

DISCUSSION

Diabetes is a multisystem disorder and complications of diabetes induce physiological changes in tissues and cells that impair the normal healing process. The pathophysiological relationship between diabetes and impaired healing is complex. The diabetic wounds get stuck in the inflammatory phase featured by continuing influx of neutrophils that release cytotoxic enzymes, free radicals and inflammatory mediators that cause extensive collateral damage to surrounding tissue. These destructive processes outbalance the healing process in such wounds and overproduction of free radicals that induce oxidative stress results in detrimental cytotoxic effects causing delayed wound healing.⁽²¹²⁾

Studies on wound healing have increased our knowledge and understanding of wound, which constitute an important clinical problem in rehabilitation medicine. Most studies concerning wound healing focus on accelerating wound and soft tissue healing, obtaining normal wound breaking strength and preventing keloid and scar formation. Some researchers found that some physical methods as constructive and adjunctive niches in facilitating and accelerating wound healing, and also improving scar quality, they include; therapeutic ultrasound, laser treatments and electro-stimulation.⁽⁷⁷⁾

Therapeutic ultrasound has been widely used over the past 50 years to treat many musculoskeletal complaints.

Low-intensity laser therapy has gained considerable recognition and importance among treatment modalities for various medical problems including wound repair processes, musculo-skeletal complications, and pain control.⁽²¹⁴⁾

The cutting-edge combination of nanotechnology with medicine offers the unprecedented opportunity to create materials and devices at a nanoscale level, holding the potential to revolutionize currently available macroscale therapeutics. Nanotechnology already provides a plethora of advantages to medical care, and the success of nanoparticulate systems suggests that a progressive increase in the exploration of their potential will take place in the near future. Nanotherapies represent a great opportunity to enhance currently available medical treatments, improving standard care and prognosis for challenging healthcare issues, like impaired wound healing.

Factors delaying wound healing include either preexisting comorbidities (diabetes, chronic peripheral vasculopathy, and immunosuppression), leading to lack of appropriate metabolism and clearance from toxic substances of the wound, and/or sudden complications, such as infections, exalting the inflammatory state. Nanobiotechnology combined with the knowledge on cellular and subcellular dysfunctional events occurring in delayed wound healing offers great opportunities for improving wound care

In an effort to provide a more clinically relevant wound-healing model, we investigated the effect of therapeutic ultrasound, laser photostimulation in comparison with dressing with silver NPs and CA nanofibers loaded with Ag NPs on diabetic wound healing. The experimental observation (rate of contraction), the histopathological examination, and the mechanical properties results from this study clearly indicate that the

use nanomaterials (Ag nanoparticles and nanofibers loaded Ag nanoparticles) facilitate diabetic wound healing.

In the present study, different types of nanomaterials (silver nanoparticles, cellulose acetate nanofibers and cellulose acetate nanofibers loaded with silver nanoparticles) were prepared and characterized to be used for wound healing in induced diabetic mice in comparison with physical therapy using laser photostimulation and therapeutic ultrasound.

Silver nanoparticles were prepared using chemical reduction method⁽²⁰⁶⁾, using trisodium citrate which act as reducing and capping agent. The obtained Ag NPs were characterized using UV-Vis spectroscopy, TEM and X-ray diffractometry.

The color transformation to yellow is the indication of Ag nanoparticles formation, change in color is due to excitation of surface Plasmon vibrations in the metal nanoparticles⁽²¹⁵⁾ which is confirmed by measuring the absorption spectrum of silver nanoparticles using UV-Vis spectroscopy as shown in figure (24) which revealed the characteristic peak at 430 nm that clearly indicate the of formation silver nanoparticles.

It is observed from transmission electron micrograph that most of the Ag nanoparticles were spherical in shape and homogeneously distributed. There is a variation in particle sizes ranging from 4.32nm to 7.27 nm with an average size 5.79 nm.

X-ray diffraction is a very important technique that commonly used to analyze the characteristic and structural details of nanomaterials. The X-ray diffraction patterns are obtained by measurement of the angles at which an X-ray beam is diffracted by the crystalline phases in the specimen. The XRD pattern of synthesized Ag-NPs showed three prominent peaks at 38° (2θ) and 42° (2θ) and 68° (2θ) indicated the presence of (111), (200) and (220) sets of lattice planes and further on this basis of they can be indexed as face-centered-cubic structure of Ag NPs. Hence from the XRD pattern it is clear that silver nanoparticles formed using trisodium citrate reduction were essentially crystalline in nature.

Silver nanoparticles are chosen in this study because silver is a powerful natural antibiotic being used since ancient times for the purpose of wound healing. When silver comes in contact with microorganisms, it leads to sudden distortion of cell wall which later on causes death of these organisms.⁽²¹⁶⁾ Surprisingly, there are minimal chances for development of bacterial resistance due to immediate death of microorganisms upon contact with silver ions. The mechanism for development of minimal resistance against silver has been well documented⁽²¹⁷⁾. This ability to create minimal or no resistance in microorganisms can lead them to replace impotent antibiotics.⁽²¹⁸⁾

It can be expected that the high specific surface area and high fraction of surface atoms of nanosilver shapes will lead to high antimicrobial activity compared to bulk Ag metal. Recent, microbiological and chemical experiments implied that interaction of silver ion with thiol groups played an essential role in bacterial inactivation^(219, 220) Surface area involves the increase of contact surface, which is an important condition for the effects of silver nanoparticles.

Cellulose acetate (CA), an acetylated derivative of cellulose polymer, is a good candidate for biomedical applications, because this polymer is hydrophilic, non-toxic,

biodegradable, and renewable with good process ability⁽²²¹⁻²²³⁾. Cellulose acetate contains a number of hydroxyl, ether, and carboxyl groups in the main backbone chains, making it highly ionic in nature and, consequently, it can be used as an excellent nanoreactor to synthesize various catalytic and functional metal nanoparticles on the polymer surface.⁽²²⁴⁻²²⁶⁾

Bringing materials to the nanometer scale not only improves their properties, but also affords it new advanced characteristics beyond bulk materials. Nanofibers, especially polymeric nanofibers, are promising for diverse applications, such as in drug delivery, tissue engineering and wound healing, due to a very large surface area to volume ratio, flexibility in surface functionalities, and superior mechanical performance. Among many approaches of fabricating nanofibers, electrospinning, which is also known as electrostatic spinning, is perhaps the most versatile process. Electrospinning has attracted much attention both to academic research and industry application because electrospinning can fabricate continuous fibers with diameters down to a few nanometers, is applicable to a wide range of materials such as synthetic and natural polymers, metals as well as ceramics and composite systems, can prepare nanofibers with low cost and high yielding.⁽²²⁷⁾

When the diameters of polymer fiber materials are shrunk from micrometers (e.g. 10–100 μm) to sub-microns or nanometers (e.g. $10 \cdot 10^{-3}$ – $100 \cdot 10^{-3}$ μm), there appear several amazing characteristics such as very large surface area to volume ratio (this ratio for a nanofiber can be as large as 10^3 times of that of a microfiber), flexibility in surface functionalities, and superior mechanical performance (e.g. stiffness and tensile strength) compared with any other known form of the material. These outstanding properties make the polymer nanofibers to be optimal candidates for many important applications. A number of processing techniques such as drawing, template synthesis, phase separation, self-assembly, electro-spinning, have been used to prepare polymer nanofibers in recent years.

The electrospinning technique has been applied to generate diverse nanofibrous textiles for various applications such as filters, skin masks, semipermeable membrane, clothing, and medical materials.⁽²²⁸⁾ Nanofibers have been applied towards medical uses such as drug delivery systems, tissue engineering scaffolds, vascular grafts, biological wound dressings, and support for the human body.⁽²²⁹⁻²³¹⁾

After the preparation of CA nanofiber and CA nanofiber loaded Ag nanoparticles, they were characterized using SEM, EDX, FTIR and the antibacterial activity of CA nanofiber loaded Ag nanoparticles was tested in vitro against Gram positive and Gram negative resistant bacteria.

Good quality nanofibers with smooth surface were obtained as proved by the SEM images presented in Figure (27). The morphology of the nanofibers shown in Figure (28) suggest that addition of silver nitrate to CA solution resulted in an increased solution conductivity and caused a slight decrease of fibers diameter, the presence of Ag NPs make the surface of nanofibers rough, as shown at high magnification (35,000X). The EDX analysis of the CA nanofibers loaded with silver nanoparticles shows a distinctive energy peak at around 3 keV, characteristic of silver as shown in Figure (29).

Fourier-transform Infrared spectra for CA nanofibers and CA nanofibers loaded with Ag NPs indicated that there are no major difference between the two samples in the major peak bands. However, it is also seen that the intensities of some peaks increased, this can

be attributed to the presence of Ag NPs. The absorption peak at 2922 cm^{-1} , suggest that the silver nanoparticles caused a reordering of CA chains by promoting the formation of peptide CH_2 links. ⁽⁸⁸⁾

As far as antimicrobial agents are considered, silver is considered material of choice, it is also established that silver enhances the epithilization. ⁽⁹²⁾ Silver and its forms are potent antimicrobials against various bacterial species. The exact mechanism how silver participates in distortion of bacteria is unfortunately still unclear. However, generally it is believed that sliver mainly makes denaturation and oxidization for cell organelles which lead to suppress the cell division.

It is well known fact when a critical amount of antibacterial compound (capable of inhibiting microbial growth) is in contact with bacterial strains the clear area near that contact is formed and these areas are referred as zones of inhibition. ⁽²³²⁾ Figures (32) and (33) shows 24 h incubated petri dishes of *E. coli* and *S. aureus* grown in presence of circular nanofiber disk. From this Figure we can clearly observe the inhibition zones around the circular nanofiber disks containing different percentages of silver (1, 3 and 5%), while as in case of pure CA (0%) nanofibers and standard antibiotics like Gentamycin and Ciprofloxacin (ABC), these inhibition zones were missing, which confirms the antibacterial property of CA loaded with Ag NPs against bacteria resistant to antibiotics. It is clear from the Figure that the antibacterial activity increased (zone of inhibition) as the silver loaded on nanofibers increased.

To study the growth kinetics of bacteria in the presence of nanofiber loaded with silver NPs, the synthesized CA NFs loaded with various concentrations of Ag nanoparticles were administered to *E. coli* and *S. aureus* to investigate the growth behavior of bacteria. It was noticed that, as the concentration of Ag NPs increased the growth inhibited. This clearly indicates that Ag NPs produced toxicity to *E.coli* and *S. aureus*. Nanofibers loaded with 5% Ag NPs was found to have the highest toxicity to the bacteria.

Ultrasound Therapy

Ultrasound is defined as a mechanical vibration above the upper threshold of human hearing ($>20\text{ KHz}$). Ultrasound can be produced in the form of continuous or pulsed waves. In the continuous mode, it is characterized by the production of biophysical and thermal effects, whereas in the pulsed mode it reduces the thermal effect due to the cyclical interruption of energy emission, while maintaining the biological effect. Accordingly, we used pulsed ultrasound (0.5 W/cm^2) 5 minutes daily, for 10 days. It has been suggested that the non-thermal effects of ultrasound, including cavitation and acoustic micro massage, are more important for the treatment of soft tissue lesions than the thermal effects. ⁽²³³⁾

Although the exact mechanism underlying its clinical effects is not known, therapeutic US has been shown to have a variety of effects at a cellular level including angiogenesis, leukocyte adhesion, growth factor and collagen production, and increases in macrophage responsiveness, fibrinolysis and nitric oxide levels. ⁽²³⁴⁾

The type of ultrasound used is dependent on the target tissues (structure and depth) and the intended effect (i.e. heating the tissues or not). Tissues with higher protein content (e.g. ligament and tendon) are able to absorb ultrasound to a greater extent than those with a low protein content (e.g. blood and fat). ⁽²³³⁾

In our study, ultrasound treatment has proved to be an effective modality supporting the treatment of full thickness wound in mice. Importantly, we have demonstrated that ultrasound of a power density of $0.5\text{W}/\text{cm}^2$ produces greater changes in the healing process in form of decreased wound dimensions than control group.

So the non-thermal effects of US, which are achieved at intensities of $<0.3\text{-}1\text{W}/\text{cm}^2$, are gaining interest. At these levels US produces two effects, cavitation and streaming. Cavitation is the formation of gas bubbles and streaming is a unidirectional, steady mechanical force. These effects cause changes in cell membrane permeability and thus the diffusion of cellular metabolites.⁽²³⁵⁾ Many laboratory-based studies have been undertaken to understand the effects of ultrasound on wound healing.

To date its effects include cellular recruitment, collagen synthesis, increased collagen tensile strength, angiogenesis, wound contraction, fibroblast and macrophage stimulation, fibrinolysis, and reduction of the inflammatory phase and promotion of the proliferative phase of healing⁽²³⁶⁾. Researchers believe that the acoustic stream flow, cavitation and the associated micro stream flow are the mechanisms responsible for the non-destructive changes in the structure and function of the cellular membrane which result in greater ion permeability of the membrane.⁽²³⁷⁾

Laser photostimulation

In an effort to provide a more clinically relevant wound-healing model, we investigated the effect of laser photostimulation on diabetic wound healing in mice. The experimental observation results from this study clearly indicate that low energy photostimulation with diode laser (650 nm, 150 mW) with a fluence of $5\text{ J}/\text{cm}^2$ 5 minutes daily, for 10 days facilitate diabetic wound healing.

Many studies^(8, 154, and 238) suggest that laser bio-stimulation occurs at fluences between 0.05 and $10\text{ J}/\text{cm}^2$, whereas fluencies above $10\text{ J}/\text{cm}^2$ have inhibitory effects in the impaired wound healing process. Many of the chronic complication of diabetes involve defects in connective tissue such as poor wound healing⁽⁸⁾. The wound healing abnormalities of diabetes results from several causes. When carbohydrates are unavailable to cells for normal aerobic metabolism, oxidation of amino acids for caloric needs results in amino acids and protein depletion. When glycogenolysis and gluconeogenesis fail to provide glucose to meet the energy requirements for fibroblasts and leucocytes, they become dysfunctional and impaired wound healing results. The poor wound healing of diabetic has been shown to be associated with a decreased amount of collagen fibrils and collagen production.⁽⁸⁾ This study demonstrates that low energy laser enhances wound healing in diabetic mice as evidenced by experimental observation.

The mechanism by which laser photo-stimulation facilitates collagen production in diabetic wound healing was not clear with the previous study. This effect may involve a variety of photo-stimulating mechanisms. It is mainly because the laser energy at certain frequencies can modulate cell proliferation and release the growth factors from fibroblasts. The other mechanism of photostimulation was that the mitochondria are the photoacceptors for light energy. The absorption of energy by the respiratory chain may cause oxidation of NADH, producing changes in the redox status in mitochondria and cytoplasm. The activation of electron transport chain results in an increase in the electrical potential across the mitochondria membrane, an increase in the ATP pool, and finally the

activation of nucleic acid synthesis. It also enhances the pro-collagen production, increased cross-linking of existing collagen molecules, acceleration of epithelial repair, and early growth of granulation tissue. ⁽²³⁹⁾

In vitro experiments support the hypothesis that low energy laser irradiation may accelerate wound healing. ⁽²⁴⁰⁾ Demonstration of increased collagen synthesis in vitro suggests that tensile strength of incisional wounds might be increased in treated wounds. Studies that show that He-Ne irradiation produce a massive transformation of fibroblasts into myofibroblasts ⁽¹⁴⁶⁾ suggest the possibility of an increased rate of wound contraction in response to laser treatment. Although these cellular and biochemical events are well documented, the application of this knowledge to wound healing acceleration has been frustrated by equivocal reports. Kana et al. ⁽²⁴¹⁾ succeeded in demonstrating accelerated healing in an incisional model, and found significant differences in an excisional model. Mester et al. ⁽¹⁵⁴⁾ has produced positive results in both models, whereas several authors have reported only negative results. Strikingly, both Braverman et al. ⁽²⁴²⁾ and Surinchak et al. ⁽²⁴³⁾ independently reported increased tensile strength in an incisional model, but failed to produce increased contraction rates in an excisional model.

It was found that the main biological effect of laser is due to the major absorbing structures. The major absorbing structures for the red visible laser wavelengths are the proteins; however, the identity of the photoreceptors responsible for the biological effects of low energy laser therapy (LELT) has not been resolved ⁽²¹⁴⁾. Several studies have suggested that either elements in the mitochondrial cytochrome system or endogenous porphyrins in the cells are the energy-absorbing chromophores in LELT. ⁽²⁴⁴⁾ The other mechanism could be due to the reason that, laser light affects the mitochondrial respiratory chain by changing the electric potential of cell membranes and consequently their selective permeability for sodium, potassium, and calcium ions or by the increasing the activity of certain enzymes such as cytochrome oxidize and adenosine triphosphatase. It also increases DNA synthesis, collagen, and pro-collagen production and may increase cell proliferation or alter locomotors characteristic of the cells.

Healing factor

By measuring the healing factors percentages for all treated groups, it was found that, the healing factor of different groups increase with increasing of the treatment period as shown in figure (46), Table (5), and the highest value obtained for diabetic mice treated with insulin and their wounds dressed with nanofibers loaded with Ag NPs.

Mechanical properties of skin of diabetic mice

Mechanical properties of skin of control and experimental groups are shown in Figure (46). It was difficult to measure the mechanical properties of the skin of the untreated diabetic mice because their skin was brittle. Wound dressing using nanofibers loaded with Ag NPs improve the mechanical properties of the treated skin of the diabetic mice. Marked improvement in wound strength and healing due to increase in formation of collagen fibers and activity of the epithelial covering in mice receiving STZ and treated with insulin in combination with dressing with nanofibers loaded with Ag NPs.

Histopathological examination

Animals receiving insulin and dressed with CA nanofibers loaded with AgNPs showed marked improvement in wound strength and healing due to increase in formation of collagen fibers and activity of the epithelial covering. For more conformation, histopathological examination of the healed skin was performed at 4th, 10th and 15th days of treatment as shown in Figures (49-66) in comparison with control skin (Figure 48).

By comparing results obtained from different treatment modalities, we found that all therapeutic tools facilitate and accelerate diabetic wound healing, and improving scar quality but with different degree, and the best method is to use nanomaterials (nanofibers in conjugation with antimicrobial nanoparticles) which act as scaffold.

Engineering dermal substitutes with electrospun nanofibres have lately been of prime importance for skin tissue regeneration. Simple electro spinning technology served to produce nanofibrous scaffolds morphologically and structurally similar to the extracellular matrix of native tissues. The engineered network has been shown to support cell adhesion, proliferation, and differentiation mimicking the fibrous architecture of the extracellular matrix

The large surface area and porosity of electrospun nanofibers enables good permeability for oxygen and water and the adsorption of liquids, and concomitantly protects the wound from bacterial penetration and dehydration. This feature shows electrospun nanofibers to be a suitable material for wound dressing, especially for chronic wounds such as diabetic ulcers or burns. The electro spinning technique can provide both degradable (collagen; chitosan) and nondegradable (PLA, polyvinyl alcohol (PVA) polymers) nanofibers for two-dimensional nanofibrous sheets. Both sorts of biomaterials have been tested in vivo showing an increased rate of wound epithelialization and dermis organization [ref], as well as good antibacterial activity against the Gram-positive and Gram-negative bacteria.⁽²⁴⁵⁾

Nanofibrous scaffolds of poly (L-lactic acid)-co-poly(ϵ -caprolactone) (PLACL) and PLACL/gelatin complexes were fabricated by Chandrasekaran et al.⁽²⁴⁶⁾ These nanofibres were characterized by fiber morphology, membrane porosity, wettability, and chemical properties by FTIR analysis to culture human foreskin fibroblasts for skin tissue engineering. The results showed that fibroblasts proliferation, morphology, and secretion of collagen were significantly increased in plasma-treated PLACL/gelatin scaffolds compared to PLACL nanofibrous scaffolds. The obtained results proved that the plasma-treated PLACL/gelatin nanofibrous scaffold is a potential biocomposite material for skin tissue regeneration.⁽²⁴⁷⁾