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Evaluation of Antimicrobial Activity and in Vivo Wound Healing Properties of Copper Nanoparticles and Copper Nanoparticles Nanoemulsion

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Abstract: Burns are a major global medical issue due to the complexity of the wound healing process and the high risk of contamination by pathogenic microorganisms. We were able to synthesize stable copper nanoparticles (CuNPs) and copper nanoparticles nanoemulsion (CuNPs-NE). Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CuNPs and CuNPs-NE were tested for *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Candida albicans*. To compare the wound-healing properties of CuNPs and CuNPs-NE, we used an experimental model of second-degree thermal burns in male Wistar rats. The experimental groups treated with CuNPs colloid solution and CuNPs-NE were compared with the untreated negative control group for the 12-day duration of the experiment. The planimetry studies (average wound size, wound healing rate, and wound diameter reduction) and histological analysis were evaluated. Our data shows that CuNPs and CuNPs-NE have strong, long-lasting antibacterial properties. Furthermore, both compounds significantly accelerate the wound healing process and more drastically reduce the wound area compared to the control. Furthermore, histological analysis confirmed a greater wound-healing effect after CuNPs-NE treatment. There is great potential in using CuNPs-NE solutions as a wound healing agent for accelerated recovery of post-trauma scar tissue.

Keywords: copper nanoparticles; nanoemulsion; antimicrobial activity; burns.

1. Introduction

Burns are a major global medical issue due to the complexity of the healing process and the high risk of contamination by pathogenic microorganisms. Furthermore, the global number of infections caused by multidrug-resistant (MDR) pathogen strains is rapidly increasing due to excessive and inappropriate use of antibiotics, which is the main cause of resistance of bacterial strains to conventional therapies. Antibiotic-resistant bacteria colonize wounds, hinder healing, and cause severe side effects [1,2]. There is a great need to discover substances

with antibacterial properties and effective but safe compounds with improved wound-healing properties.

Nanoparticles (NPs) show great potential as nano-antibacterial drugs that ensure targeted delivery of antibiotics and natural antimicrobial compounds [3,4]. The high therapeutic potential of nanoparticles in wound treatment is due to their high antibacterial and angiogenesis properties [5,6], their ability to regulate cytokine release and antioxidant activity, and selectively penetrate the stratum corneum [7-9]. Several bactericidal mechanisms of action of NPs were described in detail, such as the direct interaction with the bacterial cell wall, inhibition of biofilm formation, induction of oxidative stress, release and interaction of metal ions with pathogens' DNA and/or proteins, and alteration of gene expression [10, 11]. NPs affect the expression of key proteins involved in nitrogen metabolism and electron transport [12]. Such different modes of nanoparticle action in bacterial cells can be highly effective against MDR bacteria [13]. Furthermore, NPs possess great physicochemical properties and can be used to study antibiotic resistance mechanisms in bacteria [14].

CuNPs based drugs can effectively treat microbial infections after skin burn lesions [15]. Furthermore, copper possesses a higher biocompatibility than other metallic ions to promote skin regeneration and increase skin quality [16]. Modifying nanoliposomes with NPs for targeted dermal delivery into heating promotes skin permeability, prolongs its retention time, and accelerates the closure of the wound [17,18]. Due to these specific characteristics, polymersomes are considered promising vehicles for targeted drug delivery to improve the wound healing process [19]. The specific aim of this study was to compare the antibacterial properties and wound-healing effect of copper nanoparticles with those of copper nanoparticles nanoemulsion.

2. Materials and Methods

2.1. Preparation of copper nanoparticles and copper nanoparticles nanoemulsion.

The copper nanoparticles were synthesized using the method of chemical reduction, with L-ascorbic acid as a reducing agent and polyvinylpyrrolidone (PVP) as a capping agent. The procedure was performed in the aqueous solution following a previously established method [20,21]. Properties of CuNPs were analyzed after performing transmission electron microscopy (PEM-100, Selmi, Ukraine) and UV-Vis spectrophotometry (The Unicam UV 500 UV/Vis, Thermo Fisher, USA and DeNovix DS-11, DeNovix Inc., USA). Copper concentration was measured by atomic absorption spectroscopy (Selmi, Ukraine). When creating nanoemulsion, we used freshly synthesized and stabilized CuNPs at pH 7.3.

The oil-in-water (O/W) nanoemulsions were prepared by vigorously mixing the oil phase containing soy lecithin (1-4% w/w), Tween 80 (0,25-1% w/w), and molecular grade olive oil (5, 10 and 20% w/w) in 1:4 (v/v) surfactant to oil ratio with aqueous phase CuNPs at 400 rpm using a magnetic stirrer for 5 min. Finally, the emulsion was sonicated by an ultrasonic homogenizer at 20 kHz wave frequency for 1 min at room temperature. Obtained samples of CuNPs and CuNPs-NA were stored at 4 °C before use for no longer than 2 months.

2.2. Characterization of nanoemulsions.

The thermodynamic stability of nanoemulsions was determined as follows:

1. Heating-cooling cycles. Six cycles of 4 °C to 40 °C with storage at each temperature of not less than 48 h were studied. Stable samples were subjected to centrifugation tests to determine the nanoemulsion stability.

2. Centrifugation. To test the stability, emulsions were centrifuged at 2000 g for 30 min and were visually analyzed for phase separation.

All samples were inspected and studied for phase separation, color change, and drug precipitation. pH measurements of the emulsions were performed using a pH meter (WTW, model 330i, Weilheim, Germany). The refractive index (n) of the medium was defined as the ratio of the speed of the wave (c) in the reference medium to the phase speed of the wave (vp) in the medium: n=c/(vp).

2.3. MIC and MBC analysis of copper nanoparticles and copper nanoparticles nanoemulsion.

Minimum inhibitory concentration and minimum bactericidal concentration were determined with the standard broth dilution method by assessing microorganisms' visible growth in the agar broth. Serial dilutions of copper nanoparticles and copper nanoparticles nanoemulsion in concentrations ranging from 0.01 µM to 1.6 µM (in conversion to copper) were performed in a 96-well plate by the addition of the corresponding amount of stock solution of CuNPs (1.18 mg/ml) and CuNPs-NA (0.944 mg/ml). Microbial strains: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Klebsiella pneumoniae* (ATCC 2579), *Bacillus subtilis* (ATCC 31324), *Staphylococcus aureus* (ATCC 25923), *Candida albicans* (ATCC) 885-653 were used in the experiment. Pre-cultured microorganisms were seeded at a concentration of 10^5 CFU/ml per well. Nutrient broth (NB) (13 g of NB powder, pH 7.2) was used as a growth medium. The control groups contained only NB with inoculated appropriate microorganisms. All incubations were performed at 37 °C for 24 h. After cultivation, 100 ml of each sample was plated onto a petri dish with nutrient agar and cultivated for 24 h (as an exception, *C. albicans* was cultivated for 48 h), and a colony forming unit (CFU) count was performed. The MIC value was determined as the lowest concentration of CuNPs and CuNPs-NA that inhibits the growth of microbes compared to the control group. MBC was determined as the lowest concentration of copper nanoparticles and copper nanoparticles nanoemulsion to kill 99.9% of the initial bacteria count in the sample.

2.4. Animal procedures.

For the in vivo studies, all necessary ethical approval was obtained from the Bioethics Committee of the Institute of Animal Biology NAAS (№ 82/10.06.2020). All conducted procedures were performed in accordance with the stipulations of the Helsinki Declaration and the policy statement for the care and use of laboratory animals. Albino rats (weight; 200-250 g) were kept in separate cages at a housing temperature of 25 °C, normal daylight, and relative humidity of 50-55% with unrestricted access to food and water. The following three groups of animals were used (n=6 rats per group): untreated Control Group of animals, 0.200 mg/ml CuNPs treated group (CuNPs Group), and 0.200 mg/ml Copper Nanoparticles Nanoemulsion treated group (CuNPs-NE Group). The rats were anesthetized via intramuscular injections of 50 mg/kg body weight of ketamine hydrochloride. The second-degree burns were made with a brass block (20 mm by 40 mm) on both sides of the dorsum of the rat. The burns were treated once every 2 days with 0.5 mL of non-liposomal solution (CuNPs Group) or the liposomal form of CuNPs (CuNPs-NE Group). On day 12 post-injury, all rats were euthanized, and

samples of wounds were collected. The wound tissue samples of each animal were precut and fixed in 10% neutral buffered formalin for histology examination.

2.5. Wound examinations.

Clinical assessments, such as observations of the wound area's appearance, the wound's healing surface, the percentage of the surface area of wound contraction, and the healing time were performed. The diameters of the excised wounds were measured with a millimeter grade ruler on days 2, 4, 6, 8, 10, and 12 post-injuries. The following formula calculated the wound contraction rate:

$$\text{Wound contraction } (\%) = \frac{\text{initial area of the wound} - \text{area of wound measured}}{\text{initial area of the wound}} \times 100\%$$

The rate of wound healing over time was calculated by the formula:

$$\text{The rate of wound healing } (\text{mm}^2/\text{day}) = \frac{\text{initial area of the wound} - \text{area of wound measured}}{\text{number of days between measurements}} \times 100\%$$

2.5. Histological analysis.

The fixed tissues were dehydrated with 50% to 99.9% ethanol (V/V), embedded into the paraffin, and cut into 5 μm sections with a microtome (HM 340E Microtome, Thermo Scientific, Germany). Serial sections were stained with hematoxylin and eosin and observed under a light microscope (Primo Star, Carl Zeiss, Germany).

2.6. Statistical analyses.

Experiments were performed in triplicate for each of the two biological replicates samples. The results are reported as the average and standard deviation. One-way analysis of variance (ANOVA) was performed in the Statistical Analysis Systems (Origin 9.0) software package. Significant differences among mean values were determined by Bonferony's test at the same significance level. The level of significance was set at $P<0.05$.

3. Results and Discussion

3.1. Nanoemulsion stability.

Dispersed CuNPs with PVP were obtained through synthesis.

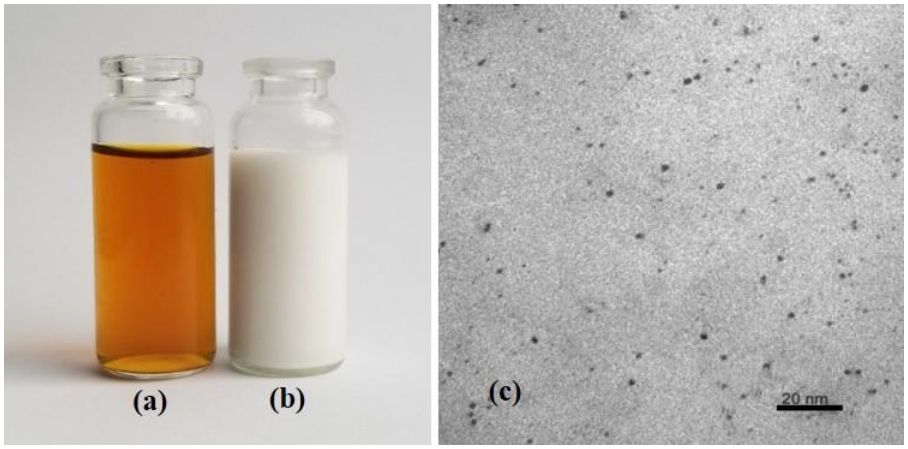

Figure 1. (a) Copper nanoparticles 0.2 mg/ml; **(b)** copper nanoparticles nanoemulsion 0.2 mg/ml; and **(c)** TEM images of copper nanoparticles.

TEM image analysis revealed spherical shapes of nanoparticles with a size range of 1-5 nm (Figure 1). The concentration of the copper in the CuNPs solution after synthesis was 1.18 mg/ml. Nanoparticles were stable for two mounts at 4 °C storage conditions.

The most important criterion of nanoemulsion is the thermodynamically stable systems formed at a specific concentration of the compound with no phase separation. The study aimed to design a stable nanoemulsion of copper nanoparticles using proper surfactant and oil concentration that could improve its bioavailability. The experimental results indicated that EC2 (10% olive oil) and EC3 (20% olive oil) formulations were more stable than EC1 (Table 1).

Table 1. Stability study of different experimental compositions of copper nanoparticles nanoemulsion.

Formulation	pH	Refractive index	Thermodynamic stability of nanoemulsion		
			Centrifugation	Heating-Cooling cycle	Inference
EC1	7.63±0.01	1.486±0.008	+	Some destabilization	Passed
EC2	7.41±0.02	1.505±0.003	+	Stable	Passed
EC3	7.53±0.01	1.501±0.004	+	Stable	Passed

Mean ± standard deviation followed by differences in the column differ from each other by Bonferony's test with 95% confidence. EC – experimental compositions: EC1 consist of olive oil 5% w/w, soy lecithin 1% w/w; EC2: olive oil 10% w/w, soy lecithin 2% w/w; EC2: olive oil 20% w/w, soy lecithin 4% w/w. The ratio of oil: surfactant (soy lecithin 1% w/w, Tween 80) (v/v) was 4:1 in all groups.

Oil-in-water nanoemulsion indicated good emulsion stability upon increasing olive oil concentration to 10% and 20% with the 4:1 ratio of surfactant to oil. For *in vivo* studies, experimental composition EC2 of Copper Nanoparticles Nanoemulsion was used.

3.2. Antimicrobial activity of copper nanoparticles and copper nanoparticles nanoemulsion.

Currently, a wide range of antibiotics is used to treat wound infections. However, due to antibiotic resistance, nano-drug medicines can improve the safety of wound treatment [22]. The results indicate that free copper nanoparticles and copper nanoparticles nanoemulsions possess remarkably powerful antimicrobial activity (Table 2).

Inhibitory concentrations tested on microorganisms were from 0.10 to 0.30 µM of CuNPs, and from 0.15 to 0.40 µM of CuNPs-NE. Minimum bactericidal concentrations of CuNPs and/or CuNPs-NE to investigate microorganisms were from 0.25 to 0.60 µM. CuNPs and CuNPs-NE have a higher antimicrobial activity than *C. albicans*, *E. coli*, and *B. subtilis* and slightly lower than *P. aeruginosa*, *K. pneumonia*, and *S. aureus*. Furthermore, significant positive effects of CuNPs on the wound healing process were observed compared to the controls (Table 2). Such wound healing effect of CuNPs is due to the antibacterial activity mechanism of copper itself [22-24]. The benefits of antimicrobials in wound treatment have been established in numerous studies, suggesting that accelerated healing due to antimicrobial activity is a mechanism of wound healing [25-27]. Ashfaq reported the effectiveness of CuNPs in polyvinyl alcohol-cellulose acetate phthalate biomaterial against gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacterial strains [28,29]. Furthermore, this study shows that free copper nanoparticles have higher bactericidal properties than copper nanoparticle nanoemulsions. We hypothesize that such a difference may be due to the free access of nanoparticles to microorganisms' surfaces compared to nanoparticles that are limited by liposomes.

Table 2. Minimum inhibitory concentration and minimum bactericidal concentration of copper nanoparticles and copper nanoparticles nanoemulsion.

Microbial Strains	MIC, µM		MBC, µM	
	CuNPs	CuNPs-NE	CuNPs	CuNPs-NE
***E. coli* ATCC 25922**	0.25	0.30	0.30	0.40
***P. aeruginosa* ATCC 9027**	0.30	0.40	0.45	0.60
***K. pneumoniae* ATCC 2579**	0.30	0.35	0.45	0.60
***B. subtilis* ATCC 31324**	0.25	0.30	0.35	0.40
***S. aureus* ATCC 25923**	0.30	0.35	0.40	0.50
***C. albicans* ATCC 885-653**	0.10	0.15	0.25	0.30

3.3. Wound healing properties of copper nanoparticles.

We compared the diameter of the burns area during post-burn treatment. We found that the surface of wounds in the group treated with CuNPs-NE had a significantly reduced diameter of burns with a statistical difference (P<0.05-0.01) in comparison with the control and Group 1 treated with CuNPs (P<0.01) (Figure 2a).

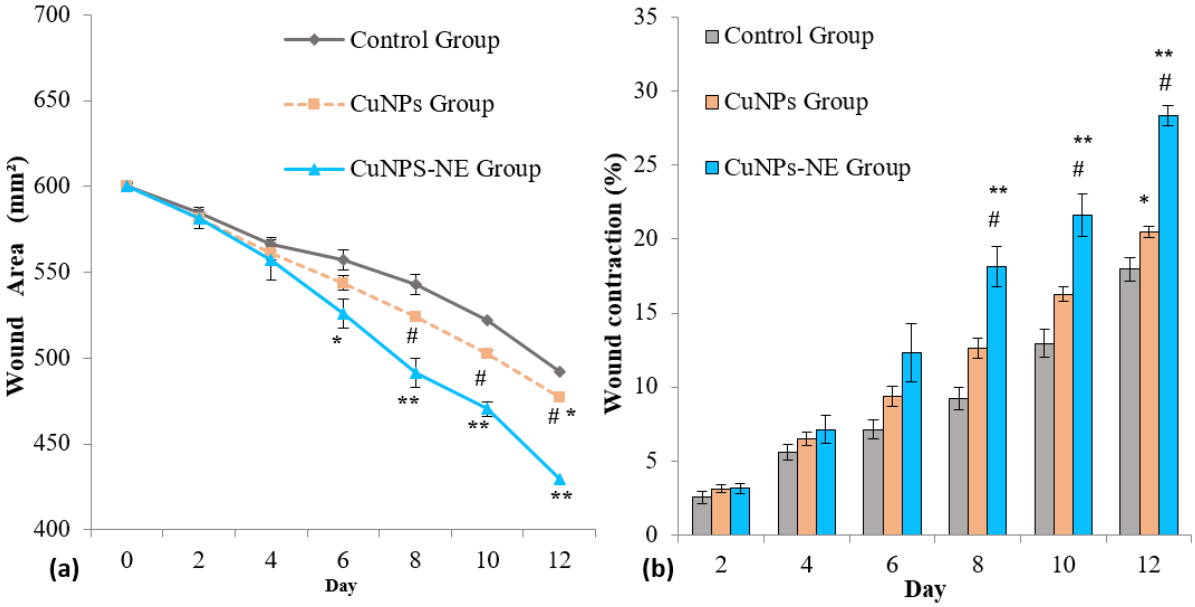

Figure 2. Effect of CuNP formulation treatments on the diameter of wound area **(a)** and percentage of wound contraction **(b)** in rats. Data are expressed as mean ± SD from six rats and analyzed by One Way ANOVA followed by Bonferroni's test. *P<0.05, **P<0.01− compared to control group; #P<0.05 − compared between CuNPs and CuNPs-NE Group.

The percentage of wound contraction was found to increase time-dependent after treatment in all groups (Figure 2b). Wound contraction was significantly higher in CuNPs-NE-treated rats throughout the study period than in control animals. Furthermore, CuNPs-NE treated wounds indicated significant wound contraction on days 6, 8, 10, and 12 when compared to the control group (P<0.05-0.01) and CuNPs treated group (P<0.05).

When comparing the rates of wound healing, our results indicate that the wound regeneration rate after treatment with CuNPs-NE was substantially higher. Figure 3 shows that wound healing rates were 1.31-1.44 times higher from day 6 to 12 in CuNPs-NE treated rats, compared to burns treated by copper nanoparticles in an aqueous solution.

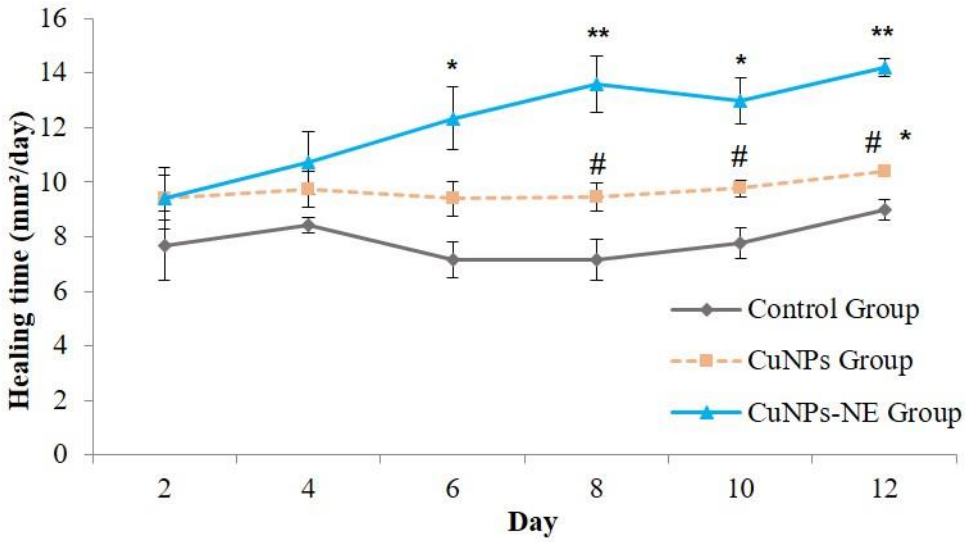

Figure 3. Comparison of the rate of wound healing in rats under CuNPs formulation treatment. All data are expressed as mean ±SD, n=6. *P<0.05, **P< 0.01 − compared to control group; #P<0.05 − compared between Group 1 and 2.

The fast wound healing in CuNPs-treated could be explained due to copper's angiogenic and mitogenic effects, its regenerative properties towards the skin, and reestablished healing of wounds by enhanced cell metabolism and proliferation [30,31]. Once inside the skin layer, copper is involved in the synthesis and stabilization of extracellular matrix skin proteins, including stimulation of dermal fibroblast proliferation [32,33], formation of granulation tissue and higher new blood vessels [34], upregulation of collagen (types I, II, and V) and elastin fiber components (elastin, fibrillins) production by fibroblasts through the induction of TGF-β [35,36]. Furthermore, copper accelerates the healing process by induction of vascular endothelial growth factor (VEGF) and angiogenesis by hypoxia-induced factor-1-alpha (HIF-1α) action. Copper enhances HIF-1α expression and HIF-1α binding to the critical motifs in the promoter and putative enhancer regions of HIF-1-regulated genes [37].

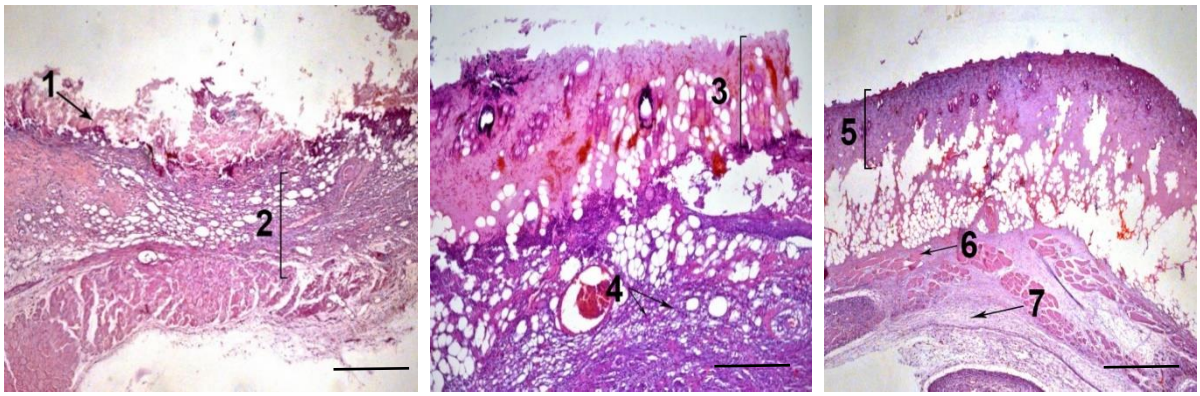

Figure 4. Images of wounds in rats 12 days post-treatment: a) control group, untreated burns; b) Group I, burns treated with Copper Nanoparticles; c) Group II, burns treated with Copper Nanoparticles Nanoemulsion. Hematoxylin and eosin stain. The tissue structural elements are numbered in the following order: 1 – epidermis (arrow indicator); 2- inflammatory infiltration in the scar; 3- scab; 4 - inflammatory zone with leukocyte infiltration (arrows); 5 - desquamation scab; 6 - epithelization (arrow); 7 - deposit of immature collagen fibers (arrow). Scale bars: 50 µm.

Combining such properties makes copper a highly promising active material for improving skin wound healing. Similar highly promising results were already reported for chitosan-based copper nanoparticles. These nanoparticles showed similar accelerated wound

healing in the excision model of adult Wistar rats after chitosan-based copper nanocomposite treatment [38]. Wound healing under copper oxide-impregnated treatment was significantly faster ($P<0.01$) than the dressing without copper or with a dressing containing silver [39]. Furthermore, phenytoin-loaded copper nanoparticles accelerated epidermal regeneration and stimulated granulation and tissue formation in the excisional rats' wound model [40].

When analyzing histological samples of healed tissue of the control group and experimental groups, several differences were observed (Figure 4).

The scab contained some necrotic tissue in the control group, and normal keratinization was observed. Burned surface partially recovered the epithelial layer, whereas the reticular layer contained more pronounced fibrosis with increased fibroblast activity. Furthermore, hypervascular focal perivascular leukocyte infiltration was observed as well. In tissue treated with CuNPs, well-formed scabs were observed, and the epidermis contained vascularization in all layers, whereas these traits were of significantly lower level in the control group. Applying CuNPs in liposomal emulsion onto the wounds led to an apparent reduction of the wound surface area, desquamation of scabs, and formation of healthy well-healed skin structures.

Histological analysis of CuNPs-treated wounds confirmed significant progress in wound healing. Foremost, such an effect was observed in the group treated with CuNPs-NE. These results indicate that the nanoemulsion of CuNPs had a greater wound-healing effect after topical application compared to CuNP colloids. That could be due to improved antimicrobial activity [41,42] and the anti-inflammatory properties of copper itself [43].

One of the many significant advantages of CuNPs in nanoemulsion is the significantly shorter wound healing period in experimental groups. These composites were shown to have an accumulative drug effect and can easily penetrate the skin via the subcutaneous barrier and subsequently improve wound healing [44-46]. Local delivery of CuNPs in nanoemulsion enhanced its content in the superficial skin layers, leading to a faster wound contraction. All this is proof that CuNPs encapsulated in liposomal emulsions are an effective approach for improving cutaneous wound healing.

In summary, CuNPs have a higher antimicrobial activity than CuNPs-NE. Vice versa, Copper Nanoparticles Nanoemulsion exhibited greater healing efficiency than CuNPs colloid. Therefore, encapsulation of Copper Nanoparticles in nanoemulsion improved the therapeutic effect of copper and promoted faster wound healing.

4. Conclusions

Evaluation of in vitro antimicrobial activity of CuNPs showed superior activity to that of CuNPs-NE. Overall, strong antibacterial properties of CuNPs and CuNPs-NE against *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *B. subtilis*, *S. aureus*, and *C. albicans* were observed. However, wound contraction was significantly higher in CuNPs-NE treated rats on 6, 8, 10, and 12 days of the study compared to the control and CuNPs treated group. Also, the diameter of the burns area during post-burn treatment was significantly reduced in the group treated with CuNPs nanoemulsion compared to burns treated with copper nanoparticles in an aqueous solution. In addition, the histological analysis confirmed a greater wound-healing effect of the nanoemulsion of CuNPs. Therefore, encapsulating Copper Nanoparticles in nanoemulsion can improve its therapeutic effect and promote faster wound healing.

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Conflicts of Interest

The authors declare no conflict of interest. The funders did not participate in the study's design, in the collection, analysis, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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