Research Results in Pharmacology

Research Results in Pharmacology 12(2): 11–19
UDC 615.262:616-001
DOI 10.18413/rrpharmacology.12.1048

Research Article

Effect of a new acetylaminohexanic acid derivative on wound healing and wound blood supply in mice with a mutation in the Agouti gene with inhibition of NOS isoform expression

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Academic editor: Mikhail Korokin ♦ **Received** 11 November 2025 ♦ **Accepted** 15 January 2026 ♦ **Published** 29 May 2026

Citation: Khentov AA, Miller AV, Pazhinsky LV, Bolgov AA, Tatarenkova IA, Sunyaykina PK, Danilenko LM (2026) Effect of a new acetylaminohexanic acid derivative on wound healing and wound blood supply in mice with a mutation in the Agouti gene with inhibition of NOS isoform expression. Research Results in Pharmacolog 12(2): 11–19. https://doi.org/10.18413/rrpharmacology.12.1048

Abstract

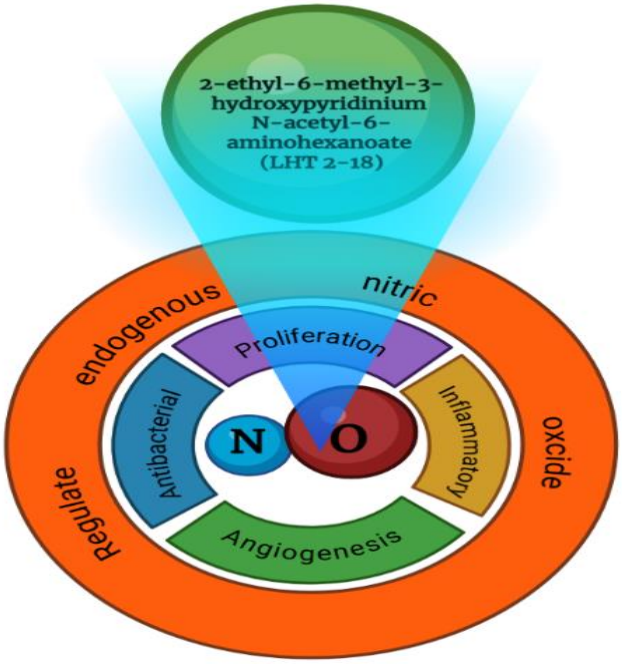
Introduction: Metabolic disorders, particularly diabetes mellitus, can lead to the formation of chronic wounds, slowing healing processes. A key factor impeding wound healing is the decreased production of endogenous nitric oxide (NO) due to a hyperglycemic microenvironment. Nitric oxide (NO) is a key signaling molecule produced by three different isoforms of nitric oxide synthase (NOS) – iNOS, eNOS, and nNOS – which regulate all phases of skin wound healing.

Materials and Methods: The effect of N-acetyl-6-aminohexanoic acid derivative in the form of a 5% carbopol-based hydrogel dosage form (carbopol 940) with 2-ethyl-6-methyl-3-hydroxypyridinium N-acetyl-6-aminohexanoate (LHT 2-18) on wound healing was studied during blockade of eNOS and iNOS nitric oxide synthase synthesis by intraperitoneal administration of a non-selective NO synthase inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) at a dose of 25 mg/kg/day and aminoguanidine at a dose of 50 mg/kg/day, respectively. The studies were conducted on 30 Agouti viable yellow (Avy/a) mice weighing 42.9±0.12 g, which indicates the development of obesity. The degree of wound healing and wound blood flow were assessed on days 3, 7, 14, and 21. Wound healing was assessed using the gravimetric method. Expression levels of eNOS, iNOS, and VEGF mRNA were analyzed using quantitative PCR.

Results: Topical application of 5% hydrogel based on the LHT 2-18 complex improves the rate of wound healing in Avy/a mice when administered L-NAME and aminoguanidine and local blood flow indices, increases the level of mRNA expression of the eNOS and VEGF genes and reduces the level of iNOS.

Conclusion: The identified regenerative effect of the LHT 2-18 complex is of interest for further study with the aim of developing an effective agent that stimulates reparative regeneration of wounds, including those caused by metabolic damage.

Graphical Abstract



Keywords

acetylaminohexanoic acid derivative; aminoguanidine; nitric oxide; N-nitro-L-arginine methyl ester (L-NAME)

Introduction

In metabolic disorders, wounds easily transit from acute to chronic states due to a severe and complex inflammatory microenvironment, including increased expression of reactive oxygen species (ROS), hypoxia, biofilm formation, and insufficient nitric oxide (NO) synthesis. Excessive oxidative stress and persistent inflammation complement each other, leading to decreased angiogenesis (Martinengo et al. 2019).

A key factor impeding wound healing is a decrease in endogenous nitric oxide (NO) production. Nitric oxide (NO) is a critical signaling molecule produced by three different isoforms of nitric oxide synthase (NOS), two of which are eNOS and iNOS, and contribute significantly to cutaneous wound healing. The wound healing process involves hemostasis, inflammation, proliferation, and remodeling (Nour et al. 2019). Wound repair and regeneration processes are regulated by cytokines and growth factors released at the wound site and involve inflammatory cells, fibroblasts, keratinocytes, and epithelial cells (Willenborg et al. 2025). All of these cells are capable of producing NO (Zahid et al. 2021). NO regulates the proliferation of epithelial cells, fibroblasts, and keratinocytes by releasing fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and transforming growth factor beta (TGF-β) (Man et al. 2022). NO also regulates collagen synthesis, collagen accumulation, and tissue contraction. A number of researchers have confirmed in vivo and in vitro experiments that deficiency of eNOS delays wound healing, deficiency of iNOS improves wound healing (Xiaoyu et al. 2008).

Current developments and approaches in wound treatment, including those of a metabolic nature, include surgical and non-surgical alternatives, such as 3D-printed topical dressings, drug therapy based on growth factors, natural and synthetic antioxidants, skin-like substitutes, bioengineered allogeneic cell therapies, and fish skin transplantation (Kaur et al. 2019; Frykberg

et al. 2020; Ahmed et al. 2022). Most of these attempts and approaches have significant therapeutic potential, but have thus far had limited clinical application.

Therefore, the search for new pharmacological agents for the treatment of difficult-to-heal wounds, including those with metabolic disorders, continues, with an emphasis on targeting the key role of nitric oxide (NO).

Among the numerous substances with pro-regenerative activity, N-acetyl-6-aminohexanoic acid is known, for which the ability to accelerate the healing of wounds of the skin and mucous membranes, as well as fractures of tubular bones has been established (Andrianova et al. 2019; Pakhomov et al. 2020; Blinova et al. 2021). To improve its protective properties, a number of its derivatives were synthesized – acexamates of zinc, sodium, magnesium, calcium, silver, ethyl thiazolylamide, 2-ethyl-6-methyl-3-hydroxypyridinium, etc. (Danilenko et al. 2025). The pharmacokinetic and pharmacodynamic characteristics associated with the pro-regenerative effect of the most active representatives of this group continue to be studied. In this study, we examine one of the hypothetical targets of the pro-regenerative effect of LHT 2-18: nitric oxide (NO).

It is known that one of the components of the LHT 2-18 complex, 2-ethyl-6-methyl-3-hydroxypyridinium, exhibits antihypoxic, antioxidant, and endothelial-protective activity, suggesting that LHT 2-18 accelerates wound healing by influencing the signaling molecule NO.

Research objective: To study the effect of 5% hydrogel based on LHT 2-18 on the dynamics of wound healing and microcirculation levels in mice with a mutation in the Agouti gene through the effect on the NO system.

Materials and Methods

Experimental animals

The study was conducted on mice heterozygous for the dominant mutation Agouti viable yellow (Avy/a) mice are characterized by a change in coat color to golden (red) and an increase in body weight.

Agouti viable yellow mice were housed under SPF conditions in the animal facility of Belgorod State National Research University (Russia) under controlled light conditions (12 h light/12 h dark) at a temperature of +22 to +26 °C, with free access to food and water. All procedures complied with the ethical principles of laboratory animal care in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS No. 170) and Directive 2010/63/EU of the European Parliament and the Council on the Protection of Animals Used for Scientific Purposes.

Animal groups

The animals participating in the experiment were randomly divided into 6 groups of 6 animals in each group (n=36):
I – Mice C57BL/6) +wound + hydrogel;
II – $A^{vy/a}$ mice + wound + hydrogel (control group);
III – $A^{vy/a}$ mice + wound + L-NAME + hydrogel;
IV – $A^{vy/a}$ mice + wound + aminoguanidine + hydrogel;
V – $A^{vy/a}$ mice + wound + L-NAME + 5% hydrogel LHT 2-18;
VI – $A^{vy/a}$ mice + wound + aminoguanidine + 5% hydrogel LHT 2-18.

Drug under study

The dosage form is a 5% carbopol hydrogel (Carbopol 940), containing 2-ethyl-6-methyl-3-hydroxypyridinium N-acetyl-6-aminohexanoate as the active ingredient (LHT 2-18) (Khentov et al. 2025). The dose of the hydrogel under study for topical application was calculated based on previously obtained results from experimental preclinical studies, taking into account interspecies dose tolerance depending on the laboratory animal species and route of administration (Danilenko et al. 2025).

Study design

After anesthesia with a combination of drugs (Zolazepam/tiletamine 0.6 mg/10.0 g + Medetomidine hydrochloride 0.5 mcg/10.0) and hair removal, a full-thickness wound measuring 15x15 mm (225 mm^2) was created in the center of the back of mice. In the study, we used an endothelial NO blocker: N-nitro-L-arginine methyl ester (L-NAME) (Sigma Aldrich, USA), at a dose of 25 mg/kg/day; an inducible NO blocker aminoguanidine (Sigma Aldrich, USA), at a dose of 50 mg/kg/day, which were administered to animals intraperitoneally for 14 days. The wounds were washed with saline and treated with hydrogel daily for 21 days at the same time and left open without a dressing.

The wound area was measured using the "weight method" (Slietsans et al. 2013; Lebedeva et al. 2023). On days 3, 7, 14, and 21, the wound area was measured, and the percentage reduction in wound size was calculated. The average mass of the "wound area" was determined on an analytical scale as follows: on the days of measurement, wound contours were drawn on transparent film, then transferred to tracing paper, then the "wound contour" was cut out and weighed. Based on the results of the studies, the percentage of wound healing was calculated using the following formula: (S - Sn) / S × 100%, where S is the initial wound area; Sn is the wound area on the day of measurement.

Microcirculatory blood flow was measured using a TSD144 sensor from BiopacSystems, Inc. (California, USA): an MP150 polygraph with an LDF150C module in the wound surface area.

Polymerase chain reaction (PCR)

RNA was extracted using ThermoFisher reagent (USA), and the RNA concentration of the obtained RNA was measured using an IMPLENNanoPhotometer® spectrophotometer. Reverse transcription was performed using the MMLVRTSK021 kit according to the manufacturer's protocol. PCR was performed using 1 μg of cDNA, 10 pmol/L of each primer, and SYBR Green I master reagent (Roche Diagnostics, Germany). The primer sequences were as follows: eNOS forward 5′-CACACTGCTAGAGGTGCTG GAA-3′, eNOS reverse 5′-TGCTGAGCTGACAGAGTAGTAC-3′, iNOS forward 5′-GCAG GTTGAGGATTACTTCTTCCA-3′, iNOS reverse 5′-GCCCTTTTTTGCTCCATAGGAAA-3′, VEGF forward 5′-GAGTATATCTTCAAGCCGTCCTGT-3′, VEGF reverse 5′-ATCTG CATAGTGACGTTGCTCTC-3′.

Statistical data processing

Statistical analysis was carried out using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). All the data obtained were subjected to adequate statistical processing. Using descriptive statistics, the data were checked for normality using the Shapiro-Wilk test. In the case of a normal distribution, the data were presented as the mean and standard deviation (M±SD) or the standard error of the mean (as in the article, M±m), while in the case of a distribution that was not normal, the median and interquartile range (Me [XQ1; XQ3], where Q1 represents the 25th percentile and Q3 represents the 75th percentile) were calculated. When analyzing the data, the intergroup differences were determined using parametric or nonparametric methods, depending on the type of distribution. In the case of a normal distribution, the Student's t-test was used to analyze the differences between two samples, and the analysis of variance was used to compare more than two samples, with the assumption of equal variances and post-hoc tests. For non-normal distributions, the Mann-Whitney U-test was used, and the Kruskal-Wallis H-test was used for multiple comparisons, with a recalculation of the critical significance level. Differences were considered significant at $p<0.05$.

The statistical analysis was conducted by using IBM SPSS Statistics 26 and Microsoft Excel 2010 software. Data are presented as mean ± standard deviation; $p < 0.05$ was considered statistically significant.

Results

Following wound modeling in Avy/a mice, rapid wound surface contraction was observed during the first three days of the experiment, not due to reparation, but rather due to contraction of the wound edges. No statistically significant differences were found between the groups during this period. Analysis of the data obtained from the study of the Avy/a animal groups revealed a significant decrease in the rate of wound healing. Thus, in the group of experimental animals Avy/a on the 21st day of the experiment, the wound surface on average decreased by 83.12%, while in the group of animals without metabolic syndrome pathology (C57BL/6 mice) this figure was at the level of 96.19% (Table 1). In groups in which the eNOS blocker L-NAME and the iNOS blocker aminoguanidine were used in A$^{vy/a}$ mice without pharmacological support, the wound healing process slowed down in the first case by 1.9 times, and in the second case it improved by 1.1 times compared to such in the control group (II), respectively (Table 1).

It can be assumed that the different healing rates in the eNOS blockade groups are due to reduced vasodilation and impaired microcirculation in the tissues adjacent to the wound. The improvement in wound healing rates with aminoguanidine may be due to the fact that blockade of iNOS synthase can reduce the hyperproduction of peroxynitrite, which obviously has a cytotoxic effect on the walls of the vascular endothelium and aggravates the inflammatory process in the wound.

Table 1. The effect of blockade of endothelial and inducible NOs on the course of wound healing using LHT 2-18 hydrogel (M± SD), n=6

Groups	Timeframe for animal examination, days			
	3	7	14	21
	Dynamics of changes in the area of aseptic flat wounds, mm², %			
I	218.37 ± 10.63	156.31±11.3 -28.42 %	60.31±2.3 -72.43	8.32±7.1 -96.19
II	217.21 ± 12.35	176.09±12.4 -18.95	90.82±11.1* -58.19	36.67±7.9* -83.12
III	220.34± 7.52	215.12±5.6^ -2.37	151.29±6.2^ -31.34	66.48±3.3^ -69.83
IV	221.72 ± 13.98	176.69±3.9 -20.31	100.27±10.8 -54.78	17.45± 6.3^ -92.13
V	216.23 ± 15.75	163.97±7.7# -24.17	72,83±3.27^# -66.32	20.74±9.4^# -90.41
VI	218.2 ± 16.13	154.58± 5.3^{y} -29.16	56.98±13.10 ^^{y} -73.89	5.94±6.6 ^^{y} -97.28

Note: I – C57BL/6 mice (wound + hydrogel); II– A$^{vy/a}$ mice (wound + hydrogel); III – A$^{vy/a}$ mice (wound + L-NAME + hydrogel); IV – Mice (A$^{vy/a}$ wound + aminoguanidine + hydrogel); V – A$^{vy/a}$ mice (wound + L-NAME + 5% hydrogel LHT 2-18); VI – A$^{vy/a}$ mice (wound + aminoguanidine + 5% hydrogel LHT 2-18). Valid with respect to groups: *– I; ^ – II; $^{\#}$ – III; ^{y} – IV. (P ≤ 0.05).

A higher wound healing rate was observed in the Avy/a group of animals treated with LHT2-18-based hydrogel against the background of eNOS and iNOS blockade. Thus, in the group of animals receiving L-NAME, the wound area was 34.98 % smaller on the 14th day, and 20.58 % smaller on the 21st day than in the untreated group, indicating the effectiveness of the studied LHT 2-18 hydrogel in wound healing. With inducible iNOS blockade and treatment with LHT 2-18 hydrogel, the wound healing rate similarly increased by the end of the experiment (Table 1).

A Doppler study on animals with wounds revealed data indicating a decrease in blood flow in Avy/a mice 1.3 times (Table 2). In the groups that received L-NAME against the background of the wound, blood flow decreased by 2.1 times by day 21, while in the groups receiving aminoguanidine it increased by 1.1 times, respectively. In experimental groups V and VI, where 5% LHT 2-18 hydrogel was applied to wounds, improved blood flow velocity was observed (Table 2).

Table 2. Dynamics of blood flow velocity in the wound during blockade of endothelial and inducible NOs against the background of the use of hydrogel LHT 2-18 (M± SD), n=6

Groups	Blood flow velocity, PU			
	3	7	14	21
I	112.9 ± 1.2	130.7 ± 2.09	215.6 ± 1.9	236.18 ± 1.3
II	73.22± 1.4	105.20 ± 1.16*	150.29 ± 1.5*	178.71 ± 0.9*
III	34.57 ± 2.1	52.11 ± 2.41^	70.91± 3.23^	83.12 ± 1.63^
IV	66.32 ± 3.63	132.25 ± 2.76$^{\#}$	165.36 ± 12.18$^{\wedge\#}$	190.52 ± 2.45^$^{\#}$
V	83.62 ± 2.12	117.54 ± 2.17$^{\#}$	157.17 ± 3.98$^{\#}$	194.23 ± 2.15$^{\#}$
VI	90.6 ± 3.42	138.16± 4.22$^{\# y}$	176.62± 3.61$^{\# y}$	210.42 ± 3.46$^{\# y}$

Note: I – C57BL/6 mice (wound + hydrogel); II– A$^{vy/a}$ mice (wound + hydrogel); III – A$^{vy/a}$ mice (wound + L-NAME + hydrogel); IV – Mice (A$^{vy/a}$ wound + aminoguanidine + hydrogel); V – A$^{vy/a}$ mice (wound + L-NAME + 5% hydrogel LHT 2-18); VI – A$^{vy/a}$ mice (wound + aminoguanidine + 5% hydrogel LHT 2-18). Valid with respect to groups: *– I; ^ – II; $^{\#}$ – III; ^{y} – IV. (P ≤ 0.05).

Next, in order to demonstrate the relationship between NO production under the influence of LHT 2-18 and wound healing, quantitative PCR was performed to determine eNOS, iNOS, and VEGF (Fig. 1A, B, C).

The expression levels of eNOS and VEGF were higher in the C57BL/6 mice group compared to such in the Avy/a mice group ($p < 0.05$, $p< 0.01$, Fig. 1A, C). The expression level of iNOS was lower in the groups that used LHT 2-18 hydrogel as wound therapy ($p < 0.01$, Fig. 1B).

In a wound model in A$^{vy/a}$ mice, LHT 2-18 hydrogel reduced the expression of proinflammatory factors iNOS, increased the expression of eNOS and VEGF, promoted angiogenesis, and accelerated wound healing. Therefore, LHT 2-18-based hydrogel can be used

as a dosage form to regulate inflammation and angiogenesis during wound healing with metabolic disorders, including diabetes, and to accelerate wound healing.

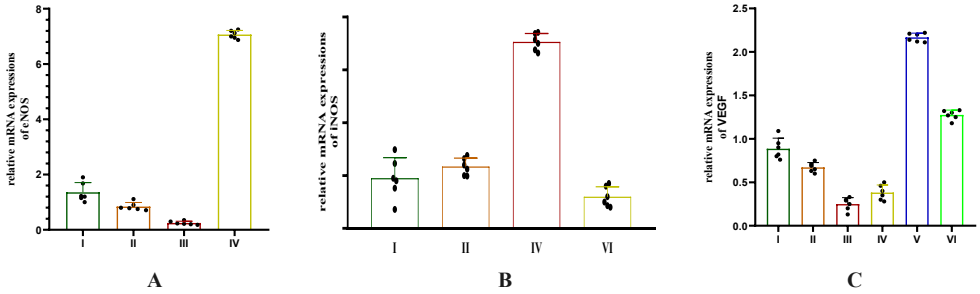


Figure 1. PCR analysis of eNOS, iNOS, VEGF, expression levels. ***Note:*** I – C57BL/6 mice (wound + hydrogel); II– $A^{vy/a}$ mice (wound + hydrogel); III – $A^{vy/a}$ mice (wound + L-NAME + hydrogel); IV – Mice ($A^{vy/a}$ wound + aminoguanidine + hydrogel); V – $A^{vy/a}$ mice (wound + L-NAME + 5% hydrogel LHT 2-18); VI – $A^{vy/a}$ mice (wound + aminoguanidine + 5% hydrogel LHT 2-18).

Discussion

The widespread occurrence of non-healing wounds, including those associated with diabetes, has a significant impact on public health and carries significant economic costs (Tehrany et al. 2023). Numerous approaches, including new drugs and technologies, are being used to achieve significant wound healing in patients with metabolic disorders. However, the search for agents with a high therapeutic effect on the healing process is ongoing and of great importance.

The study of acetylaminohexanoic acid derivatives has recently generated continued interest in the medical community due to their potentially broad spectrum of biological activity (Danilenko et al. 2023). This has led to the exploration of their pharmacological activity, including modification of their physical and chemical characteristics and a variety of dosage forms. One such acetylaminohexanoic acid derivative, the 2-ethyl-6-methyl-3-hydroxypyridinium N-acetyl-6-aminohexanoate complex (LHT 2-18), was studied.

Carbopol-based hydrogels are known to promote reparative regeneration in a moist environment, preventing the formation of secondary necrosis zones and lesion recurrence. Therefore, the studied carbopol hydrogel with LHT 2-18 has proven itself as a dosage form with a regenerative effect on the early wound healing process. It is worth noting that the agouti-mutated mice used in the experiment are characterized by a mutation in the agouti gene, which was evolutionarily responsible for fur color (Han et al. 2021) and leads to its ectopic expression in many tissues and organs, including the brain. In the hypothalamus, the agouti protein is a competitive antagonist of melanocortin receptors type 4 and leads to the development of melanocortin-type obesity. Furthermore, its effects on glucose metabolism, as well as lipid metabolism, contribute to the development of insulin resistance and dyslipidemia, key components of metabolic syndrome (Zhu et al. 2023). The type of animals chosen for the experiment allows us to predict how the drug will behave under conditions of metabolic damage, including insulin resistance and diabetes mellitus. Endothelial dysfunction is known to be an important pathogenetic link in metabolic damage, and the contribution of NO to wound healing processes is undeniable (Fig. 2).

Our experimental data on the effect of NO on local microcirculation in the wound are consistent with data from a number of authors (Wu et al. 2021). Several studies have shown that patients with diabetes mellitus and insulin resistance have impaired NO system function, with correspondingly reduced endothelium-dependent vasodilation. This may be due to reduced endothelial sensitivity to insulin and the suppressive effect of free fatty acids, free radicals, and glycated products on eNOS activity. As a result, diabetes mellitus is associated with impaired tissue circulation at the site of injury, leading to acidosis, hypoxia, and metabolic intoxication in the wound.

With blockade of eNOS and a decrease in the synthesis and release of endothelial nitric oxide, which is normally responsible for local vasodilation and antithrombotic action, a decrease in wound blood flow and the rate of wound surface contraction is observed. Consequently, prolongation of wound healing time and a negative dynamic are observed (Han et al. 2021). Our data on high rates of local blood flow in the wound, as well as the rate of contraction of the wound surface under the influence of aminoguanidine during blockade of iNOS synthase, may be associated with a decrease in the hyperproduction of peroxynitrite, which obviously has a cytotoxic effect on the walls of the vascular endothelium and aggravates the inflammatory process in the wound.

Nitric oxide (NO) stimulates the wound healing process. Various cells involved in the wound healing process produce NO via nitric oxide synthases (NOS). eNOS accelerates wound healing by promoting cell migration and proliferation of fibroblasts, endothelial cells and keratinocytes. eNOS expression was increased in the group with topical application of LHT 2-18 hydrogel, which accelerated wound healing due to cell migration and proliferation in the group. iNOS is induced by macrophages, cytokines and bacteria. iNOS is expressed by the immune response in the early stage of wound healing. The iNOS expression level was low in the LHT 2-18 hydrogel group.

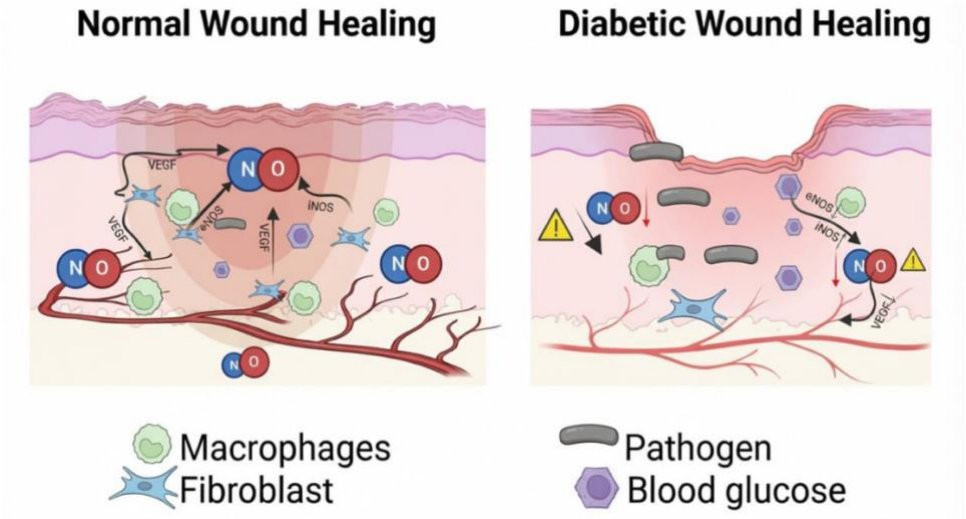


Figure 2. The physiological process of normal wound and diabetic wound, contribution of the functional system NO. Unlike normal wounds, diabetic wounds are characterized by impaired angiogenesis, excessive inflammatory macrophages (Malone-Povolny et al. 2019).

The pharmacological effect of LHT 2-18 hydrogel on wound healing process may be associated with the reduction of inflammation and oxidative stress in the wound microenvironment, which increases the bioavailability of NO, thereby allowing the wound to receive proliferative repair signals to achieve consistent healing and rapid healing. The increase in NO bioavailability when using LHT 2-18 hydrogel is confirmed by an increase in the expression level of eNOS and VEGF and a decrease in iNOS.

Conclusion

Topical application of a 5% hydrogel containing 2-ethyl-6-methyl-3-hydroxypyridinium N-acetyl-6-aminohexanoate (LHT 2-18), an acetylaminohexanoic acid derivative, reduces wound healing time by 1.7 times in mice with L-NAME-mediated endothelial nitric oxide synthase (eNOS) blockade and improves blood flow by 2.1 times. Blockade of iNOS (aminoguanidine) accelerates wound repair by 2.2 times and improves blood flow by 1.9 times.

Additional Information

Conflict of interest

The authors declare that they have no conflicts of interest.

Funding

This research was funded by the Program of Fundamental Research in the Russian Federation (FZWG-2026-0003)

Ethics statement

The study was approved by the Institutional Animal Care and Use Committee of Belgorod State National Research University (expert opinion No. 01-01i/16 dated March 18, 2025).

Acknowledgments

The authors have no support to report.

Data availability

All of the data that support the findings of this study are available in the main text.

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