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Динамика заживления и изменение бактериальной обсемененности инфицированной раны при применении пептида GHK и его структурных аналогов

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АННОТАЦИЯ

Введение. В настоящее время перспективным направлением представляется использование для ускорения раневого процесса трипептида NH_2-Gly-L-His-L-Lys-COOH (GHK), который действует на процессы регенерации ткани, обладает антиоксидантными, иммунотропными и противовоспалительными эффектами. При этом недостатком всех пептидов является их быстрая деградация протеолитическими ферментами. Одним из способов повышения устойчивости пептидных молекул является включение в их структуру D-изомеров аминокислот. Ранее нами было установлено, что GHK-D-Ala оказывал более выраженное влияние на регенеративные процессы в ране, способствовал увеличению в ране количества клеток фибробластического ряда, макрофагов на фоне уменьшения числа гранулоцитов и лимфоцитов, представлено существенное влияние D-Ala-GHK и GHK-D-Ala на показатели врожденного иммунитета и перекисного окисления липидов.

Цель. Оценить динамику заживления и бактериальной обсемененности инфицированной раны при применении пептида глицил-гистидил-лизин и его структурных модификаций с D-аланином (D-Ala).

Материалы и методы. Эксперименты выполнены на крысах линии Wistar. В работе использовали пептид GHK и его структурные аналоги D-Ala-GHK и GHK-D-Ala; вводили внутрикожно вокруг раны в дозах 0,5 мкг/кг и 1,5 мкг/кг каждые 24 ч. на протяжении 3, 7 или 10 сут. Оценивали площадь раны с расчетом коэффициента относительного ранозаживления (КОР), скорость заживления, сроки исчезновения перифокального отека, очищения раны, появления грануляций и начала краевой эпителизации. Бактериальную обсемененность определяли путем подсчета колоний на питательных средах после посева на них материала из биоптата раны.

Результаты. На 3 сут. КОР увеличился в 3,2–5,3 раза ($p < 0,05$–$0,01$) после использования пептидов D-Ala-GHK и GHK-D-Ala в обеих дозах при отсутствии эффекта после введения GHK. На 7 сут. уменьшение площади раны достигло статистически значимых различий во всех подопытных группах. К 10 сут. также во всех группах использование пептидов вызвало уменьшение площади раны при наибольшей выраженности после введения пептида GHK-D-Ala (93%, $p < 0,001$). На 7–10 сут. GHK-D-Ala увеличил скорость заживления в 4,7–5,3 раза ($p < 0,05$–$0,01$) при отсутствии существенных изменений после введения GHK и D-Ala-GHK. Также GHK-D-Ala в обеих дозах способствовал наиболее раннему исчезновению перифокального отека, очищению раны, появлению грануляций и началу краевой эпителизации. Значимое снижение бактериальной обсемененности наблюдалось после введения всех пептидов на 7 и 10 сут. при наибольшей выраженности после применения GHK-D-Ala.

Заключение. В условиях инфицированной кожной раны применение пептида GHK и его структурных аналогов D-Ala-GHK и GHK-D-Ala ускоряет ее заживление на фоне снижения бактериальной обсемененности. При этом наиболее выраженные изменения данных показателей отмечались после введения пептида GHK-D-Ala, что указывает на важное значение защиты молекулы GHK от действия карбоксипептидаз. Перспективным продолжением исследований в данном направлении может являться разработка местного средства с антибактериальным действием для стимуляции процессов регенерации в ране.

Ключевые слова: *GHK; D-аланин; регенерация; раневой процесс; бактериальная обсемененность; планиметрия*

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Effects of GHK Peptide and Its Structural Analogues on Dynamics of Healing and Bacterial Contamination of Infected Wound

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ABSTRACT

INTRODUCTION: Currently, a promising trend to accelerate wound process seems to be the use of NH_2-Gly-L-His-L-Lys-COOH (GHK) tripeptide, which acts on the tissue regeneration, possesses antioxidant, immunotropic and anti-inflammatory effects. At the same time, the disadvantage of all peptides is their rapid degradation by proteolytic enzymes. One method to increase the stability of peptide molecules is the incorporation of D-isomers of amino acids in their structure. It was previously found by us that GHK-D-Ala produces a more marked effect on regenerative processes in the wound and facilitates increase in the number of fibroblast cells and macrophages in the wound with the underlying decrease in the number of granulocytes and lymphocytes; a significant effect of D-Ala-GHK and GHK-D-Ala on the parameters of the inborn immunity and lipid peroxidation was shown.

AIM: To evaluate the healing dynamics and bacterial contamination of an infected wound when using Glycyl-Histidyl-Lysine (GHK) peptide and its structural modifications with D-alanine (D-Ala).

MATERIALS AND METHODS: The experiments were conducted on Wistar rats. In the work, GHK peptide and its structural analogues D-Ala-GHK and GHK-D-Ala were used; they were administered intracutaneously around the wound at doses of 0.5 μg/kg and 1.5 μg/kg every 24 hours for 3rd, 7th and 10th days. The wound area with calculation of the relative wound healing coefficient (RWHC), the healing rate, the time of perifocal edema disappearance, wound cleansing, appearance of granulation and onset of marginal epithelialization were assessed. Bacterial contamination was determined by counting colonies on the nutrient media after inoculation of wound biopsy material on them.

RESULTS: On day 3rd, RWHC increased 3.2–5.3 times ($p < 0.05$–0.01) after the use of D-Ala-GHK and GHK-D-Ala peptides at both doses with no effect after the injection of GHK. On day 7th, the reduction of the wound area reached statistically significant differences in all experimental groups. By day 10th, the use of peptides resulted in decrease in the wound area most evident after the injection of GHK-D-Ala peptide (by 93%, $p < 0.001$) in all groups. On days 7th–10th, GHK-D-Ala increased the healing rate 4.7–5.3 times ($p < 0.05$-0.01) with no significant changes after the injection of GHK and D-Ala-GHK. Also, GHK-D-Ala at both doses resulted in the earliest disappearance of perifocal edema, wound cleansing, emergence of granulation and the onset of marginal epithelialization in all experimental groups. Significant reduction in bacterial contamination was observed after administration of all peptides on days 7th and 10th, being most pronounced after the use of GHK-D-Ala.

CONCLUSION: The application of GHK peptide and its structural analogues D-Ala-GHK and GHK-D-Ala in infected skin wounds accelerated wound healing against the background reduction of bacterial contamination. The most pronounced changes of these parameters were observed after administration of GHK-D-Ala peptide, which indicates the importance of protecting GHK molecule against the impact of carboxypeptidases. A promising continuation of the research in this direction can be the development of local means with antibacterial effect for stimulation of the regeneration processes in the wound.

Keywords: *GHK; D-Alanine; regeneration; wound process; bacterial contamination; planimetry*

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LIST OF ABBREVIATIONS

CFU — colony forming unit
D-Ala — D-Alanine
GHK — Glycyl-Histidyl-Lysine
HR — healing rate
RWHC — relative wound healing coefficient

INTRODUCTION

Increasing the effectiveness of the regenerative mechanisms in wound processes is an actual trend in the modern biomedical research. It has now been established that the main sequentially developing regeneration stages in tissue damages are inflammatory reaction, wound healing, extracellular matrix remodeling, anti-inflammatory and anti-fibrotic response; as well as tissue remodeling [1–3]. These processes are provided by rather complex and diverse mechanisms involving all regulatory processes of an organism [4–6]. A possible way to realize this task may be investigation of peptidergic mechanisms of activating wound healing processes. Up to date, a significant amount of information has been accumulated on the use of peptide molecules and pharmacological drugs on their basis in the correction of various pathological processes [6]. Therefore, the use of tripeptide Glycyl-Histidyl-Lysine (GHK) seems promising in accelerating the resolution of wound process.

This peptide is known to act on tissue regeneration processes [7, 8], and also possesses antioxidant, immunotropic and anti-inflammatory effects [9, 10]. Upon that, the main disadvantage of all regulatory peptides is their rapid degradation by proteolytic enzymes.

One of possible ways to increase the stability of peptide molecules, and, consequently, the duration of their biological effect, is incorporation of D-isomers of amino acids in their structure. In the previous studies we found that GHK-D-Ala (D-Alanine) produced a more prominent effect on regenerative processes in the wound than GHK and facilitated an increase in the number of fibroblast cells, macrophages with the underlying decrease in granulocytes and lymphocytes [10]. A significant effect of GHK and its structural analogues D-Ala-GHK and GHK-D-Ala on the parameters of the innate immunity and lipid peroxidation in infected skin wound was also shown. The most prominent and stable effects were also observed with GHK-D-Ala [111]. The data obtained in these works necessitated the study of the integrative wound healing parameters of GHK and its structural analogues D-Ala-GHK и GHK-D-Ala, used for clinical assessment of the effectiveness of regenerative processes.

The **aim** of this study to assess the dynamics of healing and of bacterial contamination of infected wound with use of GHK peptide and its structural modifications with D-Ala.

MATERIALS AND METHODS

The experiments were conducted on 150 male Wistar rats weighing 180 g–240 g aged 6–8 months (Stolbovaya branch of the Scientific Center for Biomedical Technologies of the Federal Medical and Biological Agency). The animals were kept in standard vivarium conditions with free access to water and food, 12-hour light regime and air temperature 22 ± 2°C. Each experimental group included 10 rats.

The study used GHK peptide and its structural analogues D-Ala-GHK and GHK-D-Ala (synthesized at the Research Institute of Chemistry of St. Petersburg State University), which were dissolved in saline and administered intracutaneously (at two points around the wound, changing the injection areas daily by 90° clockwise) at doses of 0.5 μg/kg and 1.5 μg/kg in 0.1 ml 24 hours after modeling an infected wound, followed by administration of the drug at the same dose every 24 hours for 3rd, 7th, or 10th days. In the control series, animals were administered equivalent volumes of saline at similar intervals at a rate of 1 ml per 1 kg of body weight. The last administration of the drugs was carried out 24 hours before the animals were withdrawn from the experiment.

The animals were withdrawn from the experiment by taking blood from the right ventricle of the heart under ether narcosis.

All studies were conducted in compliance with the principles of the European Convention for Protection of Vertebrate Animals used in experimental research; ‘Guidelines on the conduct of preclinical trials of medical drugs’ (Moscow, 2012) and in accordance with the decision of the Regional Ethics Committee at Kursk State Medical University (Protocol No. 1 of January 16, 2014).

The infected (natural infection) open wound was modeled by inflicting full-thickness wounds of a standard

size (250 mm^2) on a shaven portion on the back of an anesthetized animal.

Immediately after modeling of the wound process, and also on the first, third, seventh and tenth day, the wounds were photographed with a Canon A410 digital camera using a millimeter grid with subsequent measurement of wound areas at these control periods. The wound areas were calculated in the ImageJ program (National Institutes of Health, USA).

For objective planimetric assessment of wound healing by the change in its area, the relative wound healing coefficient (RWHC) was used a decrease in the wound area from the initial size expressed as a percentage, calculated from the formula:

$$RWHC = (S_0 — S) / S_0 \times 100\%,$$

where S_0 — initial, and S — final wound surface (mm^2) [12].

The wound healing rate (a decrease in the wound area per day in %) was also calculated using the formula:

$$HR = (RWHC_1 — RWHC_0) / T,$$

where HR — healing rate, $RWHC_1$ — coefficient at the time of measurement (final), $RWHC_0$ — coefficient at the previous measurement, T — the number of days between measurements.

On visual examination, the clinical wound healing parameters were assessed: the time of disappearance of perifocal edema, wound cleansing, appearance of granulations and beginning of the marginal epithelialization.

For quantitative determination of the microbial contamination, the wound was pre-treated with isotonic sodium chloride solution, then with 70% ethyl alcohol to remove vegetating microflora, pus and detritus from its surface. The material was taken after withdrawal of the animal from the experiment, in a sterile box by excising a part of wound of 0.1 g–0.5 g to the full wound depth. Then the biopsy material was weighted to calculate the conversion coefficient (K) for 1 g of tissue, a suspension was prepared in the isotonic sodium chloride solution at a ratio of 1:10, and the material was inoculated on the dense nutrient medium (meat-peptone agar) by Drigalsky method. The cultures were incubated in a thermostat (37°C) for 20 hours, then at room temperature for 24 hours, after which colonies were counted with conversion to 1 g of tissue.

The colonies were counted on dishes, in which they grew isolated from each other, and their number did not exceed 300. The number of microbes in 1 g of tissue was calculated using the formula:

$$N = n \times 10 \times 10 \text{ (or } \times 100, \text{ or } \times 1000) \times K,$$

where N — number of microbes in 1 g of bioptate, n — number of microbes grown in a Petri dish, 10 — recalculation to 1 g of suspension, 10, 100 or 1000 — dilution of the material inoculated in the Petri dish, in which colonies were calculated, K — conversion coefficient of the weighted quantity per 1 g of bioptate.

Statistical processing of the obtained data was performed using the R version 4.1.0 programming language [13] in the integrated development environment RStudio Desktop ver. 1.4.1717 (RStudio, PBC, USA). To check the normality of the distribution, the Shapiro–Wilk test (the shapiro.test() function from the standard package) was used, and the Levene test (the levene. test() function from the lawstat package) was used to check the equality of variances. In case of confirmation of the hypotheses, the data were presented as 'mean ± standard deviation' (M ± SD), M and SD were calculated using the mean() and sd() functions from the standard package; in case of deviation — as 'Median [lower quartile; upper quartile]' (Me [Q1; Q3]), which were calculated using the median() and quantile() functions from the standard package. To determine the differences between the groups, Kruskal–Wallis test (the kruskal.test() function from the standard package) with Dunn's post hoc test with Benjamini–Hochberg correction (the dunnTest() function from the FSA package) was used. Dunn's test was performed only in the presence of significant intergroup differences: the p-value for the calculated Kruskal–Wallis test with 6 intergroup degrees of freedom and 70 intragroup degrees of freedom ($H_{6,70}$) was less than 0.05. The z-value and the degree of its significance (p-value) were calculated for it. Differences were considered significant at $p < 0.05$.

RESULTS

As follows from Table 1, on ***day 3rd***, no significant differences were observed in the wound area and wound healing coefficient between experimental and control groups of animals.

On day 7th, the reduction in the wound area reached reliable significant differences in all the experimental groups. The most prominent changes were observed after the introduction of GHK at doses of 0.5 (by 62%, z = -4.53, p = 0.000005) and 1.5 μg/kg (by 56%, z = -4.11, p = 0.00003), as well as D-Ala-GHK at a dose of 0.5 μg/kg (by 60%, z = -4.4, p = 0.00001), which was reflected in a reliably significant increase in the wound healing coefficient in these groups by 2.37 (z = 3.78, p = 0.00015), 2.17 (z = 3.74, p = 0.00018) and 1.94 (z = 3.13, p = 0.0017) times, respectively. The increase in this parameter after the introduction of GHK-D-Ala was not statistically significant.

By day 10th of the experiment, the use of peptides was accompanied by a significant decrease in the

wound area in all groups. The most pronounced differences relative to the control values were noted after the introduction of GHK-D-Ala peptide at doses of 0.5 (by 93%, z = -5.91, p = 0.000000004) and 1.5 μg/kg (by 92%, z = -5.33, p = 0.00000007). After the introduction of D-Ala-GHK peptide, the decrease in this parameter was least pronounced: by 58% (z = -2.19, p = 0.0028) at a dose of 0.5 μg/kg and by 59% (z = -2.03, p = 0.0042) at a dose of 1.5 μg/kg. It is noteworthy that the effect of these peptides did not depend on the dose. Against the background use of GHK, the wound area was smaller than the control values after administration at doses of 0.5 μg/kg (by 69%, z = -2.91, p = 0.00036) and 1.5 μg/kg (by 83%, z = -4.36, p = 0.000015).

Within 1–3 days, the wound healing rate in rats given GHK did not significantly differ from the control group (Table 2). With the introduction of D-Ala-GHK and GHK-D-Ala, healing accelerated in equal measure but did not reach statistically significant differences. Over the period of 3–7 days, GHK at a dose of 0.5 μg/kg increased the healing rate 2.7 times (z = 2.44, p = 0.015), and at a dose ща 1.5 μg/kg —1.4 times (z = 2.68, p = 0.007). However, on the contrary, with the introduction of GHK-D-Ala, a tendency to a decrease in this parameter was noted without reaching a reliably significant level. The use of D-Ala-GHK was accompanied by multidirectional unreliable changes in the experimental groups. At the final stage of the experiment (7–10 days), with the introduction of GHK-D-Ala, a significant acceleration of wound healing was noted. Thus, at a dose of 0.5 μg/kg, the peptide accelerated the process 4.7 times (z = 2.57, p = 0.001) and at a dose of 1.5 μg/kg — 5.3 times (z = 3.09, p = 0.0002). At the same time, no significant changes in the healing rate were noted after administration of GHK and D-Ala-GHK.

Table 1. Dynamics of Change in Planimetric Parameters of Wound Healing (n = 10) with Use of D-Ala-GHK and GHK-D-Ala Peptides, M ± SD/Me [Q1; Q3]

Groups	Timing			
	1st day	3rd day	7th day	10th day
Wound area, mm²				
Control	250.00 [249.25; 250.00]	216.60 ± 39.88	165.50 ± 47.43	85.50 [72; 155.75]
GHK 0.5 mkg/kg	250.00 [248.50; 250.00]	211.50 ± 63.93	**63.40 ± 28.32*****	**26.50 [19.5; 29.00]****
GHK 1.5 mkg/kg	249.00 [248.00; 250.00]	209.00 ± 35.05	**72.60 ± 36.41*****	**14.50 [8.5; 23.25]*****
D-Ala-GHK 0.5 mkg/kg	249.50 [248.25; 250.00]	174.60 ± 28.78	**66.60 ± 23.33*****	**36.00 [23.5; 43.50]***
D-Ala-GHK 1.5 mkg/kg	250.00 [249.25; 250.00]	174.30 ± 37.85	**97.10 ± 17.99*****	**35.50 [24.25; 59.25]***
GHK-D-Ala 0.5 mkg/kg	249.50 [248.00; 250.00]	169.00 ± 47.01	**96.70 ± 34.08*****	**6.30 [5.1; 8.60]*****
GHK-D-Ala 1.5 mkg/kg	249.50 [248.00; 250.00]	172.00 ± 47.86	**113.80 ± 42.07****	**7.20 [5.7; 16.00]*****
Kruskal–Wallis test	$H_{6,70}$ = 2.71, p = 0.84	$H_{6,70}$ = 12.47, p = 0.052	**$H_{6,70}$ = 32.96, p = 0.00001**	**$H_{6,70}$ = 51.85, p = 0.00001**
Relative wound healing coefficient, %				
Control	0.40 [0.00; 0.79]	6.80 [2.30; 25.88]	21.32 ± 27.97	36.22 [14.02; 45.85]
GHK 0.5 mkg/kg	0.20 [-0.30; 0.70]	17.56 [2.21; 35.27]	**66.68 ± 21.11*****	62.55 [33.68; 71.87]
GHK 1.5 mkg/kg	0.40 [0.00; 0.80]	13.39 [10.18; 22.30]	**65.21 ± 18.06*****	**77.98 [60.72; 86.80]****
D-Ala-GHK 0.5 mkg/kg	0.40 [0.10; 0.70]	27.33 [20.42; 38.63]	**59.79 ± 19.41*****	48.17 [36.42; 55.03]
D-Ala-GHK 1.5 mkg/kg	0.20 [0.00; 0.80]	25.74 [19.97; 39.93]	43.16 ± 9.00	61.57 [45.01; 74.50]
GHK-D-Ala 0.5 mkg/kg	0.80 [0.10; 0.80]	36.97 [15.32; 45.22]	40.31 ± 24.89	**92.00 [89.80; 94.59]*****
GHK-D-Ala 1.5 mkg/kg	0.80 [0.10; 1.09]	22.01 [19.27; 47.91]	33.04 ± 21.96	**91.98 [89.79; 94.64]*****
Kruskal–Wallis test	$H_{6,70}$ = 2.78, p = 0.83	$H_{6,70}$ = 12.47, p = 0.052	**$H_{6,70}$ = 27.85, p = 0.0001**	**$H_{6,70}$ = 47.39, p = 0.00003**

Notes: * — p < 0.05; ** — p < 0.01; *** — p < 0.001 in comparison with the control group (by post hoc Dunn's test)

Table 2. Dynamics of Wound Healing Rate (n = 10) with Use of D-Ala-GHK and GHK-D-Ala Peptides, M ± SD/Me [Q1; Q3], %

Groups	Timing		
	1st–3rd days	**3rd–7th days**	**7th–10th days**
Control	6.43 ± 8.04	5.85 [-3.12; 8.19]	3.68 ± 8.15
GHK 0.5 mkg/kg	7.52 ± 12.66	**15.87 [3.64; 18.86]****	-5.10 ± 14.53
GHK 1.5 mkg/kg	7.85 ± 7.27	**14.20 [11.03; 15.38]*****	2.83 ± 10.81
D-Ala-GHK 0.5 mkg/kg	14.64 ± 5.91	8.74 [6.36; 12.33]	-5.34 ± 12.78
D-Ala-GHK 1.5 mkg/kg	14.89 ± 7.91	4.54 [-1.91; 7.56]	5.62 ± 6.82
GHK-D-Ala 0.5 mkg/kg	15.89 ± 9.61	3.90 [-5.54; 9.74]	**17.24 ± 8.86***
GHK-D-Ala 1.5 mkg/kg	15.16 ± 9.70	1.56 [-1.43; 6.70]	**19.62 ± 7.63****
Kruskal–Wallis test	$H_{6,70}$ = 12.01, p = 0.0616	**$H_{6,70}$ = 20.04, p = 0.0027**	**$H_{6,70}$ = 34.49, p = 0.00001**

Notes: * — p < 0.05; ** — p < 0.01; *** — p < 0.001 in comparison with the control group (by post hoc Dunn's test)

The established increase in the wound healing rate with the introduction of GHK-D-Ala also manifested in the clinical parameters (Table 3). Thus, at both doses, the peptide facilitated the earliest disappearance of perifocal edema, wound cleansing, appearance of granulations and beginning of marginal epithelialization. The effects of GHK and D-Ala-GHK were less pronounced, without any statistically significant differences between them. To note, the changes in the periods of appearance of the clinical signs of wound healing were reliable with all peptides.

Table 3. Dynamics of Clinical Parameters of Wound Healing (n = 10) with Use of Peptides D-Ala-GHK and GHK-D-Ala, M ± SD/Me [Q1; Q3]

Groups / Parametrs	Disappearance of perifocal edema, day	Wound cleansing, day	Appearance of granulations, day	Beginning of marginal epithelialization, day
Control	8 [8; 8,75]	9 [8,25; 9]	7 [7; 8]	9 [8; 9]
GHK 0.5 mkg/kg	**6 [5; 6]*****	**6 [6; 6]*****	**5 [5; 5]*****	**5 [5; 5]*****
GHK 1.5 mkg/kg	**6 [6; 6]*****	**6,00 [6; 6,75]*****	**5 [5; 6]****	**6 [5; 6]****
D-Ala-GHK 0.5 mkg/kg	**6 [6; 7]***	**7 [6; 7]***	**5,50 [5; 6]***	**6 [5; 6]****
D-Ala-GHK 1.5 mkg/kg	**6 [6; 6]****	**6 [6; 7]****	**5 [5; 6]****	**6 [5; 6]****
GHK-D-Ala 0.5 mkg/kg	**5 [4; 5]*****	**5 [4,25; 5]*****	**3 [3; 3,75]*****	**4 [3; 4]*****
GHK-D-Ala 1.5 mkg/kg	**4 [4; 5]*****	**5 [5; 5,75]*****	**4 [3; 4]*****	**5 [4; 5]*****
Kruskal–Wallis test	**$H_{6,70}$ = 56,85, p = 0,00001**	**$H_{6,70}$ = 49,93, p = 0,00001**	**$H_{6,70}$ = 56,97, p = 0,00001**	**$H_{6,70}$ = 50,96, p = 0,00001**

Notes: * — p < 0.05; ** — p < 0.01; *** — p < 0.001 in comparison with the control group (by post hoc Dunn's test)

It is known that the microbial contamination plays an important role in the healing process of an infected wound. As can be seen from Table 4, on day 3rd this parameter had close values across all groups of animals. However, on day 7th it significantly decreased after the introduction of both GHK and its analogues. Upon that, the greatest decrease in microbial contamination (32%, z = 2.64, p = 0.0017) was noted with GHK-D-Ala at a dose of 0.5 μg/kg. A similar pattern of changes in this parameter, but more strongly expressed, persisted on day 10th of the experiment. Thus, the greatest decrease in microbial contamination (37%, z = 2.77, p = 0.0011) was also observed with GHK-D-Ala at a dose of 0.5 μg/kg, and the lowest effect was noted after the administration of GHK at a similar dose (8%, z = 1.56, p = 0.042).

Table 4. Parameters of Microbial Contamination of Wound (n = 10) with Use of Peptides D-Ala-GHK and GHK-D-Ala, M ± SD/Me [Q1; Q3], DlgCFU per 1g of Autoptate

Groups	Timing		
	3rd day	7th day	10th day
Control	6.48 [6.47; 6.48]	5.98 ± 5.06	5.86 [5.79; 5.90]
GHK 0.5 mkg/kg	6.48 [6.44; 6.48]	**5.83 ± 5.23*** **	**5.41 [5.30; 5.50] ***
GHK 1.5 mkg/kg	6.47 [6.46; 6.48]	**5.33 ± 4.46*** **	**4.28 [4.18; 4.37] *** **
D-Ala-GHK 0.5 mkg/kg	6.48 [6.44; 6.48]	**5.44 ± 4.79*** **	**4.30 [4.26; 4.34] ***
D-Ala-GHK 1.5 mkg/kg	6.47 [6.44; 6.48]	**5.29 ± 4.24*** **	**4.10 [4.01; 4.17] *** **
GHK-D-Ala 0.5 mkg/kg	6.48 [6.47; 6.48]	**4.08 ± 3.45*** **	**3.68 [3.51; 3.83] *** **
GHK-D-Ala 1.5 mkg/kg	6.47 [6.43; 6.48]	**5.81 ± 4.94*** **	**4.47 [4.33; 4.48] *** **
Kruskal–Wallis test	$H_{6,70}$ = 2.78, p = 0.8355	**$H_{6,70}$ = 12.47, p = 0.032**	**$H_{6,70}$ = 56.97, p = 0.00001**

Notes: * — p < 0.05; ** — p < 0.01; *** — p < 0.001 in comparison with the control group (by post hoc Dunn's test); CFU — colony forming unit

DISCUSSION

Thus, GHK and its structural analogues D-Ala-GHK and GHK-D-Ala promoted acceleration of wound healing and reduction of its bacterial contamination. To note, the obtained data are in line with the results of our previous studies, and their comparison to a large extent contributes to elucidation of the mechanisms of the identified effects. It is known that divalent copper cations are necessary for production of hydroxyl radicals, which are an important component in oxygen-dependent bactericidal mechanisms of phagocytic cells. Complete wound cleansing from necrotized elements and microbes is the main condition for the effectiveness of reparative processes. However, increased production of active oxygen species, which are important factors of secondary alteration in the inflammation focus, may be accompanied by a high activation of lipid peroxidation and an increase in the levels of malondialdehyde and acyl hydroperoxides. Upon that, the dynamics of change in the activity of these processes and their ratio may be quite complex [11].

GHK interacts with copper ions to form GHK-Cu complex that activates proliferative processes in skin wounds and positive chemotaxis of macrophages to the damaged area [14–16]. Previously, it was shown by us on a similar experimental model of a skin wound with the same observation periods that GHK and GHK-D-Ala reduced the number of granulocytes and lymphocytes and increased the number of fibroblast cells, macrophages and cellular index with higher activity of GHK-D-Ala [9]. The dynamics of these morphometric parameters obtained in histological examination of the wound bioptate, could underlie the changes in the planimetric parameters established in the present work. An early and more prominent, as compared to the control group, reduction of bacterial contamination of the wound could be due to earlier activation of free radical oxidation processes in phagocytic cells [10]. A rapid decrease in bacterial contamination could be a probable cause of an earlier noted decrease in some parameters of phagocytic activity at later observation periods [10] in result of a complete wound cleansing, which also

contributed to a positive dynamic of the integrative planimetric parameters of wound healing.

Upon that, this effect can also be due to the antibacterial action of GHK-Cu^{2+}. In particular, such an effect has been shown with local application of these complexes in the form of nanoparticles [17]. In this case, the C-terminus remains free in the structure of the GHK-Cu^{2+} molecule [9, 16] enabling attachment of D-Ala.

The increase in the effectiveness of GHK after its structural modification with D-Ala can not only be due to increase in the resistance to proteases, but also to the effect of amino acids produced in degradation of the peptide molecule. In particular, D-Ala maintains the potential of the mitochondrial membrane and prevents the formation of reactive oxygen species that damage cellular structures and nucleic acids [18–20].

CONCLUSION

Based on the data obtained, it can be concluded that the use of GHK and its structural analogues D-Ala-GHK and GHK-D-Ala accelerate healing of infected skin wound with the underlying reduction of bacterial contamination. Upon that, the most prominent changes of these parameters were observed after the introduction of GHK-D-Ala peptide, which points to the importance of protecting the GHK molecule from the action of carboxypeptidases.

A promising continuation of research in this field may be the development of a local agent for stimulating regeneration processes in the wound with an antibacterial effect based on the complex of GHK-D-Ala peptide and Cu^{2+} used in the form of nanoparticles.

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