ORIGINAL ARTICLES

EFFECTS OF SILVER NANOPARTICLE AND LOW-LEVEL LASER ON THE IMMUNE RESPONSE AND HEALING OF ALBINO MICE SKIN WOUNDS

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Abstract

The structural integrity of the skin, which acts as a barrier to keep harmful external substances out of the body, is compromised by wounds. The process of wound healing is a multifaceted and ever-changing phenomenon that entails the replacement of bodily tissues or damaged skin. It has been demonstrated that nanoparticles, especially silver nanoparticles, have anti-microbial and anti-inflammatory qualities that encourage cell migration and proliferation. Low level laser therapy has the potential to accelerate wound healing by stimulating cell regeneration after injury, reducing pain, and modulating the immune system. The aim of our study is to evaluate the healing process after treatment with silver nanoparticle and/or low level laser by measuring the serum levels of some pro-inflammatory cytokines (IL1b, IL6, and TNF-α), wound healing rate and histological analysis. Wounds were inflicted into 63 adult male albino mice (Mus musculus) and randomly divided into nine groups (7 per each). Control was left to normal healing. Other groups received a different treatment with laser, silver nanoparticle or both for 21 days. Injured skin was sampled for histopathological examination. Quantitative determination of TNFα, IL1 beta and IL6 were carried out using the sandwich enzyme-linked immunosorbent assay (ELISA) twice (day 2 and day 21). One-way and two-way analysis of variance (ANOVA) was used for statistical analysis. The results showed that the groups treated with silver nanoparticles and / or low-level laser promoted wound healing by reducing pro-inflammatory cytokines (IL1β, IL6 and TNFα) and showed significantly better wound closure with a significant reduction in wound size. At day 2 histopathological changes were very similar in different groups. When silver nanoparticles were applied, either alone or in combination with laser exposure, better granulation tissue and fibrosis also necrosis in the canter of the lesion and high score of re-epithelialization with less inflammation observed gradually till day 21. The results of this study suggested that silver nanoparticles and low-level laser have a wound healing potential, since topical treatment with silver nanoparticles and low-level leasers has effectively improved the wound healing process.

Key words: wound healing, nanoparticle, silver nanoparticle, low level laser (LLL), pro-inflammatory cytokines, AgNPs, IL6, IL1β, TNF- α.

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ВЛИЯНИЕ НАНОЧАСТИЦ СЕРЕБРА И НИЗКОИНТЕНСИВНОГО ЛАЗЕРА НА ИММУННЫЙ ОТВЕТ И ЗАЖИВЛЕНИЕ КОЖНЫХ РАН МЫШЕЙ-АЛЬБИНОСОВ

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Резюме

Структурная целостность кожи, которая действует как барьер, препятствующий проникновению вредных внешних веществ в организм, нарушается ранами. Процесс заживления ран влечет за собой замену тканей организма или поврежденной кожи. Было продемонстрировано, что наночастицы, особенно наночастицы серебра, обладают антимикробными и противовоспалительными свойствами и стимулируют миграцию и пролиферацию клеток. Низкоинтенсивная лазерная терапия может ускорить заживление ран за счет стимуляции регенерации клеток после травмы, уменьшения боли и модуляции иммунной системы. Целью нашего исследования является оценка процесса заживления после лечения наночастицами серебра и/или низкоинтенсивным лазером путем измерения сывороточных уровней некоторых провоспалительных цитокинов (IL1b, IL6 и TNF-α), скорости заживления ран и гистологического анализа. Раны были нанесены 63 взрослым самцам мышей-альбиносов (Mus musculus). Мыши были случайным образом разделены на девять групп по 7 мышей. Контрольная группы была оставлена без воздействия до нормального заживления. Другие группы получали другое лечение лазером, наночастицами серебра или и тем, и другим в течение 21 сут. Поврежденная кожа была взята для гистопатологического исследования. Количественное определение TNFα, IL1 бета и IL6 проводили с помощью иммуноферментного анализа (ИФА) дважды (2 и 21 сут). Для статистического анализа применяли однофакторный и двуфакторный дисперсионный анализ

(ANOVA). Результаты показали, что в группах, получавших воздействие наночастицами серебра и/или низкоинтенсивным лазером, заживление ран сопровождалось увеличением уровней провоспалительных цитокинов (IL1β, IL6 и TNFα). В этих группах было показано сокращение времени закрытия раны со значительным уменьшением размера раны. На 2-й день гистопатологические изменения были очень похожи в разных группах. При нанесении наночастиц серебра, отдельно или в сочетании с лазерным воздействием, наблюдалось ускоренное образование грануляционной ткани и фиброза, а также некроз в области поражения. В тих группах был получен более высокий балл реэпителизации с меньшим воспалением (до 21 сут). Результаты данного исследования свидетельствуют о том, что наночастицы серебра и низкоинтенсивный лазер обладают ранозаживляющим потенциалом, так как местное применение наночастицам серебра и низкоинтенсивного препаратами эффективно улучшило процесс заживления ран.

Ключевые слова: заживление раны, наночастицы, наночастицы серебра, низкоинтенсивный лазер, провоспалительные цитокины, AgNPs, IL6, IL1β, TNF-α.

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Introduction

Every year, millions of surgical incisions are caused during standard medical care [1]. One of the fundamental goals of clinical care continues to be promoting the healing of these inadvertent and intentional injuries, reducing the cosmetic impact on the patient, and maximizing the restoration of tissue function with the least number of scars [2]. When an injury occurs, damaged wound tissue is naturally repaired by a series of intricate cellular and biomolecular processes that bring it back to its pre-injury state [3]. The inflammatory response and related cellular migration, proliferation, matrix deposition, and tissue remodeling make up the fundamental biological mechanism of wound healing. When the healing processes proceed in a well-organized manner, the wound heals quickly and, in the case of acute wound healing, leaves little to no visible scars [4].

In healthy individuals, this mechanism functions at its best; nevertheless, there are many reasons that lead to poor wound healing, including aging, trauma, surgery, and acute or chronic illnesses like diabetes mellitus (DM) [5,6]. According to DeClue and Shornick's [7] research, the overproduction of pro-inflammatory cytokines including TNF-α, IL1β, and IL6 is linked to poor wound healing.

One of the key components of wound healing is thought to be the inflammatory response. The different inflammatory mediators are released to control the wound-healing process. Pro- and anti-inflammatory cytokines may be produced during normal wound healing, and the inflammatory response is more than sufficient. Encouraging wound healing and tissue regeneration requires safe in vivo regulation of the inflammatory response [8]. Deregulation of proinflammatory cytokines, including TNFα and IL1β, prolongs the inflammatory phase and slows healing [8,9].

Interleukin (IL)-1α/IL1β have both been promoted as "master regulators" of the wound healing response due to the large number of processes each regulates after injury or infection [10]. IL1β is released primarily by monocytes and macrophages as well as by nonimmune cells, such as fibroblasts and endothelial cells, during cell injury, infection, invasion, and inflammation [11].

IL6 is produced by neutrophils and monocytes and has been shown to be important in initiating the healing response. Its expression is increased after wounding and tends to persist in older wounds [12].

IL6 levels in wound fluids correlated with wound-healing rates [13]. TNF-α, at low levels, can promote wound healing by indirectly stimulating inflammation and increasing macrophage produced growth factors. However, at higher levels, especially for prolonged periods of time, TNF-a has a detrimental effect on healing [13,14].

NPs are tiny particles having a size range of 1–100 nm. They have unique properties such as size, shape, large surface area to volume ratio etc. NPs due to their vast range of antimicrobial property and rapid effectiveness with minimal dose are one of the choices of researchers for wound healing [15]. Conventional wound healing drugs have limited potential as they cannot penetrate the cell membrane, which a NP can [16]. Regulatory approval of Nano pharmaceuticals is slow, as the Food and Drug Administration (FDA) should approve them [16,17].

Silver is a widely used as antimicrobial agent in health care products. It has been applied for centuries in sanitization, health care, and to inhibit bacteria in food, but it has only been introduced into wound care as an antibacterial in recent years [18]. The use of silver in the past has been restrained by the need to produce silver as a compound; thereby increasing the potential effects. Nanotechnology has provided a way of producing pure silver nanoparticles [19]. This system also markedly increases the rate of silver ion release.

Laser light has the unique properties of monochromaticity (single wavelength), collimation (runs in one direction without divergence), and coherence

(with all waves in phase). These properties allow laser light to penetrate the skin's surface non-invasively [20]. Therapeutic lasers are a thermic without significant heat transfer (<0.65°C); in this way, photon energy is transferred directly to the target cells and thermal damage is avoided. Therapeutic lasers use monochromatic light in the 630 to 905 nm range, also known as the «therapeutic window» [21]. Laser therapy could enhance wound healing process by stimulating cell regeneration after injury, attenuating pain, and modulating the immune system [22].

The aim of our study is to evaluate the healing process after treatment with silver nanoparticle and/or low-level laser by measuring the serum levels of some Pro-inflammatory cytokines (IL1 β, IL6, and TNF-α), wound healing rate and histological analysis.

Materials and Methods

Silver nanoparticles

20% nano colloidal silver nanoparticles were purchased from (Paradise HealthCare)Spherical morphology with particle size ranged less than 10 nm (i.e. Aggregates as 200 atoms of Silver) [23].

Moisten infected area with colloidal silver (using cotton swabs) daily for the treated groups.

Laser treatment Exposure

Selected groups were exposed to diode laser (LSR_PS_ II # 10042504- Germany) light three times a week, for up to 21 days, with a radiation power of 650 ± 5 nm; emits 180 mW/ cm [2,24], with a 6 ml spot size at 20 cm from the injured part of the animal for 5 minutes.

Animals

In this study, 63 healthy adult male albino Swiss mice (Mus musculus) weighing 25 ± 5 g were purchased from the National Research Center (Dokki, Egypt), kept in standard polypropylene cages and acclimatized one week before the experimental work. The animals were housed at the animal house Zoology Department, Faculty of Science, Cairo University and had free access to standard pellet feed throughout, according to the test protocol, and had free access to water for the entire duration of the test. The animals were kept under standard conditions: temperature (25 ± 2ºC), relative humidity of the environment (55%) and alternating light-dark cycle (12 h / 12 h).

Animals were divided into nine groups as follows:

Group N: baseline, normal, without injury, three mice were euthanized at day3 and the others were euthanized at day 21; **Group W day 2:** positive control, injured, untreated (inflammatory phase) and were euthanized at day 2; **Group W day 21:** wounded, untreated, (remodeling phase) and were euthanized at day 21; **Group W + L day 2**: wounded treated once with laser light and were euthanized at day 2; G**roup W + L day 21**: wounded treated with laser light 3 times a week for 21 days and then were euthanized; **Group W + Silver day 2:** wound treated once with silver nanoparticle and were euthanized at day 2; **Group W + Silver day 21:** wounded treated with silver nanoparticle three times a week for 21 days and then were euthanized; **Group W + L + Silver day 2:** wound treated once with laser, followed by silver nanoparticle and then were euthanized at day 2; **Group W + L + Silver day 21:** after each laser session, the wounded were treated with silver nanoparticle three times a week for 21 days and then were euthanized.

Study Design Procedure

Wounds were inflicted on mice. Subsequently, the relevant groups were treated topically with silver nanoparticles and / or low-level laser therapy for the duration of the study (21 days). Positive control was left to normal healing. Groups designed to allow evaluating the selected parameter of the study at the inflammation period (day 2) and the remodeling period (day 21). So, wounded tissue and blood samples were collected at day 2 and 21 days after wounding, study evaluations such as healing rate, histopathological examination, cytokine assay by enzyme-linked immunosorbent assays (ELISA) were performed to confirm the effectiveness of wound healing after treatments.

Induction of wound

Before injury, each animal was partially anesthetized for a few seconds, to facilitate wounding, with an anesthetic solution of isoflurane (Anahal-Pharco-Egypt), the dorsal fur of the animals was shaved with an electric hair clipper and disinfected with 70% ethanol. A standardized full thickness open excisional wound was made using a 6 mm diameter, 2 mm deep biopsy punch (Royaltek, USA) [25,26]. Wound areas were examined on 4th, 7th, 11th ,15th,18th, and 21st day after the injury and evaluated for the percentage of wound contraction as the following equation [27]:

$$\text{Percentage of wound contraction} = \frac{\text{Wound area day 1} - \text{Wound area day } n}{\text{Wound area day 1}} \times 100$$

Histopathological Procedure

Autopsy pieces of wounded skin were collected for histopathological examination, samples fixed in 10% neutral buffered formalin. Washing within tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56°C in hot air oven for 24 h. Paraplast wax (Royaltek, USA) tissue blocks were prepared for sectioning at 4μ thickness by rotatory microtome. The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxylin and eosin stain (PHARCO) [28], then examination was done through the Olympus-USA BX43 light microscope and photographed using the Cellsens dimensions software linked to Olympus DP27 camera USA.

Blood Sampling and Cytokine Assay

Samples were collected from the bleeding orbital venous plexus after short anesthesia by Isoflurane solution, Once the required blood volume was drawn the serum was stored at -80°C then the animal was sacrificed by cervical dislocation. Quantitative determination of TNF-α, IL1β and IL6 levels was carried out with the sandwich enzyme- linked immunosorbent assay (ELISA) according to the manufacturer's instructions, optical density was measured at 450 nm using on Plate Reader (DAS-Italy), diagnostic kits for IL1β, IL6 and TNFα were purchased from Glory Science Co., Ltd-China.

Statistical analysis

All statistical analysis was executed using Statistical Package of the Social Sciences (IIBM-SPCS, version 26). Two ways analysis of variance (ANOVA) was applied to study the effect of silver nanoparticles and/or LLL on wounds and their interaction on the levels of the studied parameters (inflammatory cytokines IL1β, TNFα, IL6; wounds healing rate; histopathological examination and blood count) at day 2 and day 21. The post comparison test was used to detect the significant difference for the studied parameters among the wounded groups at definite time intervals. Data were represented as mean (M) ± standard error (SE). Significant difference was considered at $p<0.05$.

Ethical Statement

The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Sciences, Cairo University (Egypt) (CU/I/F/88/19). All experimental procedures were performed in accordance with international guidelines for the care and use of laboratory animals performed in accordance with the recommendations of the current edition of the Guide for the care and use of laboratory animals, 8th edition, 2011, USA.

Results

A low level of inflammation is necessary for faster wound healing, but its high level is destructive and delays it – this is the main objective of our study and that's how we built our methods and selected analysis to evaluate how much we achieved that.

Sixty-three healthy male adult albino mice; divided into nine groups (7 per each group) all except the normal group being circularly wounded at its dorsal as mentioned previously; one group left to normal healing and the others received a topical treatment with low level laser, silver nanoparticles, or both along the experimental period.

Wound healing monitoring, inflammatory cytokines assay and histological analysis all were considered as parameter to conclude the best effect of our treatments. Groups were designed to evaluate the selected parameters during the inflammation, maturation and remodelling phase.

Silver nanoparticles and LLL promote healing process

The re-epithelialization and closure of wounds were observed and measured regularly for 21 days in order to evaluate the progress of healing rate of all groups until the wounds were closed completely. The healing rate were calculated using the equation mentioned previously [36] (Fig. 1).

Wound closure was significantly improved within the injured mice treated with silver, LLL or both compared with the untreated group (normal healing), in untreated mice the wound closure was more severely affected than in treated mice since the wound closure percentage reached 12.74% at day 4 and increased to 28.5% at day 7 till 90.41% at day 21 after injury. In mice treated with LLL, the wound closure percentage reached 18.4% at day 4 after injury and increased to 40.4% at day 7 reached 94.4% at day21 after injury, group treated with silver only is more better as it reached 20.85 at day 4 increased to 45.6% at day7 and finally 98.8 at the end of experiment (day 21). The combined group gave the better healing percentage value as it reached 25.8% at day 4 increased to 55.2% at day7 then only at day18 completely healed 100%.

Next, statistical analysis by LSD indicated that the treatment with either silver nanoparticles or LLL induced a significant increase ($p < 0.01$; LSD) in wound closure percent at days 7 and 14 while both treatments together induced a significant increase ($p < 0.01$; LSD) at days 4, 7 and 18 after injury. Two way ANOVA revealed that the effect between groups was significant ($p < 0.001$) on wound closure percent throughout the experiment; comparison of the means in different groups at different times with Two way ANOVA Duncan Multiple Range Test (DMRT) showed at Table 1.

Среднее ± стандартная ошибка (SE) на основе анализа ANOVA; количество животных в каждой группе – 7; средние значения в одной и той же строке, использующие один и тот же надстрочный символ (символы), существенно не отличаются ($p < 0,05$) по данным Duncan Multiple Range Test (DMRT).

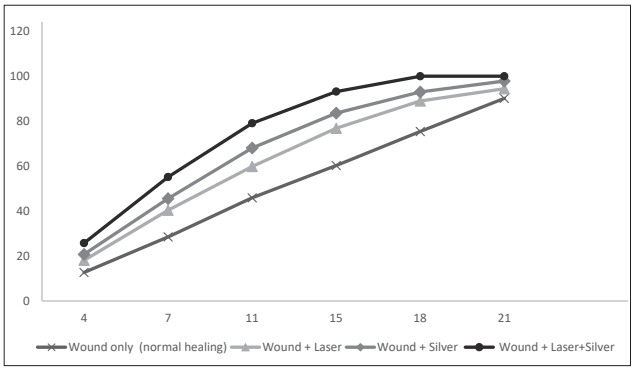

Рис. 1. Динамика уменьшения площади раны (% от исходного) во всех опытных группах в течение 21 дня после нанесения раны.

Fig. 1. The rate of wound contraction (% of initial wound area) in all experimental groups for 21 days post wounding.

Mean ± standard error (SE) based on ANOVA analysis; number of animals in each group is seven; the means in the same row which share the same superscript symbol(s) are not significantly different ($p < 0.05$) according to Duncan Multiple Range Test (DMRT). More over, we compared the appearance of healed wounds by digital photographs. We found that wounds in the Silver and LLL group showed the most resemblance to normal skin, with less hypertrophic scarring and nearly normal hair growth on the wound surface. The worst cosmetic appearance was observed in the normal healing group (Fig. 2). Healed wounds from the treated groups resembled looks as normal skin, with a thin epidermis and normal hair follicles. In contrast, untreated group showed thickened epidermis and no evidence of hair growth.

Histopathology results

Day 2

A surgical wound examination was performed in all experimental groups. Wound healing was very similar in all groups at day 2 after induction. The wound clefts were filled with necrotic tissue, abundant inflammatory cells, mainly neutrophils, edema, and hemorrhage. The wound surface was covered by a serocellular crust. The wound injury score was examined in all groups. No significant difference was found between the different groups with respect to any of the evaluated criteria; re-epithelialization, granulation tissue, inflammation, and angiogenesis (Fig. 3).

Day 21

Variation in wound healing process was found in various test groups after 21 days. The **W Day 21** group showed severe histopathological changes compared to other treated groups. In most individuals, persistence of the necrotic scab without epidermal remodeling was observed, accompanied by transmigration of large numbers of neutrophils. In the wound space, a poor organization of the filler granulation tissue was found, mixed with a strong infiltration of inflammatory cells and a poor angiogenesis process.

In group **L Day 21**, signs of re-epithelialization were found at the edge of the wound, which was characterized by hyperplasia of stratified squamous epithelial cells and partially extended to the wound surface. In the newly formed granulation tissue, less inflammation was found with better angiogenesis process compared to **W Day 21**.

Perfect wound healing was found in the **Silver Day 21** and **L + Silver Day 21** groups. Complete epidermal coverage was observed in most of the sections examined; some individuals in the **L + Silver Day 21** group showed re-epithelialization under the persistent serocellular crust. Organized fibro-vascular tissue occupied the wound clefts of the latter two groups, which were rich in collagen bundles and numerous newly formed blood capillaries.

The wound injury score showed a significant decrease in all parameters in the **W Day 21** group compared to other experimental groups. **L + Silver Day 21** group gave the highest significance observed in all the evaluation

Таблица 1
Анализ скорости заживления обработанных и необработанных ран
Table 1
Treated and non-treated wounds healing rate analysis

Группы Groups	Время наблюдения, сут Time, days					
	4	7	11	15	18	21
Рана Wounded	12.74^{n}	28.55^{K}	45.95^{I}	60.27^{g}	75.42^{e}	90.14^{bc}
Рана + низкоинтенсивный лазер Wounded + Low Level laser	18.14^{m}	40.42^{J}	59.85^{h}	76.85^{f}	89^{c}	94.42^{ab}
Рана + серебро Wounded +Silver	20.85^{m}	45.60^{I}	68.142^{g}	83.65^{d}	93.48^{b}	98.85^{ab}
Рана + низкоинтенсивный лазер + серебро Wounded + Low Level laser +Silver	25.87^{L}	55.12^{I}	79.094^{g}	93.17^{cd}	100.00^{ab}	100.00^{a}

Среднее ± стандартная ошибка (SE) на основе анализа ANOVA; количество животных в каждой группе – 7; средние значения в одной и той же строке, использующие один и тот же надстрочный символ (символы), существенно не отличаются ($p < 0,05$) по данным Duncan Multiple Range Test (DMRT).
Mean ± standard error (SE) based on ANOVA analysis; number of animals in each group is seven; the means in the same row which share the same superscript symbol(s) are not significantly different ($p < 0.05$) according to Duncan Multiple Range Test (DMRT).)

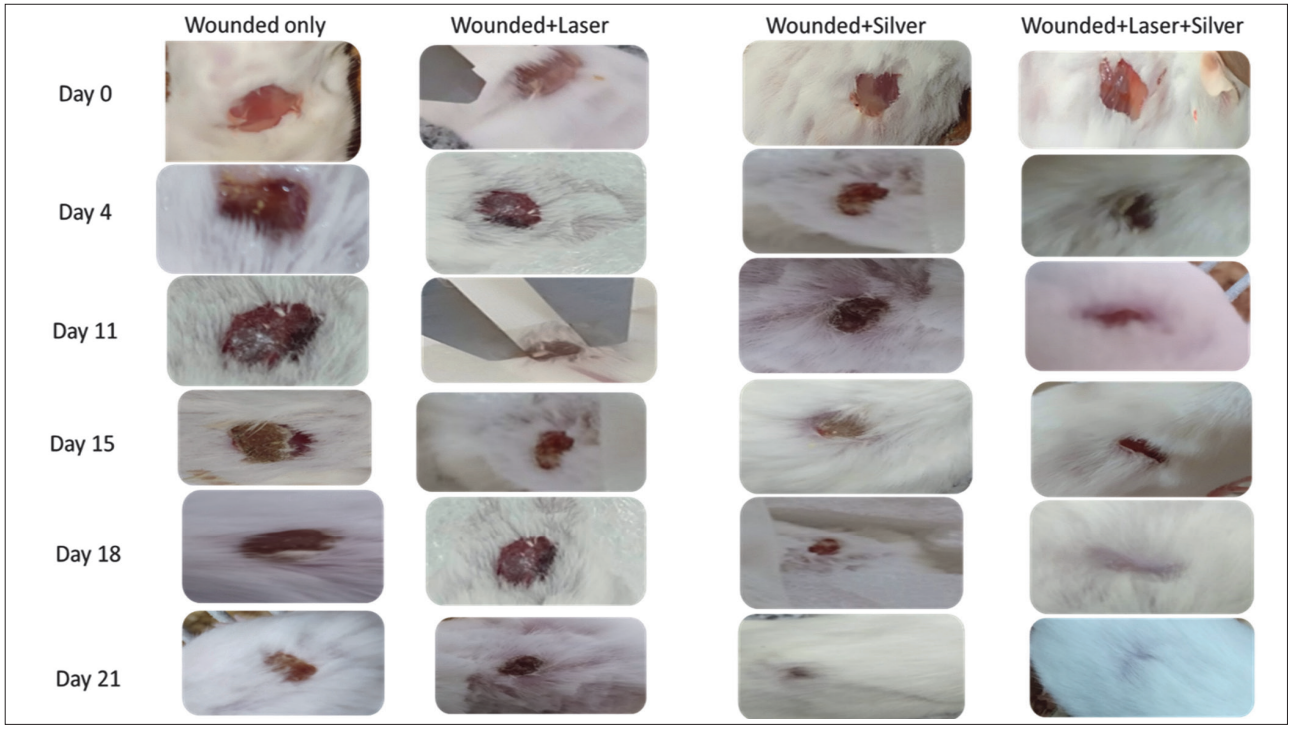

Рис. 2. Цифровые фотографии иссеченных ран всех экспериментальных групп
Fig. 2. Digital photographs of excision wounds of all experimental groups

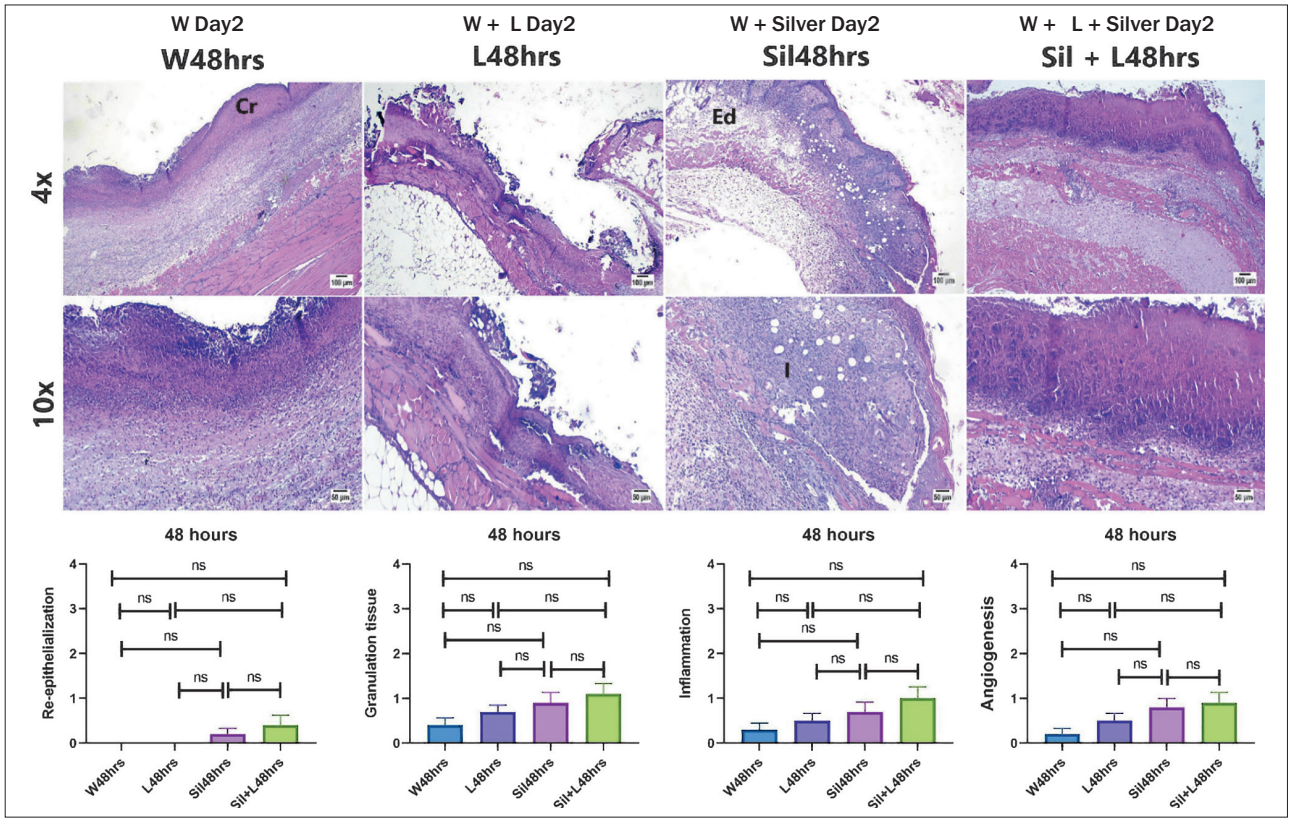

Рис. 3. Микрофотографии участков раны на 2-е сут после операции (во всех опытных группах отмечалось интенсивное острое воспаление, заполняющее раневую щель экссудатом и некротическими корочками; (Cr) корочка, (Ed) отек и (I) воспаление). Графики показывают гистопатологические параметры оценки заживления раны через 48 ч.
Fig. 3. Photomicrograph of wounded areas at day 2 post-surgery (all experimental groups displayed intense acute inflammation filling the wound gap with exudates and necrotic crusts covering; (Cr) crust, (Ed) edema, and (I) inflammation. Charts showing histopathological parameters of wound healing evaluation at 48 hours.

parameters high level of e-epithelialization, granulation tissue and angiogenesis with less inflammation score (Fig. 4).

W Day 21 and **L Day 21** groups exhibit inappropriate wound healing without epidermal covering. **Silver Day 21** and **L + Silver Day 21** groups showing compete epidermal growth and reduce the inflammation of the filling fibrovascular tissue.

Inflammatory Cytokines Assay

The data were entered, coded and analysed using the Statistical Package for the Social Science (IBM-SPSS,v.26). The experimental data were expressed as the mean ± standard error of the mean (SEM) using the ANOVA test, followed by a post-comparison test to determine which group had the best effect.

Serum TNF α

Tumor necrosis factor alpha (TNFα) levels increased significantly more in the **Wounded group** compared to the normal group ($p < 0.0001$), TNFα levels in the laser group and silver group had significantly lower concentrations than the injured group (positive control) at day 2 ($p < 0.0002$), while the combined group (**L+ Silver**) had the lowest significant concentration at day 2. Over time, the TNFα level decreased in all groups, reaching the trough level on day 21, and the combined group also had the highest significance compared to the injured group(untreated); The downregulation of the cytokine level during the experiment period showed at (Table 2).

Serum IL6

Table 3 displays the downregulation of interleukin 6 (IL6) level of during the inflammation stage and remodeling stage; levels increased significantly more in the **Wounded group** compared to the normal group ($p < 0.0001$), while IL6 levels in the laser group and silver group had significantly lower concentrations than the injured group (positive control) at day 2, while the combined group (**L + Silver**) had the lowest significant concentration at the same time (inflammation stage). Over time, the IL6 level decreased in all groups, Moreover the combined group also had the highest significance compared to the untreated group.

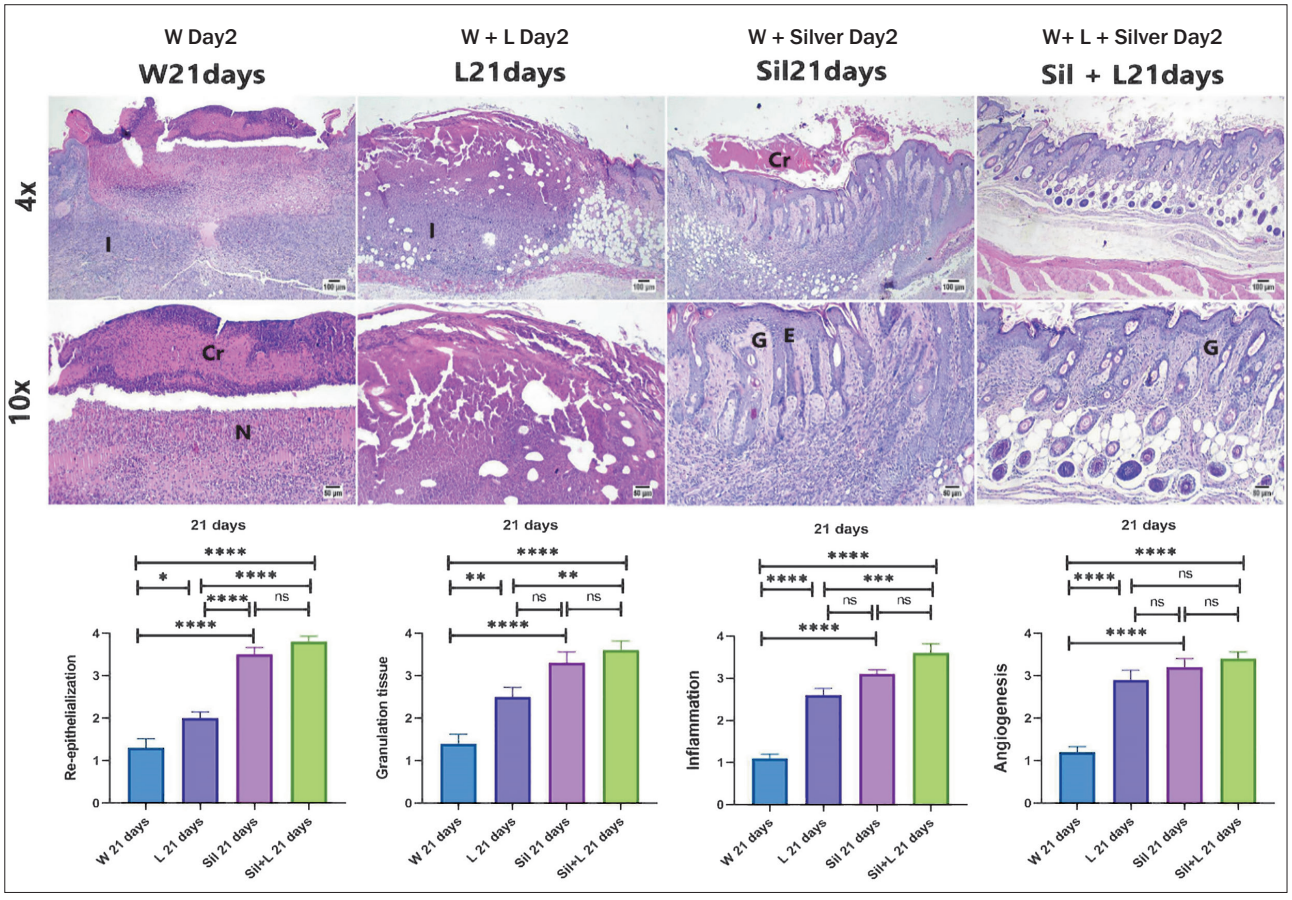

Рис. 3. Микрофотография участков раны на 21-е сут после операции. (E) Эпидермальный слой, (G) грануляционная ткань, (I) воспаление, (N) некроз. Графики показывают параметры оценки заживления раны на 21-е сут.
Fig. 4. Photomicrograph of wounded areas of different group at Day21 post-surgery. (E) Epidermal layer, (G) granulation tissue, (I) inflammation, (N) necrosis. Charts showing parameters of wound healing evaluation at day 21.

Serum IL1 β

Table 4 indicates that interleukin 1β (IL1β) levels increased significantly more in the Wounded group compared to the normal group (p <0.001), while IL1 β levels in the laser group and silver group had significantly lower concentrations than the injured group (positive control at day 2, while the combined group (**L+ Silver**) had the lowest significant concentration at day 2. Over time, the IL1 β level decreased in all groups, reaching the trough level on day 21, and the combined group also had the highest significance compared to the injured group**.**

Discussion

The natural process of wound healing entails a series of complicated cellular and biomolecular processes that restore damaged wound tissue into its original state when injury occurred [29]. During an injury, a blood clot is formed due to the damage in capillary and followed by the early phase of the inflammation, immune response to injury/wound or infection causes inflammation [31], infections related to various wounds, elevate medical expenditures and put a strain on health-care systems due to prolonged hospital admissions, treatment failures, infection persistence, and delayed wound

Таблица 2
Уровни TNFα (пг/мл) в сыворотке крови
Table 2
Serum levels of TNFα (pg/ml)

Группа Group	2 сут Day 2	21 сут Day 21
Группа без воздействия Normal	293.9 ±12.42	293.9 ±12.42
Рана Wounded	713.4 ±14.39****	486.9 ±15.27
Рана + низкоинтенсивный лазер Wounded + Low Level laser	581.2 ±31.84***	420.9±12.01
Рана + серебро Wounded + Silver	522.9± 26.62+++	389.1 ±23.44 ns
Рана + низкоинтенсивный лазер + серебро Wounded + Low Level laser + Silver	413.4± 17.34 **	300.6 ±21.7 ns
p value	<0.0001 ####	<0.0001 ++++
F	25.85	19.13

Среднее ± стандартная ошибка.
####: Достоверная разница между всеми группами на 2-е сут p < 0,0001.
++++: Достоверная разница между всеми группами на 21-е сут p < 0,0001.
****: Достоверная разница по сравнению с группой N при p < 0,0001.
***: Достоверная разница по сравнению с группой N при p < 0,0002.
+++: Достоверная разница по сравнению с группой N при p < 0,0005.
**: Достоверная разница по сравнению с группой N при p < 0,0027.
ns: Недостоверная разница по сравнению с группой N.

Means ± standard error.
####: Significant difference between all groups at day 2 p < 0.0001.
++++: Significant difference between all groups at day 21 p < 0.0001.
****: Significant difference in comparison with N group at p < 0.0001.
***: Significant difference in comparison with N group at p < 0.0002.
+++: Significant difference in comparison with N group at p < 0.0005.
**: Significant difference in comparison with N group at p < 0.0027.
ns: Non-Significant difference in comparison with N group.

Таблица 3
Уровни IL6 (пг/мл) в сыворотке крови
Table 3
Serum levels of IL6 (pg/ml)

Группа Group	2 сут Day 2	21 сут Day 21
Группа без воздействия Normal	51.09± 2.296	51.09± 2.296
Рана Wounded	303.1 ±18.32 ****	91.81 ±4.571 ns
Рана + низкоинтенсивный лазер Wounded + Low Level laser	203.1± 18.32 ***	71.22± 3.267 ns
Рана + серебро Wounded + Silver	160.8± 11.98 **	66.27 ±2.482 ns
Рана + низкоинтенсивный лазер + серебро Wounded + Low Level laser + Silver	128.3± 16.34 *	56.7± 2.134 ns
p value	<0.0001 ####	0.001 +++
F	19.58	35.85

Среднее ± стандартная ошибка.
###: Достоверная разница между всеми группами на 2-е сут p < 0,0001.
+++: Достоверная разница между всеми группами на 21-е сут p < 0,0001.
****: Достоверная разница по сравнению с группой N при p < 0,0001.
**: Достоверная разница по сравнению с группой N при p < 0,0052.
*: Достоверная разница по сравнению с группой N при p < 0,0256.
ns: Недостоверная разница по сравнению с группой N.

Means ± standard error.
###: Significant difference between all groups at day 2 p < 0.0001.
+++: Significant difference between all groups at day 21 p < 0.0001.
****: Significant difference in comparison with N group at p < 0.0001.
**: Significant difference in comparison with N group at p < 0.0052.
*: Significant difference in comparison with N group at p < 0.0256.
ns: Non-Significant difference in comparison with N group.

Таблица 4
Уровни IL1β (пг/мл) в сыворотке крови
Table 4
Serum levels of IL1β (pg/ml)

Группа Group	2 сут Day 2	21 сут Day 21
Группа без воздействия Normal	70.96± 4.937	70.96± 4.937
Рана Wounded	170±25.29***	65.77±4.9
Рана + низкоинтенсивный лазер Wounded + Low Level laser	141.9±21.28 *	62.22±3.2
Рана + серебро Wounded + Silver	102.5±22.76	59.91±6.269
Рана + низкоинтенсивный лазер + серебро Wounded + Low Level laser + Silver	80.92±20.45	52.34±3.9941
p value	0.04#	> 0.05
F	2.936	1.42

Среднее ± стандартная ошибка.
***: Достоверная разница по сравнению с группой N при p < 0,001.
*: Достоверная разница по сравнению с группой N при p < 0,012.
#: Достоверная разница в сравнении без групп на 2-е сут при p < 0,4.
ns: Недостоверная разница по сравнению с группой N.

Means ± standard error.
***: Significant difference in comparison with N group at p < 0.001.
**: Significant difference in comparison with N group at p < 0.012.
#: Significant difference in comparison within groups at day 2 at p < 0.04.
ns: Non-Significant difference in comparison with N group.

healing, which often leads to amputation and increased death [32].

The wound healing process involves multiple cellular and extracellular pathways through overlapping phases, namely hemostasis/inflammatory phase, proliferative phase, and remodelling phase [33]. The goal of the process is to restore tissue integrity and functions. Damaged blood vessels constrict during a vascular inflammatory response, and coagulation is brought on by thrombocytes congregating in a fibrin network. The wound then heals as a result of angiogenesis and re-epithelialization that take place during the proliferative stage. The remodelling phase involves reorganization, degradation, resynthesis of the extracellular matrix, and remodelling of the granulation tissue in order to achieve the maximum tensile strength [23].

Wound healing is strictly regulated by a number of cytokines and growth factors circulated at the wound site [34]. Decreased collagen deposition and growth factor production in wounds due to elevated levels of pro-inflammatory cytokines postpone wound healing [35]. Accelerating healing with minimal scars is the goal of most wound healing research [36].

Based on the experimental findings of the current study, the topical treatment application of silver nanoparticles, LLL alone or in combination gives great effect prevention of infections, decrease inflammation, complete healing and minimal scarring, moreover comparison of the rate of wound contraction among normal healing group (control) and other treated groups specially combined one, histological evaluation and other analysis which selected to monitor the progress showed a drastic difference in the healing pattern. The drastic decrease in wound contraction in control animals clearly indicated the need of therapeutic aid which could speed up the wound-healing process. Hence, in the current report, we aspired to understand the concept behind impaired wound healing in albino mice, and to come up with a therapeutic plan to accelerate healing in these persistent wounds using LLL and silver.

In the present study wound healing observed by morphological examination and photographed, wound closure in all wounded groups was measured every three days after injury till day 21, healing rate calculated [27] and give evidence that treated mice with AgNPs and/ or LLL as compared to the normal healing mice enhanced wound healing to reach 100% and 93%, respectively. Similar to our observation, Amini and his colleagues (2023) [37] have found that Ag-hydrogel nanocomposite groups consistently closed faster than control groups, and the original wound area in groups treated with Ag was significantly smaller at weeks 1, 2, and 3 post-wounding, and they confirming that Ag nanocomposite treatments accelerate the wound healing process.

AgNPs accelerate wound contraction and aid in the healing process by promoting fibroblast migration and stimulating fibroblast differentiation into myoblasts, as demonstrated by Tyavambiza et al. (2021) [38]. Also, due to the anti-inflammatory properties of AgNPs, the topical application of AgNPs in the wound area reduces the release of cytokines and lymphocytes, and reduces mast cell infiltration, which then promotes wound healing with minimal scarring [39]. Similarly, in other researches, AgNPs accelerated the rate of wound healing by activating the proliferation and migration of keratinocytes. Moreover, they aided in the differentiation of fibroblasts into myofibroblasts, which accordingly promoted wound contraction and speeded up the healing of severe wounds [38,40].

In terms of healing, the elucidation of pro-inflammatory and anti-inflammatory pathways is important for the development of strategies to defend regenerative tissue from damage caused by imbalances in cytokines, oxidants, antioxidants within the wound. Information about specific subsets of inflammatory

cell lineages and the cytokine network orchestrating inflammation associated with tissue repair has increased [40]. According to our data the treatment of wounded mice with silver and/or LLL produced a significant decrease of the elevate TNF-α, IL6 and IL1β levels as compared to the corresponding wounded controls, untreated group gave the highest significance and the highest level of the cytokine as compared to the normal levels- which illustrate the severe inflammation in the normal healing - after that cytokine level decrease to be near normal values, better values seen at the group treated with silver and LLL together.

Similar observations were obtained by Franková and his colleagues (2016) [41], who made an in vitro study by topical application of AgNPs in mice with burn wounds which results in decreasing counts of neutrophils and low levels of IL6, accompanied by an increase in the levels of IL10, TGF- β, vascular endothelial growth factor (VEGF), and interferon (IFN)- γ. They also have reported that AgNPs decreased the release of some pro-inflammatory cytokines and growth factors from normal human epidermal keratinocytes (NHEKs) and normal human dermal fibroblasts after 24 h at all the studied AgNPs concentrations (0.25, 2.5, and 25 μg/mL).

AT the present investigation the expression of TNF-α, IL6 and IL1ß in the laser group was seen in day 2 and decreased gradually till day 21.This may be due to the role of LLL on the surface epithelium cells (keratinocytes) to produce the pro-inflammatory cytokines which is needed in the acute inflammation during wound healing and also in a faster closure of the wound surface [42], after that the levels decreased gradually may occurred due to LLL anti-inflammatory effects which directly related to reduction of pro-inflammatory cytokines, as well as the amount of chemical mediators. The results indicate that LLL induces an inflammatory reaction that may modulate transcription factors linked to mRNA expression pro-inflammatory cytokines. These data are corroborated by previous studies which suggested that laser therapy can reduce the production of inflammatory mediators and events that contribute to balance the inflammation process [43].

The reason for the effective acceleration of wound healing using LLL was that the absorption of laser with specific wavelength by target tissue may result in the enhancement of fibroblast. proliferation and the promotion of collagen metabolism and granulation tissue formation also improvement of mechanical parameters and histopathological changes [42] which supported by present data. The major studies have suggested that either elements in the mitochondrial cytochrome system or endogenous porphyrins in the cells are the energy-absorbing chromophores in LLL [43,44]. It is important here to mention that photoenergy of 650nm wavelength at the given parameters possibly induced the fibroblasts to secrete the growth factors that probably acted in an autocrine manner to increase their rate of mitosis and or reduce cell death [44]. The response of low energy laser on cells may be dose dependent as well as wavelength dependent [44]. Therefore, correct energy density with an appropriate wavelength which can be easily and safely absorbed by the targeted tissues is strongly suggested.

Conclusion

Our results mandate the conclusion that the topical application of silver nanoparticles in combination with LLL following wounding had salutary effect on wound-healing progression, possibly through the decreasing of the inflammatory cytokines, activation of wound fibroblasts and elevation of collagen synthesis which accelerates wound healing rate. Testing the same optimal dose with the same power, center wavelength, laser spot size and duration, but with different doses of silver for its tissue regenerative potential is highly recommended. Although these results are very promising, more experimental studies for better understanding of silver and laser-assisted enhanced tissue regeneration are recommended.

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