RESEARCH ARTICLE

Preclinical Evaluation of Silver-Curcumin Nano- gel : A complete assessment on a new topical antimicrobial product for burn

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ABSTRACT

Nanosilver and nanocurcumin are popular nonmaterial with increasing public attention, but their synergistic therapeutic potentials in burns have never been considered. The present study aimed to provide a novel formulation from both nanomaterials (Ag – Curcumin NPs) and conduct their preclinical evaluation for burn healing. After evaluation of particle size, loading efficiency, release profile and morphology of manufactured nanoparticles by TEM and DLS techniques , a 14 days skin irritation and corrosion test on Albino rabbits was performed based on OECD 404 guideline . The Ames Mutagenicity test was performed on 4 strains of *Salmonella* Typhymurium after MIC and MBC adjustments in different doses. For clinical efficacy, skin burn model was designed and applied in rats by providing limited standard 3rd degree burns at the back of each rabbit. Manufacture lyophilized spherical nanoparticles in the range of 20- 38 nm, with low zeta potential didn't show any significant size enlargement. The nanogel was also considered as a nonirritant and non-corrosive formulation after short term and long term dermal applications (>72 hrs.) .No mutagenic effects were also identified in all strains in the test samples of Ag- Curcumin NPs (Mutation Index=0.08-0.27). This study clearly showed the safety of Ag – Curcumin NPs nanogel in low concentrations with small dimension (16-32µg/ml). Due to the safety of proposed formulation and increased rate of wound healing by Ag-Curcumin nanogel in comparison to both control groups, this combinational formulation could be considered as a safe and effective candidate for further clinical applications.

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INTRODUCTION

For the last decade, multidrug resistance has become a major concern for both gram-positive and gram-negative bacteria [1], the number of effective antibacterial molecules has dramatically decreased and antibiotic resistance has become globally a critical challenge in communities with many health problems [2]. Most of pathogenic microbial organisms have been changed into resistant variants without enough sensitivity to antimicrobial drugs[3] but the potentials of nanopharmaceuticals are still remained a promising powerful tool for the destruction of germs by killing or inhibiting the growth of bacteria, fungi and viruses [4].In fact encapsulation of antimicrobial drugs using nanotechnology has emerged as an innovative and promising alternative to existing methods that enhance the therapeutic effectiveness of nan formulations and possibly minimizes the undesirable side effects of existing pharmaceuticals[5] . Even though the efficacy and toxicity of these Nanoformulations should be well

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established because inefficient delivery could result in an inadequate therapeutic index and local and systemic side effects, including cutaneous irritation and corrosion, peeling, scaling, gut flora reduction and many other systemic unrecognized effects[6] .

Silver with efficiency against more than 650 pathogens has been newly considered as one of the most valuable alternatives to antibiotics because of its exciting potential to solve the problem of multidrug resistance [7] as well as the magic silver nanoparticles properties to enhance the therapeutic effects of other antimicrobials, allowing its use in a wide range of applications[8] . On the other hand, in recent years, considerable interest has been focused on curcumin, which is an isolated compound from turmeric. Its wide range of biological properties and its low toxicity even in high doses, makes it attractive for various disorders like tumors of skin, colon, duodenum, pancreas, breast and other skin diseases. Extensive research over the last 50 years has demonstrated that these polyphenols play important roles in the maintenance of human health and prevention of many diseases such as cancer, inflammation- based disease, skin diseases like scleroderma, psoriasis and skin cancer. Curcumin protects skin by quenching free radicals and reducing inflammation through nuclear factor-KB inhibition[9] . Curcumin treatment also reduces wound-healing time, improves collagen deposition and increases fibroblast and vascular density in wounds thereby enhancing both normal and impaired wound-healing[10] .

Curcumin has also been shown to have a beneficial effect as a proangiogenic agent in wound-healing by inducing transforming growth factor-beta, which induces both angiogenesis and accumulation of extracellular matrix, which continues through the remodeling phase of wound repair[11] .Indeed, the nanomicelles of curcumin are known as biological safe, nongenotoxic, noncytotoxic agents[12,13]which are widely used to enhance the pharmaceutical impact of other drugs from the efficacy and bioavailability viewpoints[14,15].

Based on the present background, we hypothesized a study to combine nanocurcumin and nanosilver in a novel semi- solid formulation as a promising strategy for burn healing. After the initial formulation and characterization, we have decided to evaluate its efficacy, toxicity and mutagenic properties in a preclinical setting for possible further clinical applications.

MATERIALS AND METHODS

Materials

Curcumin powder was prepared by Sami Lab, India .Nano silver colloidal solution in sizes of 10-20 nm was purchased from Nanozino Lab., Iran, which was prepared based on the reduction reaction of silver nitrate by sodium borohydride. Trinatrium citrate as stabilizing agent and all other ingredients for preparing phosphate buffer solution were purchased from Merck, Germany.

Preparation of SLNs

Curcumin loaded solid lipid nanoparticles (SLNs) were prepared using a high- pressure homogenization method[16] . In brief, active substances were dissolved / dispersed in water and tween 80 was added. In parallel side, hot oily phase was prepared by cholesterol / stearic (1:1 volume ratio) and ethanol/acetone (3:1 volume ratio), and the oily phase was added to the water phase under homogenization (13500 RPM). SLNs were prepared during homogenization and cooling of the mixture to room temperature. Prepared SLNs were lyophilized using 5% mannitol as cryoprotectant. Particle size, morphology, zeta potential and drug loading efficacy as well as drug release profile, were investigated after freeze- drying too [17] .

Synthesis of Ag- Curcumin NPs gel

1 gr carbomer (934) was added slowly to distilled water (q.s 100ml), then 2 and 3g olive oil and vitamin E was added under mixer, after obtaining a soft gel texture, 250 mg of freeze- dried curcumin SLNs and 1.6 ml of silver NPs (10nm) were added to gel very slowly to prevent the formation of air bubbles and mixed until complete homogenization and loading efficiency was checked finally.

Skin Irritation and Corrosion Test

Animal studies

Animal Housing and Maintenance for safety studies

Female rabbits were individually housed in separate quarters in solid bottom cages. Individual animals were identified by color coding, the animal number and group number also appeared on the outside of each cage to preclude mix-up. The animal room environment was controlled (targeted ranges: temperature 22°C to 25°C, relative humidity 30-20%) and monitored daily. The photo-cycle was 12 hours light and 12 hours dark. Upon arrival, all animals were submitted to a general physical examination

and all healthy ones were admitted for this study. Diet and water were offered ad libitum throughout the acclimatization and study periods. The cage cleaning schedule, air filtration and recirculation, health checks and facility maintenance were carried out in accordance with the KIAU Standard Operating Procedures, and such activities were recorded in the animal room records.

Animal Selection/Randomization

The test population of animals was selected from newly arrived animals and the method of randomization was based on the random selection of numbers generated from a set of numbers without replacement.

Application of the test substance

An acute skin irritation and corrosion study of the test article, Curcumin and silver solid lipid nanoparticles (SLNs) were carried out for topical application. The study was conducted according to OECD 404 protocol, (Acute Dermal Irritation and Corrosion Test) [18] . The test substance was applied in a single dose to the skin of the first rabbit and untreated skin areas of the test animal served as the control. The degree of irritation/corrosion was read and scored at specified intervals and was further described in order to provide a complete evaluation of the effects. The duration of the study was sufficient to evaluate the reversibility of the observations.

The skin application of Ag- Curcumin NPs gel was applied by a small area (approximately 6 cm^2) of skin and covered using a gauze patch, being held in place with non-irritating tape. Animals were observed individually at least once during the first 4 hours after dosing, periodically during the first 24, 48, 72 hours (with special attention given during the first 4 hours), and daily thereafter, for a total period of 14 days. All observations were schematically recorded with individual records being maintained for each animal. All animals were examined for signs of erythema and edema, and the responses scored at 60 minutes, and then at 24, 48 and 72 hours after patch removal. Dermal reactions were graded and recorded according to the grades in the Table 3.

Bacterial Reverse Mutation Assay (Ames test)

Microorganisms

The *Salmonella typhimurium* strains used in this study were TA100, TA98 and YG1029, YG1021 a bacterial O-acetyl transferase - overproducing strain of TA100. The YG1029 and YG1021 were cloned and its activity was first described and established by Prof. T, Naomi and colleagues [19] who provided all strains as a gift, from Biological Safety Research Center Co, Ltd in Tokyo-Japan in 2012.

MIC and MBC levels

We determined MIC value as the lowest concentration of Ag - Curcumin NPs which prevents bacterial growths. Appropriate concentrations of each Ag - Curcumin NPs, samples were determined according to their working manual, in the range of 0.015 to 31.25- µg/ml. The MIC value was considered as 99% bacterial growth inhibition concentration and the MBC value was considered as the concentration with 100% inhibitory properties in comparison to the negative control.

Bacterial reverse mutation assay

The pre-incubation Ames assay was performed according to the method of OECD 471 [20-24].Negative response was defined as no concentration- related increase in the number of revertant colonies and distilled water was used as negative control in this study [25].Before starting the main experiments, the mentioned strains were checked for their genetic integrity by histidine/ biotin dependence, rfa marker (crystal violet) and the presence of plasmid pKM101 (Ampicillin resistance) tests. During all these preliminary experiments, the strains were grown overnight in nutrient broth for 16-18h in incubator at 37°C with a density of 1 - 2 × 10^8 (CFU) ml in presence of 25 µg/ml Ampicillin for TA98, TA100 and 10 µg/ml Tetracycline for YG1029 and YG1021.At the first step MIC and MBC of the mentions strains were determined with different concentrations of (Ag-Curcumin NPs). The concentration of Curcumin-Ag NPs used in this study was 0.0015 to 3.12 µg/ plate. The top agar was prepared by dissolving 0.6 g of agar-agar and 0.6g NaCl in 100ml distilled water and sterilized aqueous solution of L- histidine and D-biotin (0.5mM/L) was added to the top agar medium immediately before applications [20] . In the next step, 100 µl of overnight cultured bacteria with concentration of 1-2×10^8 CFU/ ml were incubated at 37°C for 45 min in a sterile glass tube containing 500 µl sodium phosphate buffer (0.1 M, pH7.4) with different concentration of drug samples (Ag- Curcumin NPs in concentration based of MIC). After incubation, 2 ml of Top agar

supplemented with histidine / biotin (kept in 45 °C water bath) and added to the mixture and mixed for 3 seconds using a vortex mixer, then poured on a plate of GMA (minimal glucose agar media) [20] . Plates were incubated for 48-72 h at 37°C and the revertant colonies were counted. Three equal plates were used for each concentration and each experiment was repeated three times to get maximum accuracy and reproducibility for this sensitive method [21] . Sodium azide was used as positive control for TA100, YG1021 and YG1029 strains and 2-Nitrofluorene for TA98 strain. Distilled deionized water was used as negative control. The Mutagenic Index (M.I) was described for each assay and calculated as the ratio between the number of histidine revertants induced per plate of the test sample and spontaneous revertants of the negative control. M.I. for no mutagenic potential assumed as ≤ 1-1.6), possible mutagenic potential assumed as 1.7-1.9 and mutagenic potential assumed as ≥ 2 [25].

Animal burns, skin model and burn healing

Adult male Wistar rats, weighing 220–250 g were obtained from Razi Institute. These animals were housed in standard animal house which described above. The animals were anaesthetized with an intra-peritoneal injection of Ketamine (80 mg/kg) and xylazine HCL (10 mg/kg). The skin of dorsum was shaved and cleaned with 70% ethanol. An experimental skin burn model was induced in rats by the Edraki method [22] . Second-degree burn wounds on the dorsum of the animals were exposed to ironic cylindrical devices with a surface of 2.5 cm^2 for 10 seconds. The devices were heated in boiling water (98 ± 1 °C) at least, just 20 minutes before application to induce burn injury. Rats were randomly divided into either control or experimental groups. The control group did not receive any treatment and experimental groups were subdivided into 5 groups as follows: Group 1 received topical gel, Group 2 as positive control received burning ointment (Silver Sulphadiazine without nanoparticles), Group 3 and 4 received solution of curcumin NPs, silver NPs respectively and Group 5 as negative control received no treatment. Each rat was treated with equal volumes of topical formulation, once daily with regards to the assigned group and continued until 12 hours before sample collection.

In order to collect samples for histopathology examination, the animals were euthanized at 7 and 14 days after inducing burn injury. These samples were preserved in 10% formalin for light microscopic study. Photography was also performed until the 21th day of the study.

Histological assessments

Tissue samples of burn wounds were immediately fixed in 10% formalin histopathological analysis. Sections were embedded in paraffin and the blocks of 5 µm were prepared and stained with hematoxylin and eosin (H&E) for histopathological examination by light microscopy. All skin tissues were examined in a blinded manner by a pathologist. Inflammation, collagen deposition, angiogenesis, granulation and epithelialization were graded as 0-3 in each section [23] . The burn wounds surface area, were photographed at the 0, 5, 11, 14, 18 and 21th days of study using a digital camera; then, the healing rates were compared with the wound area of each control every day with the wound area in the first day[24] .

RESULTES

Characterization of nanoparticles

Particle size and Morphology study

· As we described in Fig 1, Dynamic Light Scattering (DLS) analysis showed lack of large agglomeration of the Ag NPs and TEM results showed the triangle Ag NPs nanoparticles (Fig.1). Electron Microscopy (SEM) (TEM) (FESEM), showed spherical shapes of curcumin loaded SLNs which were described in Fig. 2.

· Particle size studies demonstrated that particles were 38 -58nm before freeze- drying, but the particle sizes of nanoparticles were increased to 68-92 nm when 5 and 15% mannitol was applied as cryoprotectant, respectively. Therefore, 5% mannitol was chosen as optimum cryoprotectant for further experiments[24] . Particle size studies were established at desirable size distribution before and after freeze-drying which was described in Fig. 3.

· Zeta potential (mVpH) of AgNP and curcumin were adjusted at -5.83 mV and less than −33.6 respectively which provided good colloidal stabilities. According to SEM imaging, prepared SLNs showed the spherical shape and the size distribution of particles was comparable to DLS. The prepared SLNs had 82% loading efficiency and 84% of loaded curcumin was released after 48hours.

Skin Irritation and Corrosion

All skin reactions were recorded in 3 min as well as in 1, 4, 24, 48 and 72 hours from the application

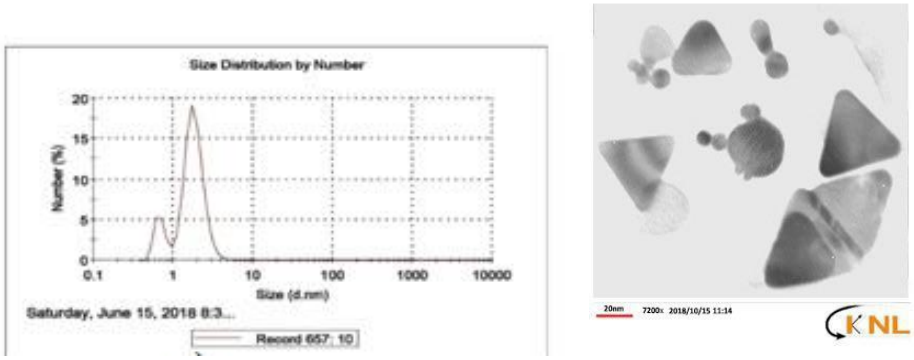

Fig. 1. Ag NPs by DLS, and TEM

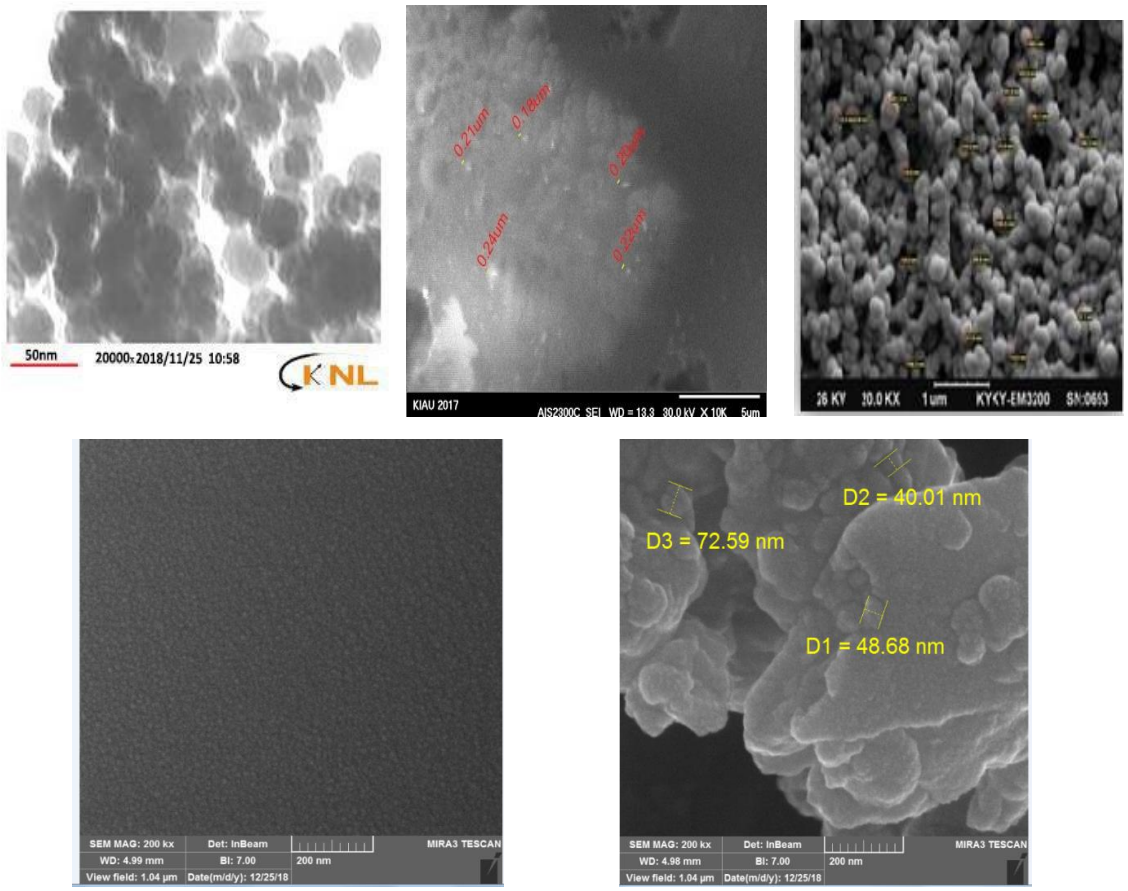

Fig. 2. FESEM, TEM, SEM Curcumin NPs

and compared with the control group and followed up for 14 days. In all rabbits Curcumin – Ag NPs gel didn't show any sign of toxicity after the first 1 hour from intervention. Very slight erythema (barely perceptible) from dermal application was shown after 24 h which were disappeared before 72 hours from first contact. In long term dermal exposures no sign of erythema and dryness were observed in both groups of cases and controls. The mild signs of toxicity were recovered to normal appearance after the removal of the patch in case groups (Fig. 4). Although the total score was 3 in this rabbit, the hairs grown naturally without any abnormal observation. At the end of the test,

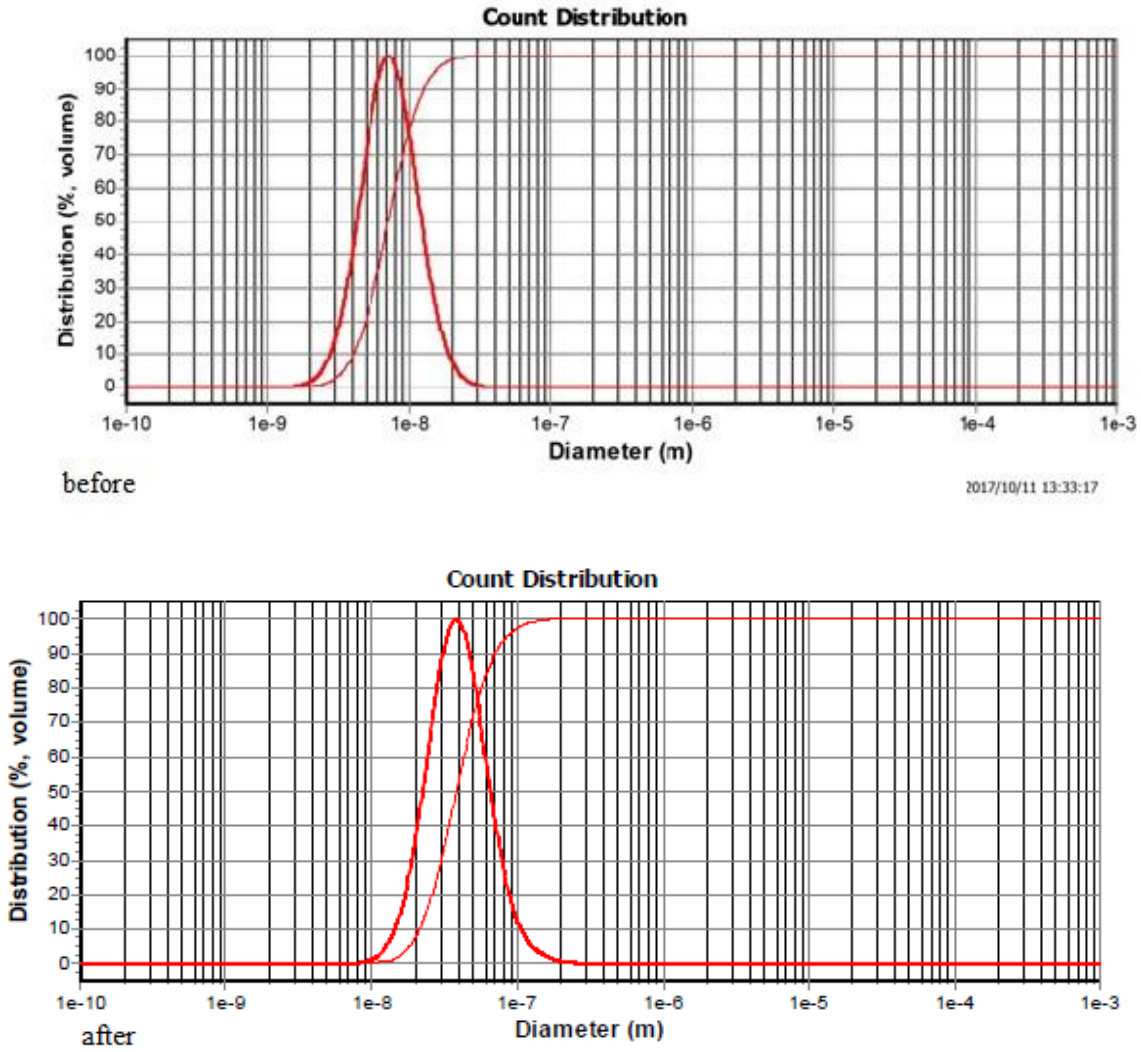

Fig. 3. Nano Curcumin, before and after freeze drying, respectively

surviving animals were weighed and compared. No significant weight changes were detected in this test (3923± 102). Based on the foregoing results, the SLNs samples didn't show any irritant and corrosive effects even after long term (14 days) exposures in treating animals when compared with control areas in Albino Rabbits.

Ames Mutagenicity Assay

MIC and MBC determination by broth serial macrodilution method

The inhibitory effects of different concentrations of Ag- Curcumin NPs on four Salmonella strains were examined. MIC and MBC in TA100, TA98, YG1029 and YG1021 were determined as 0.24, 0.24, 0.48 and 0.03µg/ml respectively.

Mutagenic potentials of Ag- Curcumin NPs by Ames tes

As described above, bacterial mutagenicity was assessed in *S.typhimurium* tester strain TA98 for detection of frame shift mutation, TA100 for measurement of base- pair substitution and YG1029, and YG1021 that was derived from TA100, with the enzymatic activity of O-acetyl transferase for its point mutation capacities via metabolic activation.The results of this experiment showed lack of mutagenic effects in all 4 evaluated strains which were described in details in Table 1.

Wound burn healing

After applying these formulations to the burned skin, the wound healing process was studied for 14

Fig. 4. Treated region in Rabbits after 24, 48, 72 hours from exposure to Nano Curcumin- AgNps and control region without any exposure.

Table 1. Ames Mutagenicity Assessment on Curcumin – Ag NPs

MI (SEM±Mean)		Number or His+ revertant colonies/plate (SEM± Mean)	NC (SEM±Mean)	PC	Strains
0.1 – 0.3		49 ± 16.3	189 ± 63	>800	TA98
0.08 – 0.16		31 ± 77	32 ± 10.6	>800	TA100
224 ±	>800	0.1 – 0.3 YG1024	55 ± 18.3 220 ± 73.3	>800	YG1029
			0.1 – 0.25	46 ± 15.3	74.6

[1]Note: Above data represents the mean number of revertant colonies ± SEM from three independent experiments, each repeated three times.
[2]Note: P. C: Positive Control; N.C: Negative Control; *Mutagenic Index (M.I.) = (The number of revertant colonies in the sample plate) / (The number of colonies in the negative control plate)

days. Rate of healing based on wound appearance and the diameter was evaluated in comparison to negative and positive controls. Results showed clearly that the healing rate could be accelerated by the application of Ag – Curcumin nanogel. Different parts of Fig 5 (a, b, c, d, e) show the wound healing procedure after using formulations in comparison to the other two control groups. The pathological studies according to Abramov's scoring system (Table 2), confirmed the above results (Table 3) and Figs. 6 (a, b, c, d, e).

DISCUSSION

The topical application of stable ,safe and effective dermal antimicrobial agents is an urgent issue in burn healing because the patients who are suffering from different burn stages are at higher risk to both local and systemic infections moreover a large group of current antimicrobial agents are practically unable to overcome the risk of recurrent infections due to the presence of multi – resistant pathogens .In fact the high prevalence of biofilm producers and multiresistant isolated microorganisms indicates the necessity of prevention programs to avoid the risk of infection in highly susceptible patients[1] .This issue could be considered as one of the major concerns for governmental and nongovernmental health promotion activities with participation of every stakeholder from policy makers down to basic researchers with clear communication to develop new strategies for resolving this problem[3] . Due to the urgent needs for developing new bactericides, fungicides and viruses[25] and due to the recent separate studies on excellent antibacterial activities of AgNP and nano curcumin[2] , their possible synergistic effects , this study aimed to development of a composite material for burn wound healing containing nanosilver nanogels along with nano curcumin with maximum efficacy and safety, to promote antimicrobial strategies in burn wounds, wound healing and infection control and on the basis of our animal developmental findings , this curcumin loaded, silver solid lipid nanoparticles (SLNs) can be employed as the next generation of nano-adhesives for rapid wound closure and aesthetic burn wound healing[26] .

Synergistic interaction of Ag- Curcumin NPs has been considered as an effective strategy against both gram-negative and gram-positive bacteria, which are causative microbes to be controlled in animals with otitis external [12]. The combined effect of old traditional medicinal ingredient Curcumin and nano sized silver nano particle is worth using these films on an industrial scale as wound dressings [13]. The synergy between silver 5 nanoparticles functionalized by curcumin has remarkable antiviral activity against RSV infection for the first time [15].These formulations could be administered either directly as solution to produce rapid alleviation in bacterial infections or as a coating on endotracheal tubes to achieve prolonged, sustained antibacterial effect[24] .

These studies suggest the beneficial effects of synergy between nano (silver - curcumin) and the potential of this compound to be developed as a potent nontoxic agent for treating skin diseases such as infections and burns.

In this research, we analyzed the toxicity potentials of Ag- Curcumin NPs gel by dermal application for the first time. Based on the foregoing results, Ag- Curcumin NPs showed any sign irritant effects even after long term (>72 hrs.) in all of test groups treated animals, when compared with skin without any exposure, according to the OECD scoring method. The test article Ag- Curcumin NPs is considered to present significant irritant and not corrosive effects after instant dermal exposure.

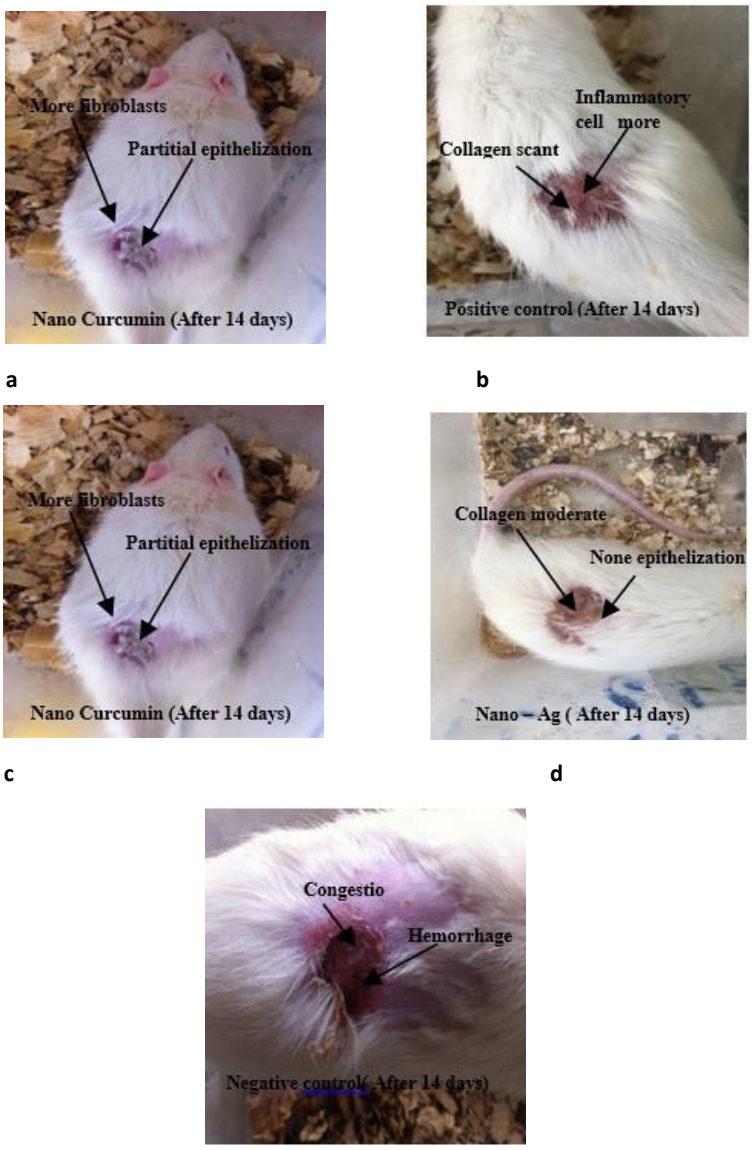

Fig. 5. a:Group 1 skin condition after 14 days, b: Group 2 skin condition after 14 days. c: Skin condition of Group 3 after 14 days, d: Group 4 skin condition after 14 days, e: Group 2 skin condition after 14 days.

Table 2. Wound evaluation (histopathologic aspect) according to Abramov's scoring system

Parameter /HPF	Grade 0	Grade 1	Grade 2	Grade 3
Epithelization (0-3)	**none**	**partial**	**complete, but thin or immature**	**complete and mature**
Angiogenesis (0-3)	**none**	**up to 5 vessels /HPF**	**6-10 vessels/HPF**	**more than 10 vessels/HPF**
Fibrosis (0-3)	**none**	**few fibroblasts**	**more fibroblasts**	**predominant fibroblasts**
Collagen (0-3)	**none**	**scant**	**moderate**	**abundant**
Inflammatory cell (1-3)	-	**1-22/HPF**	**26-50/HPF**	**>51/HPF**

Table 3. The pathology wound healing on 5 groups after 14 days

Parameter/HPF / Grade	Gel Curcumin-Ag NPs	Positive control	Nano curcumin	Nano - Ag	Negative control
Epithelization	**2**	**1**	**1**	**0**	**0**
Angiogenesis	**2**	**1**	**1**	**1**	**0**
Fibrosis	**3**	**2**	**2**	**1**	**0**
Collagen	**2**	**2**	**1**	**2**	**0**
Inflammatory cell	**0**	**3**	**1**	**0**	**2**

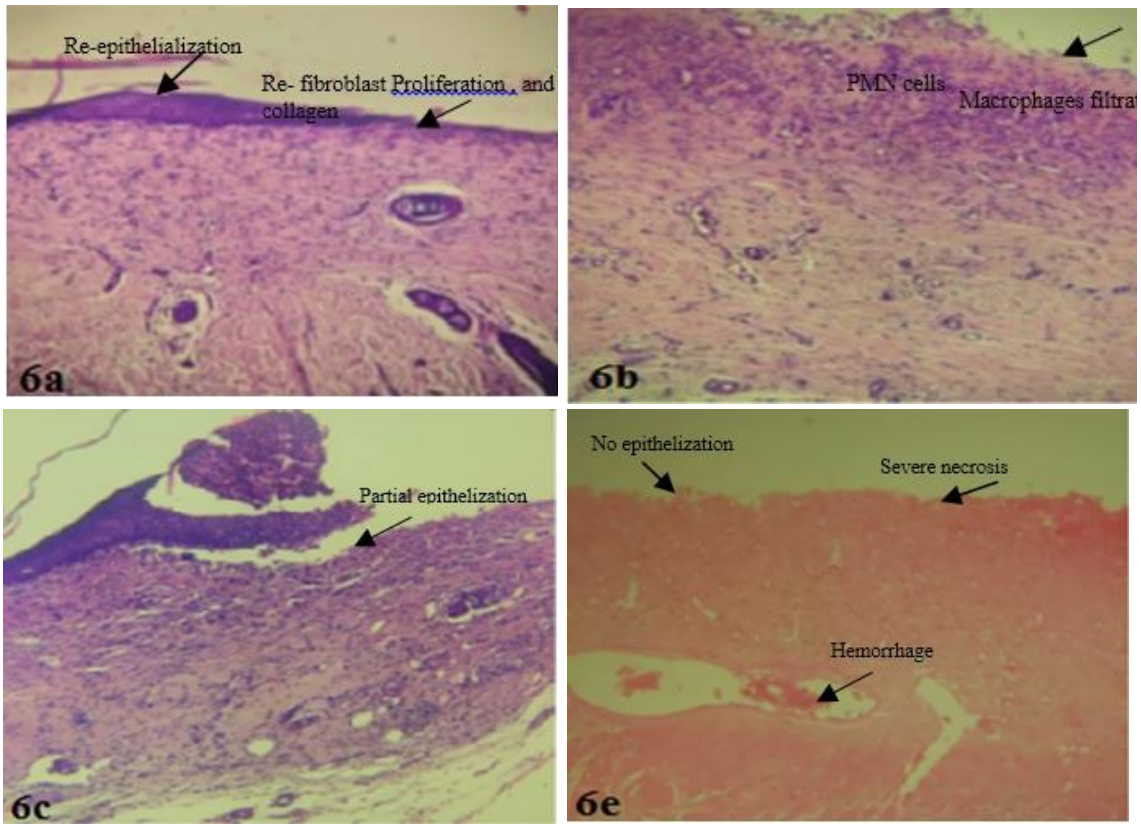

Fig.6. a: belongs+ to Group 1 after 14 days applying gel formulation and shows tissue healing with grade 1, re-epithelialization, fibroblast proliferation, and collagen fiber. (mag :200×). b: belongs to Group 2 after 14 days applying of burn ointment without nanoparticles shows Severe PMN cells and macrophages infiltration were seen in wound area : (mag:100×). c: belongs to Group 3 after 14 days applying curcumin NP_s shows partial epithelization in wound area(mag:100×) .d: belongs to Group 4 after 14 days applying Ag NP_s and shows grade 1 of angiogenesis in wound area(arrows) (mag:400×). e: is belonging to Group 5 In which no treatment was applied, after 14 days revealed no epithelization severe necrosis of epidermis , dermis and subcutaneous tissue with hemorrhage and congestion. (mag:100×) is obvious.

Our research also focused on genotoxic effects of Ag- Curcumin NPs, which can be utilized in biomedical research, pharmaceutical products and environmental cleaning applications[7,15] . Despite the widespread use of AgNPs in a wide range of biomedical products as a new group of health products, there are numerous knowledge gaps regarding their possible toxic potentials especially their mutagenic effects in humans [27].We described previously systemic toxic effects of Ag- Curcumin NPs through dermal application in animal models[28,29], but limited studies on genotoxic effects of Ag- Curcumin NPs with dermal exposure has motivated us to continue our toxicity evaluations

in this new area and this new bacterial setting for nanomaterials. We defined three distinctive test groups of albino rabbit and compared each one with negative control.

Ames test results on Ag- Curcumin NPs showed lack of any mutation in TA100, TA98, YG1029, YG1021, *S. typhymurium* strains. In addition Ag-Curcumin NPs gel reduced the mutation rates in all four strains in a concentration- dependent manner. The combination of curcumin and silver nanoparticles was evaluated as wound dressing materials in animal wound models. The application of the dressings showed significant improvement in wound healing. These two nanoparticles have synergistic antibacterial effect together. It seems that using nanotechnology could help to increase rate of wound healing.

This finding creates a new issue in the possible effective medicine for grade 2 burns of Ag-Curcumin NPs as a new pharmaceutical product which should be considered in future studies by focusing on the physicochemical properties. The consequence of this article is to confirm Ag-Curcumin NPs as a new and potent medicine to treat an intense microbial infection .To the best of our knowledge, the present study is one of the first studies about determination of cytotoxicity of Ag-Curcumin NPs in combination manner.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this

Manuscript.

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