and heat shock proteins (HSPs) in various applications

• the overall purpose of this study was therefore to investigate the potential of the niacinamide dominated, in combination with L-carnosine, hesperidin and an HSP70 homologue towards enhanced fibroblast activity and wound healing in vitro

• the data obtained has the potential to modify or complement current treatment modalities towards improved healing in vivo

• this study aimed to assess the effects of the combined use of niacinamide, L-carnosine, hesperidin and Biofactor HSP® on dermal fibroblast activity and wound healing

• the rationale for the combined use of these actives is based on the vast amount of literature relating to the various benefits of each active in plastic and reconstructive surgery, cosmeceutical sciences and experimental gerontology

• therefore, the existing knowledge was combined in a formulation that might be suited to advanced wound care

• results show that 0.31 mg/ml niacinamide, 0.10 mg/ml L-carnosine, 0.05 mg/ml hesperidin and 5.18 μg/ml Biofactor HSP® has an effect on dermal fibroblast activity in vitro

• data obtained shows that collagen synthesis was enhanced and that the mechanism does not relate to the differentiation of fibroblasts into PMF that are known to produce more collagen

• both the test and control groups contained large populations of MF and only a few fibroblasts differentiated into PMF

• cells exposed to the formulation continued with proliferation and stimulated both collagen production and wound healing in vitro

• furthermore, 0.31 mg/ml niacinamide, 0.10 mg/ml L-carnosine, 0.05 mg/ml hesperidin and 5.18 μg/ml Biofactor HSP® significantly enhanced in vitro wound closure

• the scratch models showed that the control groups reached 61.1% (SD = 3.20) confluence compared with the 99.6% (SD = 0.17) of the test groups after 24 hours

• we therefore conclude that this particular product combination enhanced fibroblast proliferation, collagen synthesis and migration without terminal differentiation

• these attributes have the potential to promote wound healing of both acute and chronic wounds in vivo through the use of a delivery system such as collagen-based matrices

In this manuscript, the prospect of altering the cellular dynamics associated with dermal fibroblasts matrix deposition and remodelling was explored through the testing of a formulation consisting of niacinamide, L-carnosine, hesperidin and an HSP70 homologue. The rationale for the formulation used in this study is motivated by the efficacy evidence of niacinamide, L-carnosine, hesperidin and heat shock proteins (HSPs) in various applications. Niacinamide, also known as nicotinamide, has been shown to stimulate collagen production, restore epidermal barrier function (14), down-regulate inflammation (3) and extend the replicative life span of cells in culture (15). More recent work by Park et al. showed that niacinamide prevents radiation-induced cellular adenosine triphosphate (ATP) loss. Furthermore, their research showed that niacinamide provides protection against radiation-induced glycolytic blockage in vitro. These protective effects in skin are believed to be the result of the mediation of cellular energy pathways as shown in human adult low calcium temperature cells (16). A recent comparison between topically applied niacinamide (4%) and hydroquinone (4%), both in a cream form, further emphasised the anti-inflammatory properties of this amide of nicotinic acid (17). Orally administered L-carnosine has been proven to reduce: the expression of TGF-β, infiltration of neutrophils, lung oedema, iNOS (the main source of NO during inflammation) and the histological evidence of lung injury, fibrotic collagen production and apoptosis. High doses of L-carnosine have been found to reduce TGF-β expression as well as extracellular matrix deposition (18). L-Carnosine is also known to reduce the expression of proinflammatory and profibrotic cytokines, interleulin (IL-6) and tumor necrosis factor (TNF-α) (19). L-Carnosine thus has the potential to induce cellular homeostasis in the healing of acute and chronic wounds. There is compelling evidence that chronic inflammation is associated with increased hyaluronic acid (HA) turnover and the formation of low molecular weight metabolites. The conservation of HA during wound healing might hold a key to improved or scarless wound healing. Meyer et al. provided further insight in their study and showed that the dermis of keloids contained noticeably diminished levels of HA (20). Hesperidin-mediated inhibition of hyaluronidase and consequent limitation of HA turnover might encourage down-regulation of proinflammatory signalling and improve overall wound healing. Hesperidin and other bioflavonoids are also known to counter capillary fragility and inhibit capillary permeability. This is particularly relevant when considering conditions plagued with a lack of capillary resistance such as diabetes, haemorrhoids, scurvy and haemorrhagic purpura (21). The functions of HSPs in wound healing have recently come under the spotlight. Kovalchin et al. conducted research on the effects of exogenously administered HSP70, HSP90 and gp96 (an HSP, also known as endoplasm) on the healing of full-thickness injuries in mice (22). The authors provided evidence that the extracellular presence of HSPs improved overall wound closure through the up-regulation of macrophage phagocytosis. Mushtaq et al. evaluated the modulatory effects of HSP70 on wound healing in a rabbit corneal epithelium organ culture (23). Their results confirmed the importance of HSP70 in epithelial regeneration.
The overall purpose of this study was therefore to investigate the potential of the niacinamide dominated, in combination with L-carnosine, hesperidin and an HSP70 homologue towards enhanced fibroblast activity and wound healing in vitro. The data obtained has the potential to modify or complement current treatment modalities towards improved healing in vivo.

**Materials and methods**

**The combined effects on cell viability and proliferation**

Dermal fibroblasts were obtained from donated human skin from Southern Cryoscience (Pty) Ltd, a licensed tissue bank, after consent by the donors (approval number H005-10). The tissue was mechanically fragmented as described by Keira et al. (24). These fragments were washed thrice in a 1% Pen-Strep-Fungizone and transferred to 25 cm² culture flasks. Incubation at 5% CO₂ and 37°C for 24 hours followed in Dulbecco’s minimum essential medium/Ham’s F-12 (DMEM/F12) (Lonza) with, 3·15 g/l glucose, L-glutamine and 10% foetal bovine serum (FBS). Fifth passage cells were used and seeded into 24-well plates (density of 1 × 10⁵ cells per well), maintained with 1 ml medium and incubated at 5% CO₂ and 37°C for 24 hours.

Test group media were prepared as per the cosmeceutical formulation by adding niacinamide (3·0% w/w), L-carnosine (1·0% w/w), hesperidin (0·5% w/w) and Biofactor HSP® (0·05% w/w) to the DMEM/F12 containing 3·15 g/l glucose, L-glutamine and 10% FBS. These concentrations were used in a prototype product of Southern Rejuvenation Technologies (Pty) Ltd. The HSP70 homologue, Biofactor HSP®, was purchased from Vincience (Sophia Antipolis, France) and is a yeast extract rich in HSP70. Niacinamide, L-carnosine and hesperidin were obtained from Cosmetichem (Pty) Ltd, Bryanston, South Africa. The medium of the control group consisted of the supplemented DMEM/F12 as described above. The test group medium was used to prepare three dilutions, namely, 1:10, 1:100 and 1:1000. The medium containing the test formulation will be referred to as the enhanced medium whereas the non-formulation containing medium will be referred to as the normal medium in the text to follow. The study was initiated after a 24-hour incubation period. The culture media of both the control and test groups were substituted with the supplemented and normal medium, respectively. The culture medium of both the control- and formulation-exposed test groups was replaced after 24 hours of exposure with a 50 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazolium bromide solution and incubated for 3 hours at 5% CO₂ and 37°C. The absorbance at 570 nm was read using a Bio-Tek ELx800 plate reader. A standard curve was prepared as described above in 24-well culture plates. The culture media of both the control and test groups were substituted with the normal and enhanced medium, respectively. The enhanced medium was again used in three dilutions, namely 1:10, 1:100 and 1:1000. Cell layers were washed with phosphate-buffered saline (PBS) after the exposure periods and fixed for 1 hour with 1 ml Bouin’s solution. The fixative was removed, the plates were washed and staining with 1 ml Sirius Red solution for 1 hour followed. The Sirius Red solution was withdrawn and 0·01 N HCl was used to remove the unbound dye. The plates were rinsed with water, allowed to dry and microscopically examined. The dye was dissolved in 0·2–0·3 ml NaOH (0·1 N) for 30 minutes. These extracts were then transferred to a 96-well plate and the absorbance was read at 570 nm using a Bio-Tek ELx800 plate reader. A standard curve was prepared and analysis was repeated after obtaining the optimal dilution of the enhanced medium. Both test and control groups were used and the cells were exposed for 24 and 96 hours, respectively. This was performed in order to determine whether the induced fibroblast collagen production and cellular differentiation were dose-dependant. The data was graphically depicted to represent the Sirius Red absorbance versus sample type.

**The combined effects of fibroblast differentiation**

Cell morphology was evaluated by crystal violet staining, exposure to the control and optimal enhanced media was either for 24 or 96 hours without replacement or addition of any media in the latter. Classification of the cells was based on morphotypes as either MF or PMF. MF are known to be small spindle shaped or epithelioid varying in size from small to large. PMF are typically larger than MF with irregular spindle shapes, extreme epithelioid cells and also shows signs of degeneration and fragmentation (5). Staining was performed by removing the medium from the wells, washing the cells gently with PBS, staining for 10 minutes with 0·5% crystal violet (in 145 mmol/l NaCl, 0·5% formal saline, and 50% ethanol) and the subsequent washing of the cells three times with water (26). The cells were counted and differentiated as either MF or PMF. Cell morphology and structure were studied through inverted light microscopy using a Zeiss Axiovert system.

**The combined effects on in vitro wound healing**

Control and test cells were uniformly seeded into 24-well tissue culture plates at a density of 1 × 10⁵ cells per well, cultured to confluence and wounded by scraping with a 200 μl yellow pipette tip. Following PBS washes to remove the floating cells, cells were allowed to migrate into the simulated wound area in the presence or absence of the optimal enhanced formulation. The proliferation and migration were photographed at intervals of 6 hours over a period of 24 hours.
through a digital camera connected to an Olympus CKX31 inverted microscope (27). A revised mask was generated for each subsequent image in order to clearly delineate the border of each wound at each interval. Both the phase contrast and masked images were used for data analysis. Overall, wound closure was monitored and analysed by using two measures namely, wound confluence and wound width. The percentage of wound confluence was graphed over time in order to evaluate the characteristics of wound closure in vitro. Wound width (μm) was also graphed over time.

Statistical analysis

Statistical analysis was performed by means of a t-test and P-values <0.05 were considered to be statistically significant. Data obtained from the scratch wound assay was analysed through the use of SPSS Statistics 17.0 (SPSS Inc., Chicago, IL) and a one-way analysis of variance (ANOVA) and Tukey–Kramer post-hoc test for multiple comparisons. Significance values of <0.05 were considered to be statistically significant.

Results and discussion

The combined effects of niacinamide, l-carnosine, hesperidin and Biofactor HSP® were evaluated at various concentrations. Results of the cell viability assay depicted in Figure 1, showed that the 1:100 dilutions of this formulation enhanced dermal fibroblast proliferation by 3% (SD = 11.44). This increase in cell proliferation was slightly more compared with the control (P = 0.67). Both the 1:10 (84.2 ± 1.17%) and 1:1000 (87.5 ± 6.88%) dilutions resulted in significant decreases in cell viability with P-values of 0.00 and 0.03, respectively.

The results (Figure 2) of the picrosirius red caliometric assay show that the 1:100 dilutions resulted in the highest collagen yield of all the test groups. Collagen production increased by 17.0% compared with the control group with P = 0.24 and SD = 18.20. The 1:1000 dilution also showed an increase (1.3%) in collagen production although insignificant (P = 0.80). A decrease in collagen (31%) production was witnessed when a 1:10 sample dilutions with a P < 0.01 were tested. This decrease in collagen yield is attributed to a decrease in cell viability.

Data from the cell viability assay along with the caliometric detection of collagen production suggested the 1:100 dilution to be the most suitable for the promotion of dermal fibroblast activity. The most significant increase in collagen production without cell viability was pursued. The concentrations of the actives in this dilution were calculated and found to be the following: niacinamide (0.31 mg/ml), l-carnosine (0.10 mg/ml), hesperidin (0.05 mg/ml) and Biofactor HSP® (5.18 μg/ml). These concentrations were thus regarded as the optimum concentrations and were used in the rest of the in vitro assays.

Cells were differentiated as either MF or PMF based on their morphology at 10× magnification (Figure 3). The majority of the cells in the test and control groups were small spindle-shaped MF. Only a small portion of the cells in both groups differentiated into PMF having either large spindle shapes or were found to be large and epithelioid.

MF morphotypes represented 91.72% (SD = 2.58) of the total cells counted in the test groups compared with the 90.95% (SD = 1.86) of the control fibroblast population (Figure 4). There was no significant difference (P = 0.70) in these percentiles. Similarly, no significant difference (P = 0.73) was found in the expression of PMF morphotypes in both the test and control populations. PMF represented 9.04% (SD = 2.58) of the total population of the test groups. This compared well with the 8.40% (SD = 1.65) of PMF found in the test groups. The ratios of MF/PMF were thus 0.09 and 0.10 for the test and control groups, respectively.

The combined effects of niacinamide, l-carnosine, hesperidin and Biofactor HSP® homologue on cellular proliferation and migration were assessed using scratch wound assays. Wound closure of both the test and control groups was monitored over 24 hours at 6-hour intervals. The scratch models showed that the control groups (Figure 5) reached 61.1% (SD = 3.20) confluence compared with the 99.6% (SD = 0.17) of the test groups (Figure 5) after 24 hours.

Figure 1 Results of the cell viability assay indicate that the 1:100 dilutions resulted in a slight increase in fibroblast proliferation whereas the cells exposed to the 1:10 and 1:1000 dilutions showed a significant decrease in cell viability with P-values of 0.00 and 0.03, respectively.

Figure 2 The most striking increase in collagen yield, induced by the combination of l-carnosine, hesperidin, niacinamide and Biofactor HSP® was the result of the 1:100 dilution (P = 0.24).
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Figure 3  Test and control group fibroblasts were photographed under 10× magnification. The majority of the cells in the test (B and D) and control groups (A and C) were small spindle-shaped MF. Only a small portion of the cells in both groups differentiated into postmitotic fibrocytes having either large spindle shapes (arrow in D) or were found to be large and epithelioid (arrows in A–C). Scale bar = 50 μm.

Figure 4  The ratios of MF/postmitotic fibrocytes (PMF) were found to be 0.09 and 0.10 for the test and control groups, respectively. Both the test and control groups contained large populations of MF and only a few fibroblasts differentiated into PMF.

Data comparison shows the average wound width (Figure 7) reduced every 6 hours with a final wound width of 21.1 μm (SD = 4.55) for the test groups and 106.3 μm (SD = 15.81) for the control groups. The test groups showed a considerable decrease in average wound width. Data of the ANOVA tests presented with significance values of <0.05 for both the wound width and cellular confluence. Similarly, no significant differences were found (P < 0.05) upon comparison of each time interval between the control and test groups for both the wound width and cellular confluence.

Conclusion

This study aimed to assess the effects of the combined use of niacinamide, l-carnosine, hesperidin and Biofactor HSP® on dermal fibroblast activity and wound healing. The rationale for the combined use of these actives is based on the vast amount of literature relating to the various benefits of each active in plastic and reconstructive surgery, cosmeceutical sciences and experimental gerontology. Therefore, the existing knowledge was combined in a formulation that might be suited to advanced wound care. Previous research suggests possible synergy of these actives, and this relate to enhanced collagen synthesis, fibroblast phenotype retention, epidermal regeneration, anti-inflammatory and antioxidant activity. It
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Figure 5 Results of the control groups showed that incomplete wound confluence was reached 24 hours after wounding. The control group reached 61-1% (SD = 3-20) confluence after the incubation period (scale bar = 32 μm). Cell exposed to the formulation (test group) showed that almost complete wound confluence was reached 24 hours after wounding. The wounds progressed every 6 hours to reach 99-6% confluence (scale bar = 32 μm).

Figure 6 The scratch models showed that the control groups reached 61-1% (SD = 3-20) confluence compared with the 99-6% (SD = 0-17) of the test groups after 24 hours.

Figure 7 Data obtained showed a considerable decrease in average wound width in the test groups. Final average wound widths of 21-1 μm (SD = 4-65) and 106-3 μm (SD = 15-81) were reached by the test and control groups, respectively.

was therefore anticipated that advanced wound healing might result in the combination of the products and that the different actives may promote optimal collagen production without negating fibroblast longevity in the frail wound environment.

Results show that 0-31 mg/ml niacinamide, 0-10 mg/ml l-carnosine, 0-05 mg/ml hesperidin and 5-18 μg/ml Biofactor HSP® has an effect on dermal fibroblast activity in vitro. Data obtained shows that collagen synthesis was enhanced and that the mechanism does not relate to the differentiation of fibroblasts into PMF that are known to produce more collagen. The exposed fibroblasts retained their morphotypes in vitro. The preservation of MF in the wound is especially essential to ensure healthy proliferative cell populations in an already compromised milieu. The ratios of MF/PMF were found to be 0-09 and 0-10 for the test and control groups, respectively. Both the test and control groups contained large populations of MF and only a few fibroblasts differentiated into PMF. Cells exposed to the formulation continued with proliferation and stimulated both collagen production and wound healing in vitro.

Furthermore, 0-31 mg/ml niacinamide, 0-10 mg/ml l-carnosine, 0-05 mg/ml hesperidin and 5-18 μg/ml Biofactor HSP® significantly enhanced in vitro wound closure. Results showed a considerable decrease in average wound width in the test groups. The scratch models showed that the control groups reached 61-1% (SD = 3-20) confluence compared with the 99-6% (SD = 0-17) of the test groups after 24 hours. It is thus evident that the combined action of niacinamide, l-carnosine, hesperidin and Biofactor HSP® can prove beneficial in vitro. We therefore conclude that this particular product combination enhanced fibroblast proliferation, collagen synthesis and migration without terminal differentiation. These attributes have the potential to promote wound healing of both acute and chronic wounds in vivo through the use of a delivery system such as collagen-based matrices.

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