REGENERATIVE EFFECTS OF GLY-HIS-LYS AND GLY-HIS-LYS-D-ALA PEPTIDES IN INFECTED SKIN WOUNDS

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Skin wound healing mechanisms and new ways of improving their efficiency represent an important focus in medicine. In this regard, regulatory peptides, which exhibit physiological polyfunctionality and modulate cell growth and differentiation, are of special interest. This study evaluates the effects of Gly-His-Lys (GHK) and Gly-His-Lys-D-Ala (GHK-D-Ala) peptides in the infected skin wound healing. The wounds were modeled in rats (n=150) by full-thickness dorsal skin defects. The peptides were administered intracutaneously at daily doses of 0.5 or 1.5 μg/kg. The healing was assessed on days 3, 7, and 10 by histomorphometric examination of the wounds with adjacent intact skin. GHK-D-Ala administered at daily doses of 0.5 μg/kg had pronounced positive effect on regeneration processes in the wound, as indicated by significantly reduced numbers of granulocytes and lymphocytes with increased representation of fibroblastic lineages and macrophages, and the resulting higher cellular index ($p < 0.05$–0.001). At higher doses of GHK-D-Ala (1.5 μg/kg), the beneficial effects were less pronounced. According to the comparative morphological examination, the highest positive effect was achieved with 0.5 μg/kg of GHK-D-Ala. Thus, local administration of the GHK peptide with extra D-alanine at carboxy-terminus significantly mitigated the inflammatory reaction and facilitated the healing of infected skin wounds in rat model.

Keywords: Gly-His-Lys-D-Ala, GHK-D-Ala, infected wound, regeneration, inflammation

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Compliance with ethical standards: the study was approved by Ethics Committee of the Kursk State Medical University (Protocol № 1 of January 16, 2014). The study was carried out in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and the Guidelines for conducting preclinical drug trials (Moscow, 2012).

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РЕГЕНЕРАТИВНЫЕ ЭФФЕКТЫ ПЕПТИДОВ GLY-HIS-LYS И GLY-HIS-LYS -D-ALA ПРИ КОЖНОЙ ИНФИЦИРОВАННОЙ РАНЕ

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Исследование механизмов регенерации при раневом процессе и поиск новых путей повышения эффективности заживления являются одними из актуальных направлений в медицине. Поэтому представляется целесообразным изучение репаративных эффектов регуляторных пептидов, обладающих физиологической полифункциональностью и оказывающих влияние на процессы роста и дифференцировки клеток. Целью исследования было изучить влияние пептидов Gly-His-Lys (GHK) и Gly-His-Lys-D-Ala (GHK-D-Ala) на процессы регенерации в условиях инфицированной кожной раны у крыс. Рану моделировали на 150 животных путем нанесения на участке спины полнослойной раны, пептиды вводили в дозах 0,5 и 1,5 мкг/кг подкожно в области раны раз в день в течение 3, 7 или 10 суток. Для оценки течения раневого процесса изучали гистологические и морфологические препараты участков раны с прилежащей интактной кожей. GHK-D-Ala в дозе 0,5 мкг/кг оказывал более выраженное, чем GHK, влияние на регенеративные процессы в ране, что отразилось в значимом снижении числа гранулоцитов и лимфоцитов и повышении числа клеток фибробластического ряда, макрофагов и клеточного индекса по сравнению как с контрольной группой ($p < 0,05$–$0,001$), так и с животными, которым вводили GHK в эквивалетной дозе ($p < 0,05$–$0,001$). При увеличении дозы до 1,5 мкг/кг эффекты GHK-D-Ala несколько ослабевали. По результатам сравнения исследованных показателей наибольшая активация регеративных процессов в ране выявлена после введения GHK-D-Ala в дозе 0,5 мкг/кг. Таким образом, присоединение D-аланина к С-концу пептида GHK способствовало ослаблению воспальной реакции и усилению регенеративных процессов при местном введении в условиях инфицированной кожной раны.

Ключевые слова: Gly-His-Lys-D-Ala, GHK-D-Ala, инфицированная рана, регенерация, воспаление

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Regenerative mechanisms of the wound healing and new srategies for its enhancement constitute one of the topical areas in medicine. Skin repair involves all three regulatory systems of the body — nervous, endocrine, and immune [1, 2]. These systems are known to act through regulatory signaling molecules, which exhibit physiological polyfunctionality and modulate cell growth and differentiation [3].

A naturally occurring NH_2-Gly-L-His-L-Lys-COOH tripeptide (Gly-His-Lys, GHK) can stimulate tissue regeneration processes [4–6] and confer antioxidant, immunotropic, anti-inflammatory, and neurotrophic effects [7–10]. The curative effect of GHK in skin wounds is significant but moderate [11], probably due to the rapid degradation of the peptide by proteolytic milieu of the wound. To overcome this limitation, we modified the structure of GHK by adding D-alanine (D-Ala) moiety to its carboxy-terminus in order to increase its resistance to proteolysis and thus reinforce its action.

The study aimed at comparative evaluation of the influence of GHK and its structural modification GHK-D-Ala on healing processes in the infected skin wounds in rat model.

METHODS

The experiments were carried out on male Wistar rats, 180–240 g body weight, aged 6–8 months, obtained from the "Stolbovaya" branch of the Scientific Center for Biomedical Technologies of the Federal Medical and Biological Agency of Russia. The animals (n = 150) were kept under standard conditions with ad libitum access to chow and water, at 12-h light cycle and 22 ± 2 °C. The animals were randomly assigned to 15 groups, 10 animals in each group.

The infected wound was modeled by introducing a 250 mm^2 full-thickness skin defect to a shaven area of the dorsum in anesthesized animals.

The study used peptides GHK and GHK-D-Ala synthesized in the Institute of Chemistry, Saint Petersburg State University. The peptides were dissolved in saline and administered intracutaneously at two points around the wound, changing the injection sites clockwise by 90° each time. The first injection made at 24 h after wound modeling was followed by repeated injections of the same medication every 24 h for 3, 7 or 10 days. The treatment groups received either 0.5 or 1.5 μg/kg daily doses of either GHK or GHK-D-Ala; the control groups received sham injections of saline pro rata 1 mL per 1 kg body weight.

The animals were withdrawn from the experiment by puncture of the right cardiac ventricle under ether anesthesia. The healing process was assessed by histological examination of wound autopsies on days 3, 7, and 10 post-wounding. The specimens were collected by dissection of the wound area with adjacent intact skin, formalin-fixed, paraffin-embedded, and sectioned using routine techniques. The 7 μm sections were stained with hematoxylin and eosin (H & E) for light microscopy.

Morphological examination of the slides was carried out with a Nikon Eclipse Ci microscope (Nikon; Japan) equipped with a digital camera. The histological assessment accounted for the degree of inflammatory reaction, landmarks of granulation tissue development and marginal epithelization, as well as the structural maturity of the newly formed epithelium.

Morphometric assessment for the course of healing process was carried out by differential cell counts in histological slides. The cells were counted at ×400 magnification of a representative area beneath the leukocyte-fibrinous scab to a total of 100 cells, and the shares of particular cell populations (fibroblastic lineages, macrophages, granulocytes, and lymphocytes) were expressed as percentages.

A quantitative index for the extent of healing and regeneration phase was calculated as follows:

$$CI = \frac{Fb+M}{Gr+L} \times 100\%,$$

where CI stands for "cellular index" derived from differential cell counts including Fb — fibroblast lineages (prefibroblasts, fibroblasts, and fibrocytes), M — macrophages, Gr — granulocytes (neutrophils, eosinophils, and basophils), and L — lymphocytes. At CI > 100%, regeneration processes are considered dominant, whereas at CI < 100 inflammatory processes are considered dominant.

Statistical processing of the data was carried out in R v.4.1.0 [12] using the RStudio Desktop v. 1.4.1717 integrated development environment (RStudio, PBC; USA). The initial processing involved Shapiro–Wilk test for normality (shapiro.test() function of the R base package) and Levene's test for equality of variances (levene.test() function of the lawstat package). Subsequent two-group comparisons used one-way ANOVA (aov() function of the R base package) with a post hoc Dunnett test (DunnettTest() function of the DescTools package); the data are presented as means and standard deviations (M ± SD) calculated using mean() and sd() functions of the R base package. Multiple comparisons used Kruskal–Wallis test (kruskal.test() function of the R base package) with a post hoc Dunn's test (dunn.test() function of the dunn.test package); the data are presented in the median [lower quartile; upper quartile] format (Me [1Q; 3Q]), calculated using median() and quantile() functions of the R base package. The differences were considered significant at $p < 0.05$.

RESULTS

The results of histological examination of skin defects on day 3 post-wounding were similar in all groups. The defect was filled with polymorphocellular infiltrate showing a predominance of leukocytes beneath the clearly distinguished superficial leukocyte-necrotic layer. The boundary with the intact dermis had signs of edema: sparse "empty" vessels with expanded lumina and thinned walls, and swollen fibers forming a network growing denser towards the leukocyte-necrotic layer.

On day 7 post-wounding, skin defects in the control group had the leukocyte-necrotic layer non-ubiquitously demarcated from the infiltration zone. The deeper layers presented with initial stages of the granulation tissue histogenesis, with cellular composition dominated by neutrophils, histiocytes, and lymphocytes. With the use of 0.5 μg/kg doses of either GHK or GHK-D-Ala, the leukocyte-necrotic layer was preserved, although non-ubiquitously, and was significantly reduced compared with the control. The discontinuities of the leukocyte-necrotic layer showed fibrin deposits clearly demarcated from the infiltration zone. The underlying young connective (granulation) tissue contained the fully perfused dilated rounded capillaries. The signs of edema included the moderately thinned capillary endothelium and prominent interstructural spaces. Cellular composition of the granulation tissue was dominated by macrophages, fibroblasts and lymphocytes. With 1.5 μg/kg doses of either GHK or GHK-D-Ala, the signs of edema were reduced and the leukocyte-necrotic scab was fragmentary. Formation of the granulation tissue and a network of thin collagen fibers were observed in the wound area, accompanied by round-cell infiltration. In addition, the higher-dose groups presented with the onset of wound epithelization at the boundaries with intact skin, in the form of marginal epidermal thickening with preserved stratification.

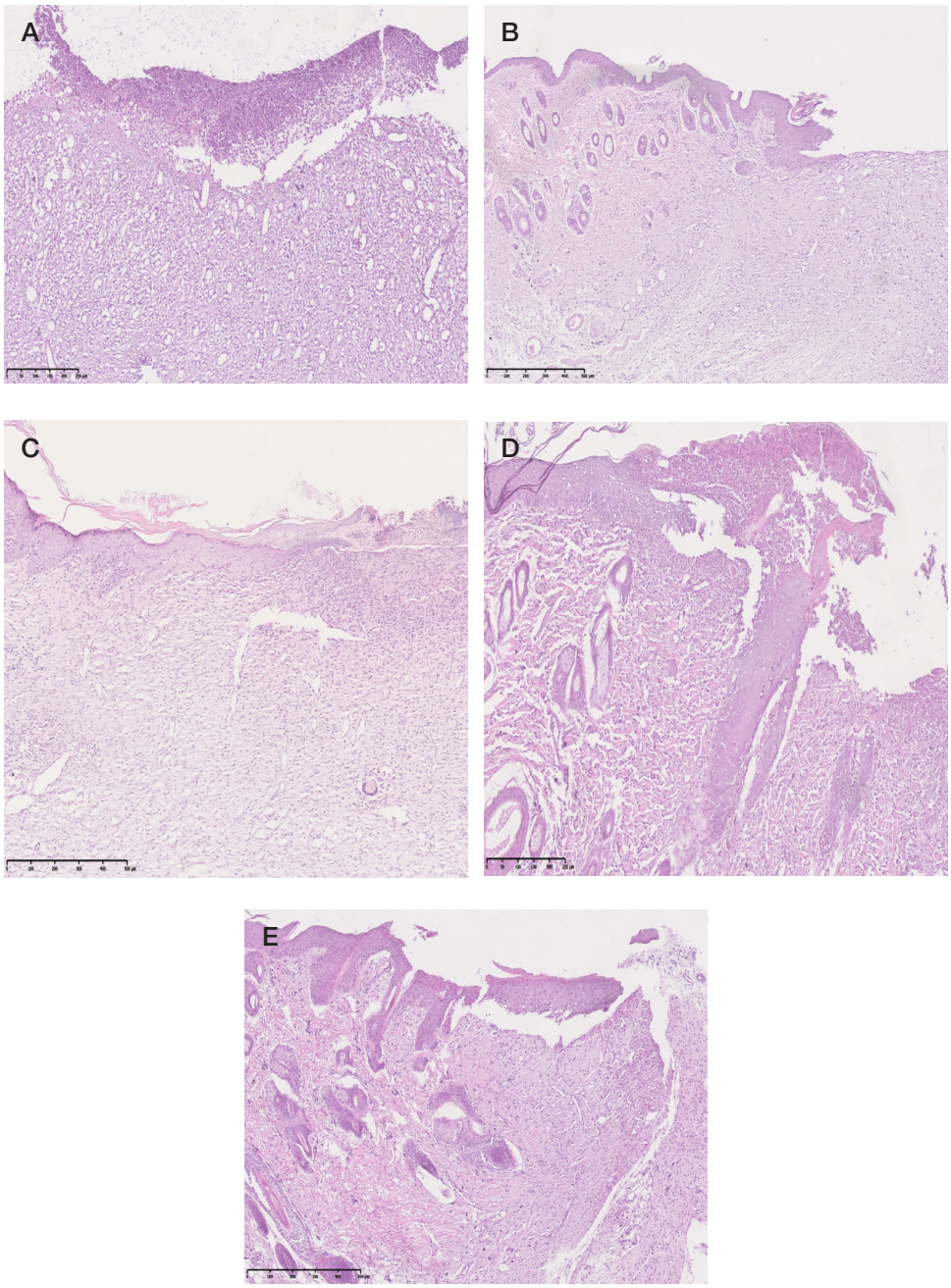

Fig. 1. Microphotographs of the skin wound area on day 10 post-wounding (H & E, magnification × 40). **A**. Control. **B**. GHK peptide, 0.5 μg/kg daily. **C**. GHK-D-Ala peptide, 0.5 μg/kg daily. **D**. GHK peptide, 1.5 μg/kg daily. **E**. GHK-D-Ala peptide, 1.5 μg/kg daily

On day 10 post-wounding, the control group continued to present with inflammatory reactions. In deeper layers of the wound, connective tissue acquired a more mature appearance in terms of cell types and fibrous patterns. Amid the persisting mild lymphocyte infiltration, a fully-formed mature connective tissue appeared above the hypodermis. The boundary of the defect with adjacent intact skin exhibited a thickening of the basal epidermal layer and a major portion of the wound was coated in detritus (Fig. 1A).

In the 0.5 μg/kg GHK group on day 10 post-wounding, the leukocyte-necrotic layer was observed in the central portion of the wound only. The underlying young granulation tissue showed rounded dilated capillaries, as well as less perfused vertical capillaries. The fibers were more mature than in the control. The low degree of edema and mild polymorhous infiltration persisted amid the predominance of fibroblast lineages. The deeper layers presented with fully-formed mature connective tissue. The adjacent intact skin at the immediate boundary with defect had thickened epidermal layers and showed marginal expansion of the newly formed epithelium with irregular stratification (Fig. 1B).

In the 0.5 μg/kg GHK-D-Ala group on day 10 post-wounding, the leukocyte-necrotic layer was missing. The underlying young connective tissue with underperfused rounded dilated capillaries showed visual predomination of fibroblast lineages and noticeable expansion of the epithelial flap over its surface. The epithelium showed regular structure except in the corneal layer. The scale of epithelial growth matched the size of the defect, with full epithelization observed in certain sections. The dermis of intact skin around the wound retained the signs of edema albeit less prominent than in the control (Fig. 1C).

In the 1.5 μg/kg GHK group on day 10 post-wounding, the leukocyte-necrotic layer was preserved albeit significantly reduced in size and frequently shed off during histological

Table. Dynamics of morphometric indicators upon injections of Gly-His-Lys-D-Ala peptide (M ± SD / Me [1Q; 3Q], *n* = 10) assessed by histological examination on days 3, 7, and 10 post-wounding

Indicator	Group	Time point		
		Day 3	Day 7	Day 10
Fibroblast lineages, %	Control	15.9 ± 2.47	15.9 ± 3.54	20.6 ± 6.67
	GHK 0,5 μg/kg	9.5 ± 3.31***	15.2 ± 1.55	22.5 ± 3.50
	GHK-D-Ala 0,5 μg/kg	17.00 ± 2.49###	43.20 ± 4.44***.###	34.80 ± 1.93***.#
	GHK 1,5 μg/kg	11.7 ± 1.77**	22.50 [20.50; 23.00]***	34.8 ± 1.93***
	GHK-D-Ala 1,5 μg/kg	12.1 ± 3.60**	20.5 ± 1.43**	30.1 ± 3.38***.$$$
Macrophages, %	Control	13.3 ± 3.97	17.6 ± 3.06	16.5 ± 8.18
	GHK 0,5 μg/kg	20.7 ± 4.57**	24.4 ± 3.12***	19.4 ± 3.86
	GHK-D-Ala 0,5 μg/kg	34.60 ± 3.10***.###	23.30 ± 4.57**	33.90 ± 3.14***.##
	GHK 1,5 μg/kg	27.1 ± 7.03***	34.3 ± 3.09***	54.2 ± 4.52***
	GHK-D-Ala 1,5 μg/kg	21.4 ± 5.68***	23.9 ± 4.20**.$	26.4 ± 3.17***.$$$
Granulocytes, %	Control	55 ± 3.50	36.2 ± 2.30	20.2 ± 5.85
	GHK 0,5 μg/kg	46.5 ± 2.22***	36.50 [36.00; 37.75]	28.1 ± 1.20***
	GHK-D-Ala 0,5 μg/kg	13.00 [12.00; 14.00]***.###	13.50 [11.25; 17.50]***.###	4.00 [3.00; 5.00]***.#
	GHK 1,5 μg/kg	18.2 ± 2.53***	23.50 [22.25; 24.75]***	15.5 ± 5.23*
	GHK-D-Ala 1,5 μg/kg	44.00 ± 3.65***.$$	24.2 ± 2.30***	18.4 ± 1.71
Lymphocytes, %	Control	15.8 ± 4.76	30.3 ± 4.06	42.7 ± 8.07
	GHK 0,5 μg/kg	23.3 ± 1.25***	24.50 [24.00; 26.00]**	30 ± 3.94
	GHK-D-Ala 0,5 μg/kg	35.00 [34.25; 36.75]***.#	18.40 ± 3.41***.##	27.70 ± 4.47***
	GHK 1,5 μg/kg	43.0 ± 5.10***	21.4 ± 4.58***	19.9 ± 4.46***
	GHK-D-Ala 1,5 μg/kg	22.5 ± 2.46***.$$$	31.4 ± 3.10$$$	25.5 [25.00; 26.75]***
Cellular index, %	Control	37.9 [35.6; 44.9]	51.0 ± 10.35	61.2 ± 20.92
	GHK 0,5 μg/kg	43.4 ± 4.69	63.9 [58.7; 68.8]**	72.9 ± 12.14
	GHK-D-Ala 0,5 μg/kg	108.33 [104.08; 112.77]***.###	206.7 ± 54.09***.###	225.6 ± 46.76***.###
	GHK 1,5 μg/kg	65.0 ± 17.35***	128.31 ± 20.26***	184.89 ± 28.27***
	GHK-D-Ala 1,5 μg/kg	51.1 ± 10.68*	80.7 ± 12.88***	130.1 ± 8.71***.$

Note: * — $p < 0.05$; ** — $p < 0.01$; *** — $p < 0.001$ compared with the control group; # — $p < 0.05$; ## — $p < 0.01$; ### — $p < 0.001$ compared with the GHK 0.5 μg/kg group; $ — $p < 0.05$; $$ — $p < 0.01$; $$$ — $p < 0.001$ compared with the GHK 1.5 μg/kg group

processing; the exposed regions presented with distinct fibrin deposits. The underlying young connective tissue was rich in underperfused rounded dilated capillaries. The epithelial flap outgrowth followed the boundary between the leukocyte-necrotic layer and the young connective tissue. The epithelium showed regular structure except in the corneal layer. The scale of epithelial growth matched the size of the defect, with full epithelization observed in certain sections. Beneath the epithelial outgrowth, the granulation tissue was structurally similar to the typical connective tissue of the dermis (Fig. 1D).

In the 1.5 μg/kg GHK-D-Ala group on day 10 post-wounding, the leukocyte-necrotic layer was fragmentary. The boundary with adjacent intact skin exhibited a thickening of the basal and granular epidermal layers. The epithelial flap outgrowth followed the boundary between the leukocyte-necrotic layer and the underlying young connective tissue, exceeding the dimensions of outgrowth observed in the control group. The epithelium showed regular structure (except the most distal regions). The dermis retained the signs of edema albeit less prominent than in the control (Fig. 1E).

The observed progression of the inflammatory reactions to regenerative phase was confirmed by morphometric study (Table). Granulocyte counts for 0.5 μg/kg GHK-D-Ala at all time points were significantly lower compared with both the control (3–5-fold; $p < 0.05$–0.001) and 0.5 μg/kg GHK (3.5–7-fold; $p < 0.05$–0.001), consistently with the milder secondary alterations in the wound area and the enhanced regeneration efficiency achieved with 0.5 μg/kg GHK-D-Ala. For 1.5 μg/kg doses, the trends were similar albeit with reduced difference between the peptides. Overall, granulocyte counts in all GHK/GHK-D-Ala groups were significantly lower than in the corresponding control groups at all time points of the experiment.

Macrophage counts for 0.5 μg/kg GHK-D-Ala were significantly higher compared with the corresponding control groups at all time points ($p < 0.05$–0.001) and compared with 0.5 μg/kg GHK groups on days 3 and 10 ($p < 0.05$). The 1.5 μg/kg doses of both peptides significantly increased the macrophage counts at all time points compared with the control ($p < 0.05$–0.001) with somewhat more pronounced effects observed for GHK.

Migration of fibroblast lineages to the site of injury, thought to reflect the induction of regeneration processes, was most pronounced for 0.5 μg/kg GHK-D-Ala at all time points of the experiment compared with both the controls and corresponding doses of GHK. The use of 1.5 μg/kg doses of the peptides had similar effects on fibroblast counts ($p < 0.05$–0.01) without significant differences between GHK-D-Ala and GHK ($p > 0.05$).

The dynamics of lymphocyte counts in the course of the experiment was generally consistent with the timing of transition from the inflammatory reaction to regenerative phase (for other cell types, the transition was detectable as well). Administration of GHK-D-Ala at 0.5 μg/kg significantly increased the lymphocyte counts compared with both groups of comparison on day 3 ($p < 0.05$–0.001), whereas on days 7 and 10 the effect was reverse. On the other hand, administration of GHK at 1.5 μg/kg significantly influenced the lymphocyte counts at all time points ($p < 0.05$–0.001 compared with the control), while the effects of GHK-D-Ala were less pronounced.

According to the calculated cellular index, the most pronounced effects at all time points of observation were achieved with daily injections of GHK-D-Ala at 0.5 μg/kg doses ($p < 0.05$–0.001). The use of GHK at this dose had no significant impact on regeneration ($p > 0.05$). Of note, daily injections of GHK at 1.5 μg/kg doses afforded a more robust regenerative response on days 7 and 10 post-wounding and a more pronounced overall effect compared with 1.5 μg/kg doses of GHK-D-Ala.

Thus, intracutaneous administration of GHK-D-Ala at daily doses of 0.5 μg/kg provided maximal regeneration benefits, exceeding those achieved with similar doses of GHK. Increased doses of the peptides (1.5 μg/kg) leveled the difference by reducing the positive effects of GHK-D-Ala and enhancing the positive effects of GHK. Overall, the highest morphometric indicators of regeneration processes in this study were obtained with 0.5 μg/kg of GHK-D-Ala.

DISCUSSION

The results indicate enhanced regeneration and decreased inflammatory response in the infected skin wounds treated with GHK peptide reinforced by chemical addition of D-alanine to its carboxy-terminus. The beneficial effects of GHK are well known and multiple hypotheses on their mechanisms can be found in the literature. For instance, GHK has been considered as a transport molecule for Cu^{2+} cations essential for the completion of phagocytosis, which ensures the effective physiological debriding and proper healing of the wound. In particular, it has been demonstrated that Cu^{2+} binds the amino-terminus of GHK through interactions with glycine and histidine [13]. Our study features a structural modification of the GHK tripeptide, which leaves the amino-terminus intact; accordingly, the Cu^{2+} cations can interact with the specified amino acids, rendering GHK capable of its role in cation transfer. Besides, GHK has been implicated as a mediator molecule orchestrating the positioning of cells within extracellular architecture of the dermis, facilitating cell motility and stimulating the production of signaling and structural molecules including certain growth factors and decorin [4, 14].

The higher degree of healing benefits attributed to the structurally modified tripeptide molecule may be explained by its increased resistance to the degrading action of carboxypeptidases. Our data are consistent with the reported impact of GHK-D-Ala on the phagocytic activity of granulocytes and lipid peroxidation processes in the infected skin wounds in rats [15]. The curative activity of GHK observed by us in this study corresponds to the expectations based on published evidence [11].

Comparative analysis between different studies should take into account the routes of administration. The peptides are administered locally in order to make them available to cells at the site of injury. However, the peptides may enter systemic circulation and influence other organs and systems of the body. In particular, when administered intraperitoneally at comparable doses, GHK exerts an analgesic and anxiolytic effect, which may contribute to the reduction of the stress response and increase the regenerative potential by modulating the cutaneous neuroimmune interactions [2]. However, the chemical addition of D-alanine to the carboxy-terminus of GHK has been shown to neutralize such effects [16, 17]. In this regard, it can be assumed that the enhanced pro-regenerative efficacy of GHK-D-Ala observed by us in this study is not directly related to the neurotrophic effects described for GHK.

The conserved direction of the pro-regenerative and anti-inflammatory effects described for GHK upon administration of GHK-D-Ala are indicative of the common mechanisms. For instance, apart from the aforementioned findings, GHK has been shown to stimulate the expression of vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2) [18]. In addition, the unmodified tripeptide inproves the rates of skin wound healing and stimulates skin renewal by stimulating the release of growth factors from platelet granules (notably of the transforming growth factor β, TGFβ), which promote the recruitment of immune cells to the site of injury [19]. Besides, GHK inhibits the production of pro-inflammatory cytokines, including tumor necrosis factor — (TNFα) and interleukin 6 (IL6), by fibroblasts, which leads to a decrease in cutaneous inflammatory reactions and prevents the formation of hypertrophic scars [20].

CONCLUSIONS

The study shows that modification of GHK peptide through addition of D-alanine at carboxy-terminus may increase its curative effect in infected skin wounds. The modified peptide administered at daily doses of 0.5 or 1.5 μg/kg successfully stimulated tissue repair and alleviated the inflammatory response on days 3, 7, and 10 post-wounding in rat model. The GHK-D-Ala peptide facilitated an increase in fibroblast and macrophage counts accompanied by a decrease in granulocyte and lymphocyte counts. The results highlight the use of structural modifications of GHK peptide to potentiate its efficiency in skin wound healing. Further efforts will be required to elucidate its mechanisms and the role of peptide regulation in regenerative processes.

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