Research Article

Potential Wound Healing of PLGA Nanoparticles Containing a Novel L-Carnitine–GHK Peptide Conjugate

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The development of a product that has simultaneous wound healing, anti-inflammatory, and antimicrobial properties is desirable for wound healing medicine. In this study, glycine–histidine–lysine (GHK) peptide as a skin repair accelerator was coupled to L-carnitine with antibacterial and anti-inflammatory properties to investigate its new wound healing properties in a nanoparticle (NP) platform. The conjugate was synthesized by solid-phase synthesis method with Fmoc chemistry, purified by preparative HPLC, and was identified with ESI-Mass technique. The conjugate was then loaded into PLGA NPs, which was prepared using solvent evaporation technique. Zetasizer results showed a mean size and zeta potential of 193.15 nm and –30.2 ± 3.8 mV for the conjugate-loaded PLGA NPs. Scanning electron microscopy (SEM) exhibited spherical-shaped morphology for the NPs with uniform size distribution (PDI = 0.265). Conjugate release from the NPs was about 10% at initial time, which increased to more than about 70% at 200 hr, exhibiting a sustained release trend. Additionally, the treatment of fibroblasts with the nanoconjugate indicated its biocompatibility. Compared to GHK, L-carnitine and free conjugates, the most satisfactory wound healing activity was observed for conjugate-loaded NPs upon wound closure in a rat model of skin injury repair. Furthermore, more epithelialization and neovascularization were observed for L-carnitine–GHK nanoconjugate. Taken together, the L-carnitine–GHK conjugate-loaded PLGA NP will be a promising candidate for wound healing management.

1. Introduction

The skin is considered a protective barrier against the external environment. In this regard, wounds in acute or chronic phases appear whenever integrity of the skin is destroyed [1–3]. Wounds as an important clinical issue have received a great deal of attention before leading to mortality. Furthermore, chronic wounds are reported to be more difficult to manage due to pain, infection, and prolonged hospitalization [3, 4]. For instance, some standard of care has been established to treat diabetic foot ulcers as chronic wounds including surgery, debris, dressing, infection control, wound off-loading, and vascular assessment [5, 6].

The wound healing process is a general phenomenon comprising a series of molecular and cellular events and also three phases including inflammatory, proliferative, and remodeling [1]. This consecutive process originates through the interaction of cells in the dermis and epidermis, alongside inflammatory cell recruitment, fibroblasts, and keratinocytes. Growth factors as effective wound healing promoters stimulate fibroblasts to migrate into the wound site, which are essential for wound repair [7]. During the proliferative phase, the migration of keratinocytes starts from the edge of the
wound into the wound bed. The proliferative phase is accomplished until the wound bed is entirely healed [8]. Wound infections are caused by the growth and spread of microbes, as well. In this regard, fast and appropriate cure plays a vital role in the process of wound healing [9].

Bioactive peptides, being biocompatible, highly active, specific, and stable, have gained attention in wound-healing research [10]. Peptide-based compounds with wound healing properties fall into two categories: (i) antimicrobial peptides (AMP) and (ii) non-antimicrobial peptides [11–14].

The tripeptide glycine-L-histidine-L-lysine (GHK) has received a lot of attention in the field of wound repair and regeneration [15]. Some important properties of the peptide that have been reported in this field include displaying anti-inflammatory effects; attracting endothelial cells to the injured area; modulating the activity of both metalloproteinases and their inhibitors; stimulating collagen and glycosaminoglycan and its ability to modulate the expression of a vast number of genes [16, 17].

GHK is a tripeptide, which naturally occurs in human plasma, saliva, and urine with a strong affinity for copper ions which readily form the GHK–Cu complex in the body [18]. The GHK sequence is present in the alpha 2(I) chain of type I collagen and proteins of extracellular matrix (ECM), which is released by proteolytic enzymes into the site of the wound [19]. Furthermore, the GHK sequence is also present in glycoprotein SPARC, which is found in sites of remodeling. In addition, GHK is able to increase the expression of growth factors by fibroblasts, which are key cells in skin regeneration. Growth factors are contributing factors in proliferation, angiogenesis, and epithelialization and accordingly, GHK can play a crucial role in wound healing [19, 20].

Another common, well-known compound used in the cosmetic industry is L-carnitine [21]. The carnitine (?-hydroxy-γ-N-trimethylaminoobutyric acid) is a quaternary ammonium salt, existing as two bioactive stereoisomers: L-carnitine and D-carnitine. The L-form naturally occurring in the body is synthesized by lysine and methionine, while the D-form inhibiting the activity of the L-form is considered toxic. Being a quaternary ammonium salt, L-carnitine is effective against Gram-positive bacteria [22]. It also exhibits anti-inflammatory effects as a result of reducing major inflammatory cytokines including nuclear factor-kappa B (NF-κB) and tumor necrosis factor-alpha (TNF-α) [23]. Evidence has shown that L-carnitine derivatives heal wounds through beneficial antioxidant and anti-inflammatory on endothelial dysfunction [24].

In recent years, research has been focused on the development of nanotechnology-based formulations in wound healing activity [25, 26]. Enormous beneficial properties of nanomaterials are reported in the field of wound healing, including accelerating healing, preventing bacterial infections, providing a moist environment for wounds, and decreasing frequent dressing changes.

Furthermore, bactericidal and bacteriostatic activities, promotion of rapid wound closure, and modulation of inflammatory responses have been reported for some metal oxide NPs in wound dressing applications. Nanomaterials have been used as delivery vehicles for therapeutic agents [27]. In other words, encapsulation of drugs, genes, and growth factors into nanomaterials as a vector has offered new benefits. Indeed, the use of nanomaterials has improved bioavailability, stability, targeting, and sustaining delivery of drugs as well as antimicrobial agents [27]. For example, gene delivery in a stable form has been carried out using nanoparticles for wound treatment. Additionally, wound bacteria have been eradicated by some photoabsorbent NPs, which transform light to heat or ROS [27]. It is known that nanomaterials with high adsorption capacity, small size, and superior surface-to-volume ratio are able to change the wound microenvironment from nonhealing to healing state by stimulating some cellular and molecular pathways [28].

Recently, polymeric biomaterials have been used as nanocarriers to deliver therapeutic agents for wound healing. Numerous antibiotics, antioxidant, and anti-inflammatory agents have been loaded in various polymer nanomaterials, such as chitosan, polycaprolactone, cellulose derivatives, and their effects on wound healing have been investigated [29–33]. For example, the potential of PLGA polymer NPs has been used to load drugs for wound healing. The biocompatible and biodegradable NPs not only have increased the stability, solubility, and efficacy of drugs, but also have released therapeutic agents in a controlled manner [34, 35].

Herein, we have synthesized a novel compound namely L-carnitine–GHK conjugate in the hope of developing a new biocompatible peptide-based conjugate displaying improved wound healing properties. Furthermore, the conjugate was loaded into PLGA NPs to compare them all in efficiency. Taken together, the conjugate-loaded PLGA NPs offer more skin repair efficiency than others that can be introduced into topical strategies for the management of chronic wounds.

2. Materials and Methods

2.1. Materials. L-Carnitine salt was purchased from Merck company while PLGA copolymer (MW 7,000–17,000) and PVA (MW 30,000–70,000) were obtained from Sigma company. All reagents and solvents for peptide synthesis were provided kindly from peptide chemistry laboratory (Khajeh Nasir Uni).

2.2. Peptide Synthesis, Conjugation, and Characterization. The peptide was synthesized using standard Fmoc solid-phase protocols as shown in supportive information [36]. L-Carnitine was conjugated to GHK peptide on resin before deprotection and purification procedure. Purification of the peptide and conjugate was done using reverse-phase HPLC (ODS-C18 column, 250×4.6 mm, 3–5μm) with flow rate of 1 ml min⁻¹, wavelength of 218 nm, injection volume of 20μl and gradient system of solvent A (1% acetic acid in 100% water) and solvent B (acetonitrile: solvent A, 80:20), running from 95% solvent A to 20% solvent A at 20 min and again 95% at final time. Finally, the molecular weight of the conjugate was characterized by mass spectrometry (+) ESI-MS.

2.3. PLGA Nanoparticles Preparation. PLGA NPs were synthesized using solvent evaporation technique (oil/water) [37].
In the other words, PLGA and l-carnitine Peptide conjugate were dissolved in 2 ml of dichloromethane (DCM) following drop wise injection into 20 ml volume of PVA solution (1%) under homogenization by ultrasonic probe for 5 min.

2.4. Nanoparticle Characterization. Surface charge, particle size, and size distribution of NPs were measured using dynamic light scattering (DLS) (Malvern Zetasizer, Malvern, UK). Furthermore, size and surface morphology of NPs were evaluated by emission scanning electron microscope (FESEM) (SU 8040, Hitachi, Japan). Before imaging the NPs, the fresh sample which dried overnight at 25°C onto a silicon wafer was coated with chromium.

2.5. Loading Estimation of Peptide Conjugate. The amount of the conjugate in the loading flask and release medium was determined using a spectrophotometer (JASCO, V-530) at 220 nm. Absorbance of the conjugate in centrifuged supernatant compared to initial absorbance was measured to calculate the conjugate loading, indirectly.

2.6. In Vitro Release Profile of Peptide Conjugate. Release of the conjugate from the PLGA NPs was performed in phosphate buffer solution (PBS) medium containing 0.5% SDS by the dialysis membrane (cut-off of 5 kDa). The dialysis bag containing 10 mg of dissolved PLGA NP in 2 ml of PBS was suspended in 25 ml medium under stirring at 30 rpm and 37°C for 7 days. At different time intervals, 0.5 ml of sample was removed and replaced by 0.5 ml of fresh medium. Drug release percentage was quantified as mentioned in the loading estimation section.

2.7. Stability Study. In order to study the stability of NPs in wound fluid, it was not easy to obtain sufficient fluid and it dried out quickly. So, the stability was performed in phosphate buffer saline (pH 7.4) in terms of particle size variation using DLS.

2.8. Cell Viability Assay. To access cytotoxic effect of GHK, l-carnitine and conjugates as well as NP, an MTT assay was conducted for NIH-3T3 albino mouse fibroblastic cell line (Pasteur Institute, Tehran). Briefly, cultured cells in complete Dulbecco’s modified Eagle’s medium (DMEM) were seeded into a 96-well plate at 3 x 10^4 cells/well and incubated for 24 hr at 37°C with 5% CO2.

Thereafter, the cells were treated with culture medium containing 100 IU/ml penicillin, 100 μg/ml streptomycin, and a concentration of materials equivalent to that used for animal testing. After 24 hr and 6 days, the supernatants of each well were replaced with 100 μl of fresh DMEM. To each well, 50 μl of 3-(4,5-dimethyl thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) was added and incubated for 4 hr at 37°C followed by dissolving formazan crystal by 100 μl DMSO during shaking for 15 min. Finally, absorbance was measured at 570 nm using a microplate reader (Bender MedSystem, Vienna, Austria). The difference in absorbance of treated and negative control groups was used for the calculation of cell viability percentages. Results of the sixth day was reported compared to the first day [38].

2.9. Wound Healing Experiment. All animal experiments and protocols were carried out in accordance with the medical research ethics committee of Tehran University of Medical Sciences. For this, male Wistar rats (240 ± 25 g) were anesthetized intraperitoneally using a solution of 10% ketamine (50 mg/kg) and 2% xylazine (10 mg/kg) and then the dorsum of each rat was depilated with a AGC2 electric shaver and disinfected with Betadine antisepic solution (10% povidone-iodine). Rats were divided into six groups and wounds of about 1.5 cm in diameter were created on the back of each rat to the depth of the skin basal layer. On the day of wounding, the wounds were topically exposed to 0.2 ml of sterile aqueous solutions of GHK, PLGA NP, l-carnitine–GHK, and l-carnitine–GHK-loaded PLGA NP. As positive control, the rat was also exposed to 0.4 g of Mimosa 10% healing cream while PBS treatment was considered as negative control. The wounds were subsequently dressed with sterile gauze. On days 3, 6, 9, and 11 postwounding, dressings were changed and the doses were repeated. After injury, wounds were imaged and the wound area of each rat was measured on the day of wounding as well as days 3, 6, 9, 11 postwounding using ImageJ 1.51. The percentage of wound healing was estimated using Equation (1):

\[
\text{Wound contraction: } \frac{\text{The wound area of the first day – the wound area of the } n\text{th day}}{\text{The wound area of the first day}} \times 100.
\]

Comparison and statistical analysis were performed by one-way ANOVA. Furthermore, values of \( p < 0.05 \) were considered statistically significant.

3. Results

Schematic representation of peptide synthesis and l-carnitine conjugation was shown in supporting information (Scheme S1). Furthermore, potential wound healing of
the conjugate in the PLGA nanoparticle platform has been exhibited in the graphical abstract, provided in the supplementary materials.

3.1. Peptide Conjugate Characterization. The purity of GHK-l-carnitine conjugate after solid phase synthesis and purification on semipreparative RP-C18 column were determined by liquid chromatography. Analytical HPLC chromatograms of the compounds synthesized are shown in Figure S1 and Figure S2. GHK and l-carnitine–GHK were synthesized with high quality of 99.9% and 91.4% purity. According to Figure S3, the peaks at 341.2 and 681.5 (m/z) corresponding to [M+1] and [2M+1] respectively, confirmed the presence of GHK. The formation of l-carnitine–GHK is also confirmed by peaks at 484.3 and 242.9 (m/z) which are related to [M] and [M/2], respectively (Figure S4). All measurements were performed in 1% TFA in distilled water.

3.2. Synthesis of Conjugate-Loaded PLGA Nanoparticle and Characterization. PLGA NPs loaded with l-carnitine–GHK peptide conjugate were prepared using solvent evaporation in the presence of the PVA stabilizer. Results of size and morphology for the prepared NPs are presented in Figures 1(a) and 1(b), respectively.

The SEM measurement showed a round morphology of the NPs with a size of 19 ± 2.8 nm (Figure 1(a)), while the hydrodynamic size, PDI, and zeta potential of conjugate-loaded PLGA NP were found to be 193.15 ± 5.0 nm, 0.265 and −30.2 ± 3.8 mV, respectively (Figure 1(b)). Furthermore, encapsulation efficiency of conjugate-loaded NP was found to be 85.3% ± 4.5%, respectively. As shown in Figure S5, NP showed good stability for more than 10 days at the temperature of 4°C without significant change in particle size.

3.3. Release Profile of l-Carnitine–GHK Peptide from PLGA Nanoparticle. Release study is important as the limited conjugate release from NPs would reduce the wound healing efficacy. As shown in Figure 2, initial burst release of conjugate within 24 hr (about 30 ± 3%) was followed by sustained release behavior over a period of 200 hr with a final release percentage of about 70% ± 4%.

3.4. Biocompatibility of Peptide Conjugate and Nanoparticles. To ensure biocompatibility of conjugate and NPs before animal study, the MTT toxicity test was performed at the assumed concentrations for the wound healing test. Cells were also treated with conjugated components as controls at the same molar concentrations. As shown in Figure 3, more than 86% cell viability was observed after 24 hr cell treatment with the conjugate and control groups. Higher viability percentages after 6 days of treatment showed no cytotoxicity, which overall confirmed the biocompatibility of the conjugate, its components as well as NPs (Figure 3).

3.5. Wound Contraction Measurements. A full-thickness excisional model in the mouse was used to evaluate the effects of conjugate and materials on wound healing trends by tacking during 14 days. The results of wound measurements as well as the rate of wound healing are represented in Figure 4. According to the photographs of the wounds on days 1, 3, 6, 9, and 11, the size of each wound decreased over time, and depending on the wound healing agent used, the extent to which wounds were covered by epithelial cells was different (Figures 4(a) and 4(b)). Furthermore, 11 days after injury, the wound area (mm²) of each rat
was plotted per day (Figure S6). According to Figure S6, which represents the relative ability of each wound healing agent throughout the whole treatment period, it was observed that GHK-\(\alpha\)-carnitine-loaded PLGA NP, having the steepest negative slope, exhibits the maximum. The \(\alpha\)-carnitine–GHK conjugate was the second most efficacious and \(\alpha\)-carnitine had minimal therapeutic influence on the wound area. PBS treatment as negative control had the lowest efficacy than others. Interestingly, unloaded-PLGA NP showed more potential in healing activity rather than \(\alpha\)-carnitine upon wound closure.

According to the reported anti-inflammatory, antibacterial, and healing effects of GHK peptide and \(\alpha\)-carnitine on wounds, we investigated wound closure-associated histology, hence animals were evaluated on day 14th for histological analysis.

3.6. Histological Analysis. Microscopic findings of blank groups on day 14 post injury showed a wound area with a thick crusty scab and the presence of inflammatory cells without re-epithelialization. Inflammatory response was considerably higher than other treatments (Figure 5). Whereas, after treatment of wounds with PLGA NP compared to the blank group, not only crusty scabs and incomplete epithelialization were observed, but also inflammation was reduced due to relative infiltration of inflammatory cells.

Finally, wound healing with GHK-\(\alpha\)-carnitine nanoconjugate resulted in a granular tissue formation and a crusty scab as well as complete epidermal layer for the wound. Moreover, neovascularization was also higher in this group in comparison to the other treatments.

4. Discussion

In the last decades, a lot of research has been focused on developing wound-healing agents which are biocompatible with accelerated therapeutic efficacy. In this regard, some studies have developed nanotechnology-based healing agents for chronic wounds [25].
In our study, a peptide conjugate-loaded nanopolymer as a novel wound healing agent was fabricated. L-carnitine–GHK peptide conjugate as a therapeutic was encapsulated into PLGA NPs as a carrier. PLGA as a FDA-approved copolymer can be used for controlled delivery of different biomedical materials. Other reported advantages of the polymer include inherent wound healing activity using lactate byproduct, drug protection or stability enhancement, as well as solubility improvement [39]. There is the straight relationship between physical/chemical properties of a NP and its stability. As no significant change in zeta potential and size of NPs was found during 2 weeks’ monitoring, it can be concluded that no aggregation occurs and NPs are stable.

Previously, wound-healing activity of the GHK peptide has been proven [17]. However, to further increase therapeutic outcomes, a conjugate of L-carnitine and GHK peptide was designed, taking advantage of the combined benefits of both substances. The conjugate in NP and non-NP forms was characterized and, the success of the conjugation and purification as well as desired characteristics of the NP was proved based on the results.

The larger size of NPs measured by DLS than that by SEM is related to the hydrodynamic diameter of PLGA in solution. A negative zeta potential of PLGA NP is attributed to the presence of carboxylic groups in terminal position. Before evaluating the wound healing efficacy, in vitro drug release in physiological condition was performed. Diffusion and degradation are known as mechanism of release from PLGA NPs [40].

The biphasic release profile of the conjugate from NPs including initial burst and subsequent sustained release, ensured an adequate drug concentration for in vivo study.

We are the only researchers who formulated a stable nanoconjugate to investigate the wound healing activity of the GHK peptide conjugate in the NP form, as no significant size variation was observed in the stability study. However, in order to study the stability of NPs in wound fluid, it was not easy to obtain sufficient fluid and it dried out quickly. So, the stability was performed in phosphate buffer saline (pH 7.4) in terms of particle size variation using DLS.

Furthermore, by visual comparison, although GHK peptide did not offer satisfactory results, its coupling with L-carnitine improved its healing ability. In other words, on the 11th day of post injury, wound healing percentages were calculated in this order: conjugate-loaded-PLGA NPs > conjugate > GHK > PLGA > L-carnitine > PBS, respectively. Wound healing was improved about three times by exposure to the conjugate-loaded NP on day 11 in comparison with the untreated group. Therefore, an obvious accelerated wound

**Figure 5:** H&E-stained microscopic sections of healed incisions in treatment groups; black thick arrows: crusty scab; white thick arrow: epidermal layer; thin arrows: infiltration of inflammatory cells; arrowheads: neovascularization.
healing was observed for the conjugate-loaded PLGA NP compared to other groups. In order to show microscopic healing activity of the L-carnitine–GHK conjugate, histological analysis was also performed. Hence, two- and four-fold increase in epithelialization were observed for topical treatment by unloaded L-carnitine and L-carnitine–GHK conjugate-loaded PLGA NP than untreated group at day 14 post injury, respectively. Furthermore, the number of inflammatory cells was decreased to about 1.8- and 3.5-fold upon the same treatments, respectively. Anyway, angiogenesis after exposure of wounds to them was elevated two and four times, respectively.

In recent years, some scientists have focused on the use of drug-loaded polymer NPs to accelerate wound healing. Compared to a study of simvastatin-loaded PEG/PVP NP by Alven et al., lower wound healing percentage by nanoconjugate in our study can be attributed to lower nanoconjugate dose, loading percentages as well as different drug release profiles. However, complete epithelialization was clear after wound creation in two studies [32].

In the present study, improving neovascularization was observed using a histopathology section in the group treated by nanoconjugate. Formation of blood vessels or angiogenesis appeared after nanoconjugate treatment, which is in accordance with study of insulin-loaded chitosan for wound healing reported by Ribeiro et al. [41].

Our study was in accordance with the results of the study, curcumin-loaded chitosan/carboxymethyl cellulose NPs on wound healing by Shende et al., increasing the rate of wound closure. Synergistic action on wound healing was due to the chitosan effect. Anyway, in our study, this synergistic effect could be attributed to lactate, a digestive by-product of PLGA [42].

Some studies have shown PLGA and related lactate release accelerate skin wound healing. Accordingly, lactic acid pool upon PLGA degradation is an additional factor, which accelerates wound healing activity in synergetic relation with conjugate, confirming our results [43]. As shown, the use of PLGA for NP preparation gives nanoconjugate a sustained release profile.

In this area, Hasan et al. [31] have developed clindamycin-loaded PLGA-PEI NPs to treat infected wounds. Similar to our study, the NP treatment accelerated the reduction in wound size as well as the number of inflammatory cells. However, further investigation is needed to compare the antimicrobial properties. In addition, the fast epithelialization using the nanoconjugate in the present study confirmed the effect of ferulic acid-loaded PLGA NPs on diabetic wound healing during wound closure assessment, a research done by Bairagi et al. [44].

The biocompatibility of the nanoconjugate in the present study is nearly comparable to most studies of PLGA-based NPs developed for wound healing, making it a potential candidate for wound healing management [45].

We speculate that the regeneration mechanism of wound healing may be a combination of mechanisms related to GHK peptide and L-carnitine effects, in which modulation of metalloproteinase activity, fibroblast activation, stimulation of epidermal basal cell; prompting angiogenesis; antioxidant and anti-inflammatory activities are attributed to peptide portion while, dermal angiogenesis, improvement of mitochondrial β-oxidation and osmoprotectant activity can be attributed to L-carnitine portion, as reported in refs. [24, 43, 46]. However, further studies are needed to elucidate the signaling pathway or exact mechanism of peptide conjugate for wound healing as well as antimicrobial properties.

5. Conclusion

In conclusion, for the first time we synthesized the L-carnitine–GHK peptide conjugate with high purity of about 91% using SPPS method to investigate wound-healing efficacy in a NP platform. Excellent biocompatibility was obtained for the conjugate which was loaded into PLGA NPs with high encapsulation efficiency of 85% and a stable particle size of about 200 nm. Surprisingly, a three-fold increase in wound closure efficacy of the conjugate resulted after encapsulation into PLGA NP compared to the untreated one upon reduced wound size. Furthermore, less inflammation, more epithelialization, and neovascularization were observed during the healing trend for the nanoconjugate. Our finding reveals the fact that incorporation of L-carnitine into GHK can highly improve GHK’s wound repair properties. The L-carnitine–GHK conjugate-loaded PLGA NP will be a promising candidate for wound healing in clinics.

Data Availability

All the experimental data are included in the manuscript.

Conflicts of Interest

The authors declare that they have no conflict of interests.

Acknowledgments

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Supplementary Materials

Scheme S1: Solid phase synthesis of L-carnitine–GHK. Figure S1: Analytical HPLC chromatogram of GHK. Figure S2: HPLC chromatogram of GHK-L-carnitine. Figure S3: ESI-MS Spectra of GHK-OH. Figure S4: ESI-MS Spectra of L-carnitine–GHK-OH. Figure S5: Tracking the stability of nanoparticles through size measurement using Zeta sizer. Figure S6: Representation of wound healing trends in the whole treatment period of the materials in the study. (Supplementary Materials)

References


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