GHK-Cu-liposomes accelerate scald wound healing in mice by promoting cell proliferation and angiogenesis

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ABSTRACT

Glycyl-l-histidyl-l-lysine (GHK)-Cu is considered to be an activator of tissue remodeling, and has been used in cosmetic products. In this study, we prepared liposomes encapsulating GHK-Cu and analyzed their effect on human umbilical vein endothelial cells (HUVECs) proliferation and scald wound healing in mice. The nano-scaled GHK-Cu-liposomes promoted HUVECs proliferation, with a 33.1% increased rate. Flow cytometry analysis showed increased cell number at G1 stage and decreased cell number at G2 stage after GHK-Cu-liposomes treatment. Western blotting indicated that the expression of vascular endothelial growth factor (VEGF) and fibroblast grow factors-2 (FGF-2) were both enhanced, as well as cell cycle-related proteins CDK4 and CyclinD1. In a mice scald model, angiogenesis in burned skin treated with GHK-Cu-liposomes was better compared with free GHK-Cu, and immunofluorescence analysis showed enhanced signal of CD31 and Ki67 in GHK-Cu-liposomes treated mice. Moreover, the wound healing time was shortened to 14 days post injury. Our results provide the evidence that GHK-Cu-liposomes could be utilized as a treatment for skin wounds.

INTRODUCTION

The use of endogenous peptidic antibiotics to treat skin and tissue infection has the potential for clinical use. The human tripeptide glycyl-l-histidyl-l-lysine (GHK), which was first isolated from plasma, has been shown to help wound repair.[1] GHK has a very high affinity to chelate Cu^{2+}, and the formed GHK-Cu could promote angiogenesis, and increase skin oxygen as well as the auxiliary antioxidant enzymes such as superoxide dismutase (SOD).[2] GHK-Cu could also be applied to uninjured skin to increase skin collagen, reduce skin irritation and redness, improve overall appearance and reduce age spots, photodamage and hyperpigmentation, etc. Thus, GHK-Cu can be considered as an activator of tissue remodeling, and has a long history of safe use in cosmetic products.

Recent studies demonstrated that the GHK tripeptide regulates a large number of human genes, which may contribute to the pleiotropic health-promoting effects of its copper complex.[3] In addition to regulating genes involved in wound healing and skin remodeling, GHK also increases the production of collagen, dermatan sulfate, chondroitin sulfate, and the small proteoglycan, decorin.[4] Proliferative markers of epidermal stem cells, integrins and p63 were stimulated by GHK-Cu and GHK at low concentrations.[5,6]

Bacterial biofilms secrete powerful proteases that can degrade peptides in minutes. In diabetic and venous stasis skin ulcers, GHK is broken down quickly. When GHK-Cu injected into wounded tissue, it is cleared rapidly from injection area.[2] Thus, encapsulating GHK-Cu in liposomes may prevent it from being degraded and

enable it to exert its activity. Liposomes can be targeted and have a sustained-release effect, delaying the half-life and improving the stability of the encapsulated drugs.[7] Ligand-modified liposomes can be targeted preferentially to selective receptors on diseased cells and improve the therapeutic index.[8]

In this paper, we prepared GHK-Cu-liposomes and observed its effect on cell proliferation, cell cycle and the expression of vessel formation- and cell cycle-related factors. The murine scald model was used to evaluate the effect of GHK-Cu-liposomes on wound healing *in vivo*.

MATERIALS AND METHODS

Cells and animals

Human umbilical vein endothelial cells (HUVECs) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 100 U/L penicillin and 100 mg/L streptomycin at 37 ℃ and 5% CO_2. Cells with 70-80% confluence were used in the experiment.

SPF (Specific pathogen free) Kunming mice were provided by Dalian Medical University Animal Experiment Center. All the animal experiments were under the regulation and inspection of the animal ethics committee of Dalian Medical University.

Primary Reagents

DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) and DOPG

[1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycero1) sodium salt] were obtained from Japan Nippon Oil and Fats (Tokyo, Japan). GHK-Cu (purity more than 98%) was purchased from CP Biochem Co., Ltd. (Chengdu, China). Cell Counting Kit 8 (CCK-8) was purchased from Dojindo Company (Tokyo, Japan). All primary antibodies were rabbit polyclonal antibodies. VEGF, FGF-2 and CyclinD1 were from Proteintech Group (SanYing Biotechnology, Wuhan, China), CDK4 was purchased from Bioworld Technology, Inc. (St. Louis Park, MN, USA), Ki67 and CD31 were from Abcam (Cambridge, MA, England). β-actin was purchased from Beijing TransGen Biotech Co. Ltd. Second antibodies were goat anti-rabbit antibody, horseradish peroxidase (HRP)-labeled antibody was purchased from Proteintech Group, Alexa Fluor 594-labeled antibody was from Life Technologies (Willow creek, OR, USA). Electrochemiluminescence detection kits were from Thermo Fisher Scientific (Rockford, IL, USA).

GHK-Cu-liposomes preparation and characterization

Liposomes composed of DOPC: DOPG: CH (3:3:4, molar ratio) were prepared by lipid film hydration, as described previously.[9] Briefly, 100 μmol of lipid dissolved in 2 mL of a chloroform/diethyl ether mixture (1:1 v/v) was added to a rotary evaporator to form a lipid film under reduced pressure. Five milliliters of 100 mM GHK-Cu solution was then added and the lipid film was vortexed until a suspension was formed. A tip-type ultrasonic homogenizer and an extruder device with a polycarbonate membrane 100 nm in pore size were used to control the size and

lamellarity of the GHK-Cu-liposomes. The mean diameter and zeta potential of the prepared liposomes were determined with a Malvin 2000 laser particle size analyzer. Unencapsulated free GHK-Cu was removed by a PD-10 desalting column (BD, Amersham) with PBS as the elution buffer.

For morphology analysis, liposome sample was negatively stained with 2% phosphotungstic acid for 2 min and observed with a JEM-2000-ex transmission electron microscope.

Effect of GHK-Cu-liposomes on the growth of HUVECs

The proliferation of HUVECs under different treatments was assessed using the CCK-8 assay. After culture in 96-well plates in complete medium for 24 h, the cells were incubated with 10, 50 and 100 μM GHK-Cu and GHK-Cu-liposomes for 24 h, and cell viability was analyzed with CCK-8 according to the manufacturer's instructions. Briefly, following incubation at 37 °C for 12 h, 10 μL CCK-8 was added to each well, and the plates were incubated for 1-2 h. The Optical density (OD) at 450 nm was read with a microplate reader (Multiskan Ascen, Thermo Fisher Scientific, Waltham, MA, U.S.A.), and the values were compared with that of the control group. The proliferation rate was calculated as follows: [(OD450 treated cells-OD 450 blank control)/(OD450 control-OD450 blank control)-1]×100%. Three independent experiments were performed.

Measurement of Cu content in HUVECs

To detect GHK-Cu delivery to the cells, 100 μM of GHK-Cu or GHK-Cu-liposomes were added to the cells at 70-80% confluence in 6-cm dishes. After incubation for 3, 6, 12, 24 and 48 h, the cells were washed with PBS, dissolved in 200 μL of concentrated nitric acid overnight, and diluted with 5 mL of MilliQ water. The Cu content was measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES, optima 2000 DV, Perkin Elmer, Fremont, CA, USA).

Flow cytometry analysis of cell cycle

For analysis of the cell cycle, HUVECs were incubated with GHK-Cu-liposomes for 24 h. After treatment, cells were harvested and washed with PBS, and resuspended with 70% cold ethanol, put in the dark at 4°C overnight according to the instruction of cell cycle detection kit (Keygentec, Shanghai, China). After incubation, cells were treated with RNaseA for 30 min at 37°C and labeled with 400 μL PI and samples were immediately analyzed by flow cytometry (BD Beckman Coulter, Epics XL).

Western blotting analysis of protein expression after GHK-Cu-liposomes treatment

After treatment with GHK-Cu-liposomes and GHK-Cu (10μM) for 24 h, total proteins were extracted from HUVECs using RIPA buffer (Sigma). Equivalent amounts of proteins were subjected to SDS-PAGE and transferred to a PVDF membrane. Blots were probed with the desired primary antibodies (VEGF, FGF-2, CDK4, and CyclinD1), followed by the treatment with horseradish

peroxidase-coupled secondary antibody. Immunoreactive protein bands were visualized using an enhanced chemiluminescence detection kit on a ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA). β-actin was used as an internal control. The signal strength of the strips was quantified with Image-Pro Plus software, and the relative expression of the protein was expressed as the ratio of the target protein to β-actin.

Mice scald model and GHK-Cu-liposomes treatment

To establish the mice scald model, 2.5 cm^2 sized back hair of mice was removed by treatment with 8% Na_2S for 2 min. The area was then rinsed immediately with double-distilled H_2O and the naked skin was allowed to dry. The next day after hair removal, under ether anesthesia, a small pad of 1 cm^2 was dipped into boiling water, then rapidly placed on the hairless patch for 3 seconds to generate a shallow II degree burn model [10]. Scalded mice were randomly divided into four groups (PBS, PBS liposomes, GHK-Cu, and GHK-Cu-liposomes groups) of five mice each. Mice were daubed once a day with PBS, PBS liposome, 100 μM GHK-Cu, and 100 μM GHK-Cu liposomes, respectively. The wound healing status was observed daily, a healing standard was expressed as skin with closed wounds, dry surface, and the eschar area back to normal.

Haematoxylin and eosin (H&E) staining and immunofluorescence analysis

Two days after treatment with GHK-Cu-liposomes, mice were scarificed under

anesthesia and the skin patch was cut off. The mice skin were washed with PBS and fixed with formalin. The formalin-fixed paraffin-embedded mice skin tissues were sliced into 3-μm thick sections and baked for 2 h at 60 °C, then deparaffinized with xylene and rehydrated. The slices were stained with H&E through graduate change of alcohols for dehydration. Blood vessels in H&E staining slices were counted under a microscope. Values are expressed as mean vessel numbers in nine random fields of view (40×objective).

For immunofluorescence analysis, the tissue sections were incubated in 3% H_2O_2 and then dip into 10 mM sodium citrate buffer to heat for 20 min using a microwave oven for antigen retrieval. After cooled down, the slices were blocked with 1% BSA and incubated with CD31 and Ki67 antibody overnight at 4 °C. The slices were washed with PBS for 10 min at room temperature before incubating with Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody at 37 °C for 1 h. The nuclei were stained with DAPI (Life Technologies, Carlsbad, CA). The fluorescence signals were visualized with an Olympus IX71 fluorescence microscope.

For cell immunofluorescence obervation of Ki67, after 24 h incubation with PBS, PBS-liposomes, GHK-Cu, and GHK-Cu-liposomes, HUVECs were fixed with 4% paraformaldehyde (PFA) and treated with 0.2% Triton X-100 for 10 min seperately, washed with PBS twice after each treatment, immunofluorescence assay was carried out as above.

Statistical analysis

Data are shown as mean ±SD and were analyzed with the Student's t-test. $P < 0.01$ was considered statistically significant.

RESULTS

Characterization of GHK-Cu-liposomes and ICP-AES analysis of GHK-Cu absorption in HUVECs

After liposomes were prepared with the film method, they were observed by transmission electron microscopy. Liposomes with uniform size and single vesicle structure with double membrane could be clearly visualized (Figure S1), indicating the homogeneity. The average diameter was 117.2 nm and the zeta potential was -37.88 mV. To detect the uptake of GHK-Cu-liposomes by cells, we measured the Cu concentration in HUVECs at different time points. The ICP-AES results indicated that GHK-Cu-liposomes were absorbed by HUVECs, and the uptake increased with time (Figure S2). The uptake of GHK-Cu-liposomes was higher than that of free GHK-Cu (data not shown).

Effect of GHK-Cu-liposomes on HUVECs proliferation

As shown in Figure 1A, both GHK-Cu-liposomes and GHK-Cu could promote HUVECs proliferation, but the GHK-Cu-liposomes had a better effect and the boost was dose-dependent. The proliferation rate was the highest at 100 μM, with 33.1%. The VEGF and FGF-2 expression levels in cells treated with GHK-Cu-liposomes were higher than those in GHK-Cu or PBS liposome treated cells at 24 h (Figure 1B).

Compared with PBS and PBS-liposomes control, the expressions of VEGF and FGF-2 were both increased more than twice (Figure 1C).

Cell immumofluorescence result indicated that Ki67 showed strong colocalization with nucleus after treatment, demonstrating that GHK-Cu-liposomes promote cell proliferation in HUVECs (Figure 2).

Effect of GHK-Cu-liposomes on HUVECs cell cycle

We checked the expression of cell cycle-related factors, Western blotting results indicated that the expression levels of CDK4 and CyclinD1 were both enhanced after treatment with GHK-Cu-liposomes at 24 h (Figure 3A). Flow cytometry analysis showed that the cell percentage at G1 stage reached to the highest of 57.49%, while the number in G2 stage decreased to 4.78% (Figure 3C).

Effect of GHK-Cu-liposomes on angiogenesis

Two days after treatment with GHK-Cu-liposomes, mice were scarificed under anesthesia and skin sections were prepared. As shown in Figure 4, more blood vessels were formed with GHK-Cu-liposomes treatment compared to the GHK-Cu and PBS-liposomes, indicating the strong angiogenesis activity of GHK-Cu-liposomes. The increased expression of CD31 in vessel endothelial cells in immunofluorescence analysis further confirmed the proangiogenic ability of GHK-Cu-liposomes (Figure 5). In accordance with *in vitro* cell result, Ki67 expression was also increased in mice skin wound after GHK-Cu-liposomes treatment.

Effect of GHK-Cu-liposomes on burn healing in mice

The healing effect of the GHK-Cu-liposomes was superior to free GHK-Cu and other treatments (Figure 6). During the early stage of wound healing, GHK-Cu-liposomes group showed wound integrity and reduced inflammatory reaction. In the later stage of wound healing, we found that GHK-Cu-liposomes could retard scar-forming and fasten tissue repair. We also evaluated the healing days of the wounded mice, the minimum time for healing was 14 days from being scalded (Table S1).

DISCUSSION

Liposomes have been tested extensively in experimental animals as drug delivery vehicles. Liposomes offer the potential to enhance the therapeutic index of anticancer agents, either by increasing the drug concentration in tumor cells or by decreasing toxicity to normal host tissues. Doxorubicin and paclitaxel have been encapsulated in liposomes for cancer treatment. [11] For drug delivery to skin, liposomes can exert different functions after topical application. They can improve drug deposition within the skin at the site of action, where the goal is to reduce systemic absorption and thus minimize side effects. [12] The localising effect of liposomes is dependent on lipid composition and preparation method. Maghraby et al showed that stratum corneum lipid liposomes show the best results compared with phospholipid liposomes and O/W (oil in water) emulsion and hydroalcoholic drug solution. [13]

As discovered by Pickart and Thaler in 1973, [14] the tripeptide GHK and its copper complex (GHK-Cu) function as an activator of tissue remodeling. Copper is an essential trace element, critical for a variety of biological processes; for example, for hemoglobin synthesis, enzyme activation, (i.e., superoxide dismutase), or more generally as a key component of mitochondrial, cytoplasmic and nuclear enzyme systems. [15] It is a key factor in the production of collagen and elastin. [16] GHK-Cu possesses a wide array of actions that suppress the acute phase inflammatory and scar-forming events after wounding. Cosmetic GHK-Cu products are also applied to uninjured skin to improve overall appearance, including increasing skin collagen, and reducing skin irritation, redness, age spots, photodamage and hyperpigmentation. [2]

GHK-Cu at low concentrations is a powerful attractant for capillary cells that build new blood vessels, [17] and macrophages and mast cells that remove damaged cellular debris and secrete proteins important for tissue healing. Mast cells can stimulate wound contraction. [18,19] GHK-Cu helps re-establish blood flow into damaged tissues through a mixture of three actions: angiogenesis, anti-coagulation and vasodilation. GHK-Cu increases the expression of basic fibroblast growth factor and vascular endothelial growth factor from wound repair fibroblasts, both of which aid blood vessel formation. [20] However, direct application of GHK-Cu to the wound will enable it to come into contact with acidic material on the surface of the skin. Thus, encapsulation of GHK-Cu in liposomes protects it from degradation by acidic substances in the wound. The other advantage of liposome encapsulation of GHK-Cu is that it can protect the peptide from degradation by powerful proteases secreted by

bacterial biofilms and proteinaceous growth factors, as GHK is quickly broken down by wound proteases found in diabetic and venous stasis skin ulcers. [2]

In our previous work, we found that GHK-Cu-liposomes induce hair follicles in a mouse alopecia model. Burn surgeons have long observed that the influx of hair follicles into a burned area predicts a good healing response. It is now established that dermal hair follicles provide a major source of stem cells used for dermal healing. [21] Peled et al claimed that GHK increases proliferation of stem cells, while GHK-Cu increases their progression into differentiated cells. [22,23] Choi et al showed that GHK promotes the survival of basal stem cells in a skin equivalent model, and that the number of proliferating cells is also increased by the addition of GHK. [6] The additional function of GHK-Cu-liposomes we found in this study was to enhance HUVECs proliferation by 33.1% compared with GHK-Cu. ICP results indicated that although the Cu content delivered by GHK-Cu-liposomes is higher than that by free GHK-Cu, there is no big difference for the GHK-Cu uptake after 24 h with liposome encapsulation. But the Western blotting analysis clearly proved that GHK-Cu-liposomes could effectively promote the expression of cell cycle-related proteins, CDK4 and Cyclin D1, which exert funtion in G1/S stage, suggesting more DNA was synthesized. We also used flow cytometry to check the percentage of different stages. Cell number at G1 stage increased and decreased at G2 stage, suggesting that more cells divided after GHK-Cu-liposomes treatment. In accordance with Western blotting result, immumofluorescence analysis *in vitro* and *in vivo* indicated that Ki67 showed strong expression in nucleus, demonstrating that

GHK-Cu-liposomes promoting cell proliferation. Compared with free GHK-Cu, liposome-encapsulated GHK-Cu may be localized in different compartments in cell. This observation suggests that the lipids comprising liposomes may play a role in the promotion of proliferation.

VEGF and FGF-2 are two important vascular growth factors that can promote the formation of blood vessels. VEGF can specifically induce vascular endothelial cell proliferation, angiogenesis, and increase vascular permeability, which leads to the leakage of intravascular molecules to provide the matrix for the migration of vascular endothelium and angiogenesis. FGF-2, which originates from the neural ectoderm and mesoderm cells, can promote proliferation, differentiation and migration, and can also affect the stages of wound repair. [24,25] There are synergistic effects between FGF-2 and VEGF. [26,27] In our study, Western blotting analysis showed that GHK-Cu-liposomes and GHK-Cu could promote both growth factors, that the effect on VEGF and FGF-2 expression increased with time and that GHK-Cu-liposomes had a greater effect than free GHK-Cu. In a mice scald model, more blood vessels could be observed in H&E staining and immunofluorescence analysis after GHK-Cu-liposomes treatment, and the wound healing time was shortened post injury, indicating a stronger effect on angiogenesis.

Tissue remodeling is a poorly understood process that restores normal tissue morphology after various types of injuries. This remodeling process stops the initial inflammatory event and scar-forming processes after injury, then slowly removes the scar tissue and cellular debris arising from injuries. Our *in vitro* and *in vivo*

experiments proved that GHK-Cu-liposomes could promote the proliferation of HUVECs, increase the expression of angiogenesis-related factors and fasten burn wound healing. Our results suggest that liposome encapsulated drugs provide the potential to enhance their function for skin injury treatment.

ACKNOWLEDGEMENTS

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Conflict of Interest: All authors declare no conflict of interests.

Figure captions

Figure 1. Effect of GHK-Cu-liposomes on HUVECs proliferation. (A) CCK-8 analysis of different concentrations of GHK-Cu-liposomes on cell proliferation. HUVECs were incubated with 10, 50 and 100 μM GHK-Cu and GHK-Cu-liposomes for 24 h, and the proliferation rate was determined by the CCK-8 assay. (B) Western blotting analysis of VEGF and FGF-2 after GHK-Cu-liposomes treatment. β-actin was used as the loading control. (C) Relative quantitative analysis of protein expression strength, ** $p<0.01$.

Figure 2. Immunohistochemistry analysis of Ki67 expression in HUVECs after GHK-Cu-liposomes treatment. HUVECs were incubated with GHK-Cu-liposomes for 24 h and fixed with 4% PFA. Anti-Ki67 rabbit polyclonal antibody was used as the primary antiboday and Alexa Fluor 594-conjugated goat anti-rabbit IgG was used as the secondary antibody. The fluorescence signals were visualized with a fluorescence microscope. Bar=50μm.

Figure 3. Effect of GHK-Cu-liposomes on cell cycle in HUVECs (A) Western blotting analysis of CDK4 and CyclinD1 expression after GHK-Cu-liposomes treatment for 24 h in HUVECs. (B) Relative quantitative analysis of protein expression strength, ** $p<0.01$. (C) Cell cycle phase distribution by flow cytometry.

Figure 4. Effect of GHK-Cu-liposomes on angiogenesis in the skin of scalded mice. (A) Blood vessel formation in mice skin wound H&E slice after GHK-Cu-liposomes treatment for 48 h (B) Quantitative analysis of blood vessel numbers, ** $p<0.01$. Bar=200μm.

Figure 5. Immunofluorescence analysis of CD31 and Ki67 expression in mice after GHK-Cu-liposomes treatment. Mice slices were prepared 48 h after treatment, antigen retrieval was carried out and then skin sections were incubated with rabbit anti-CD31 and Ki67 polyclonal antibody overnight at 4 ℃. After incubating with Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody at 37 ℃ for 1 h. The fluorescence signals were visualized with a fluorescence microscope. Bar=200μm.

Figure 6. Effect of GHK-Cu-liposomes on mice wound healing. After the mice burn model was established, GHK-Cu-liposomes were applied to the scalded skin once a day. For observation of angiogenesis in scalded mice skin, mice wound healing status was photographed every other day.

Supplementary data

Figure S1. Characterization of GHK-Cu-liposomes. (A) Diameter of GHK-Cu-liposomes. (B) Image of GHK-Cu-liposomes under TEM observation (40,000×)

Figure S2. ICP-AES analysis of Cu delivery to HUVECs by GHK-Cu-liposomes. After incubation with 100 μM GHK-Cu-liposomes or GHK-Cu for 3, 6, 12, 24 and 48 h, HUVECs were harvested by dissolving the cells in diluted nitric acid, the amounts of internalized Cu were detected.

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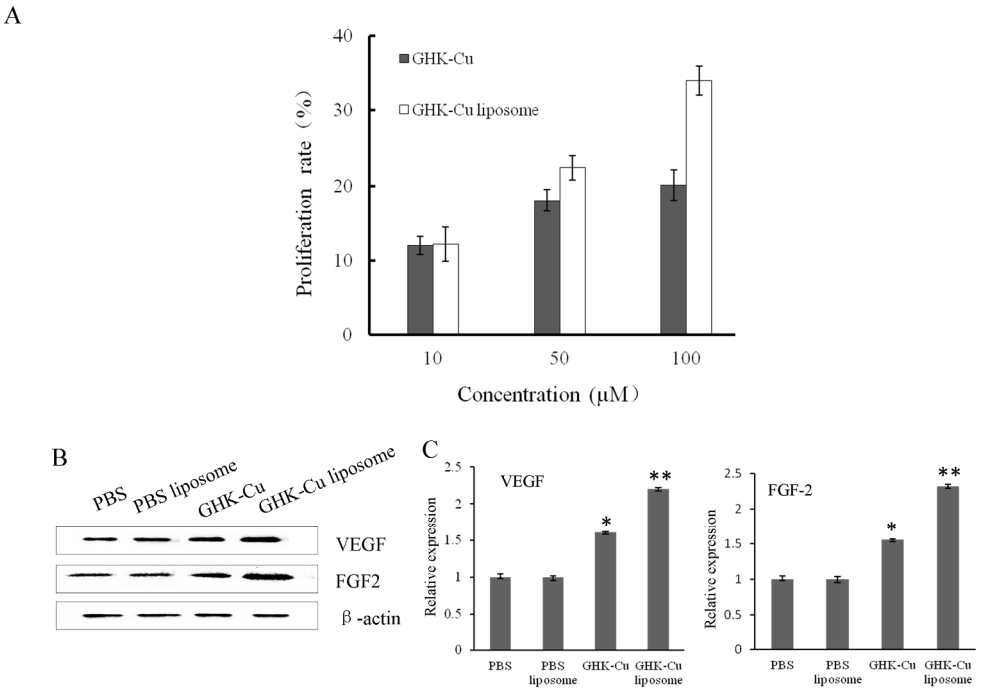

Figure1. Effect of GHK-Cu-liposomes on HUVECs proliferation

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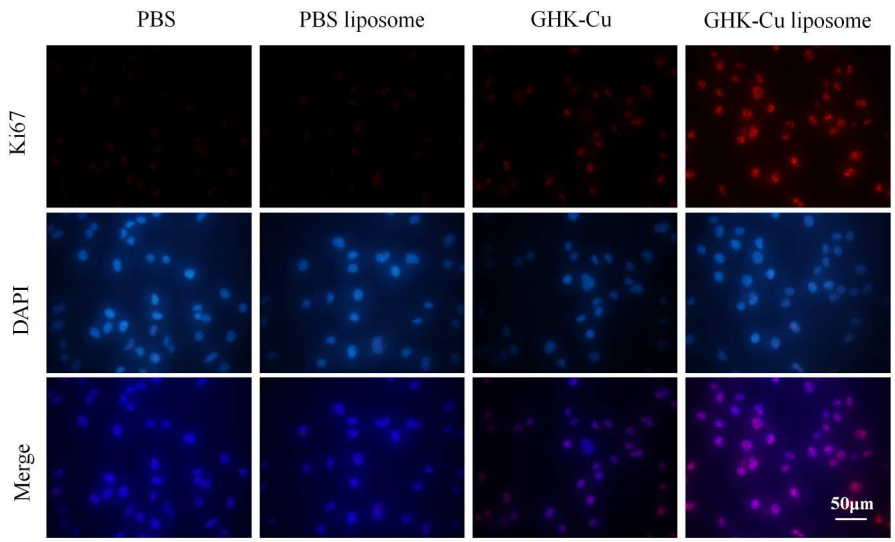

Figure 2. Effect of GHK-Cu-liposomes on expression of Ki67 in HUVECs

Figure 2. Immunohistochemistry analysis of Ki67 expression in HUVECs after GHK-Cu-liposomes treatment. HUVECs were incubated with GHK-Cu-liposomes for 24 h and fixed with 4% PFA. Anti-Ki67 rabbit polyclonal antibody was used as the primary antiboday and Alexa Fluor 594-conjugated goat anti-rabbit IgG was used as the secondary antibody. The fluorescence signals were visualized with a fluorescence microscope. Bar=50μm.

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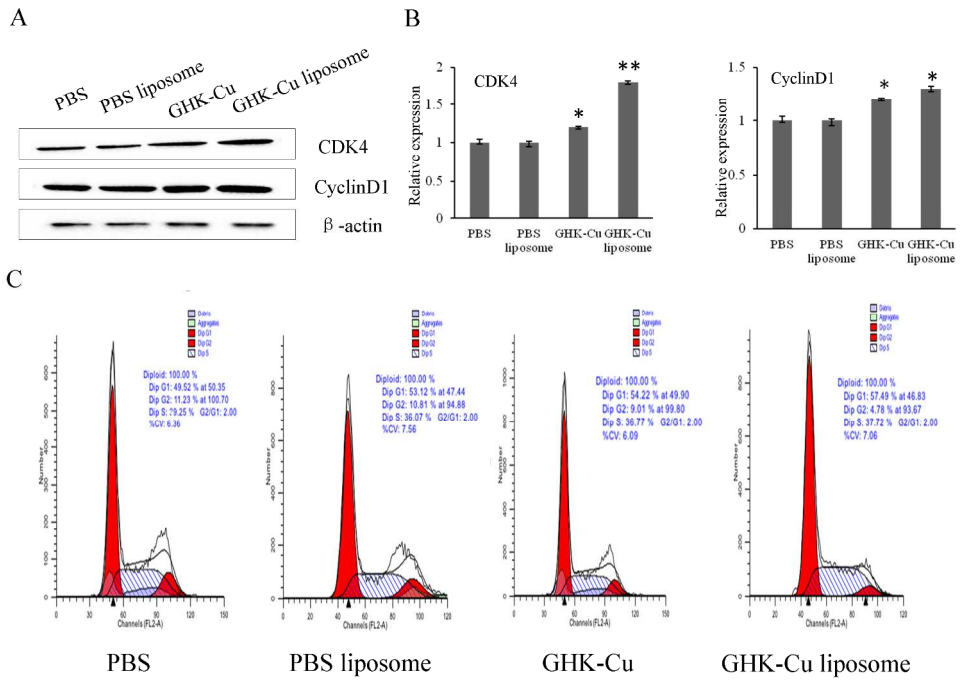

Figure 3. Effect of GHK-Cu-liposomes on cell cycle in HUVECs

Figure 3. Effect of GHK-Cu-liposomes on cell cycle in HUVECs (A) Western blotting analysis of CDK4 and CyclinD1 expression after GHK-Cu-liposomes treatment for 24 h in HUVECs. (B) Relative quantitative analysis of protein expression strength, ** p<0.01. (C) Cell cycle phase distribution by flow cytometry.

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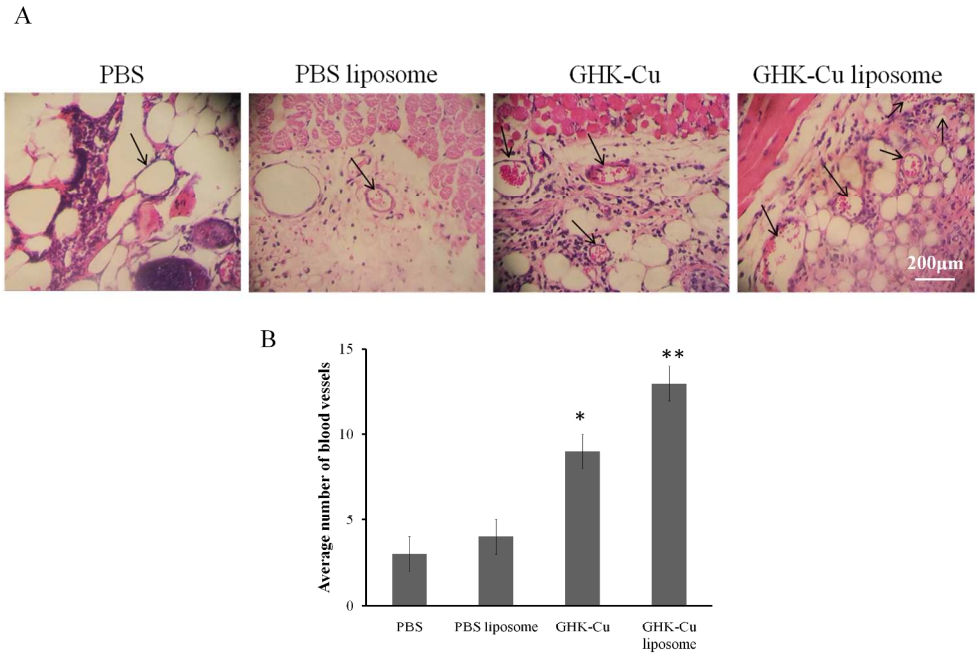

Figure 4. Effect of GHK-Cu-liposomes on blood vessel formation

Figure 4. Effect of GHK-Cu-liposomes on angiogenesis in the skin of scalded mice. (A) Blood vessel formation in mice skin wound H&E slice after GHK-Cu-liposomes treatment for 48 h (B) Quantitative analysis of blood vessel numbers, ** p<0.01. Bar=200μm.

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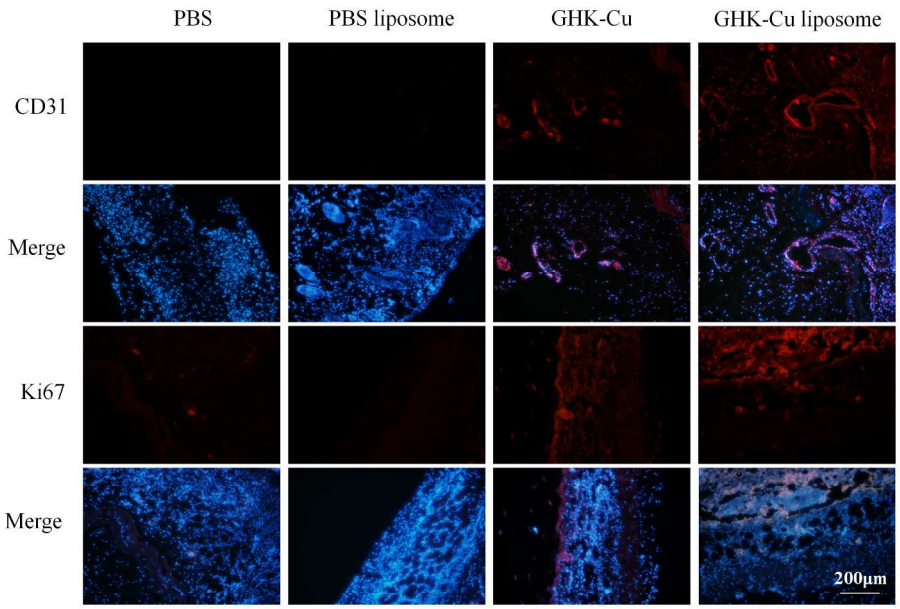

Figure 5. Effect of GHK-Cu-liposomes on expression of CD31 and Ki67 in mice

Figure 5. Immunofluorescence analysis of CD31 and Ki67 expression in mice after GHK-Cu-liposomes treatment. Mice slices were prepared 48 h after treatment, antigen retrieval was carried out and then skin sections were incubated with rabbit anti-CD31 and Ki67 polyclonal antibody overnight at 4 °C. After incubating with Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody at 37 °C for 1 h. The fluorescence signals were visualized with a fluorescence microscope. Bar=200μm.

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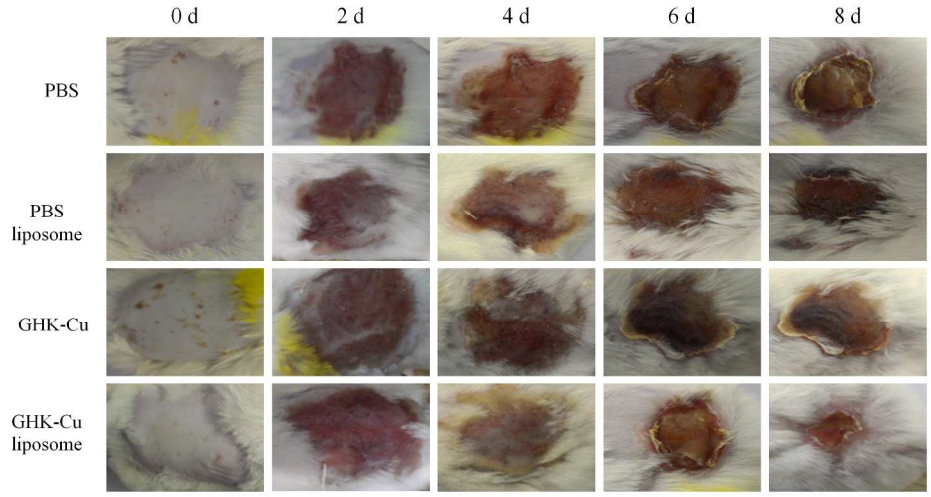

Figure 6. Effect of GHK-Cu-liposomes on burn healing in mice

Figure 6. Effect of GHK-Cu-liposomes on mice wound healing. After the mice burn model was established, GHK-Cu-liposomes were applied to the scalded skin once a day. For observation of angiogenesis in scalded mice skin, mice wound healing status was photographed every other day.

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