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Research Article

Novel PLA/ZnO Nanofibrous Nanocomposite Loaded with Tranexamic Acid as an Effective Wound Dressing: *In Vitro* and *In Vivo* Assessment

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Background: Chronic wounds contribute to the majority of clinical cases, associated with significant morbidity, and place a massive financial burden on healthcare systems. Thus, various bandage mats have been designed to facilitate wound healing in clinical applications. Polylactic acid (PLA) nanofibers, as suitable drug carriers, are highly desirable to prepare a controlled environment for wound healing in dressing tissue. Zinc oxide (ZnO) nanoparticles as an effective antibacterial agent for wound treatment prevent bacterial invasion and wound infection.

Objectives: In this project, for the first time, a new (PLA)/(ZnO) nanofibrous nanocomposite loaded with tranexamic acid (TXA) has been introduced as a useable dressing in wound healing. Furthermore, the antibacterial properties, coagulant assay, and wound healing assays of nanocomposite are evaluated.

Material and Methods: PLA/ZnO nanofibrous nanocomposites were loaded with tranexamic acid fabricated by electrospinning method at distinct concentrations. The prepared structure was characterized using field emission scanning electron microscopy (FESEM), energy-dispersive X-ray spectroscopy (EDS), and Fourier transform infrared spectroscopy (FTIR). Further, antimicrobial properties of tissue were investigated against *Escherichia coli* and *Staphylococcus aureus* bacteria. Also, the coagulation assays, *in vitro* cytotoxicity, and *in vivo* skin wound healing model in mice were evaluated.

Results: Morphological analysis of the prepared nanofibrous nanocomposites showed uniform bead-free nanofibers with an average size of 90 nm diameter. The structure exhibited proper antibacterial activities against *Escherichia coli* and *Staphylococcus aureus* bacteria, and a good blood clotting effect. *In vitro* cytotoxicity assay of the structure approved that this mat has no cytotoxic effect on human dermal fibroblast cells. *In vivo* wound healing examination in mice observed over 7 and 14 days showed a faster rate of wound healing over the control.

Conclusions: Novel electrospun PLA/ZnO nanocomposites loaded with tranexamic acid can be prepared by the electrospinning method and used for wound treatment. This structure displayed the effect of two agents in wound healing, including antibacterial nanoparticles and antifibrinolytic drugs to accelerate wound closure.

Keywords: Nanofibrous nanocomposites, Polylactic acid, Tranexamic acid, Wound healing, ZnO nanoparticles

1. Background

Nowadays chronic wounds are growing as a national and global problem which are widespread especially among diabetic patients (1). Also, chronic wound is persistent, thus it is under high risk of bacterial attack and represents a significant burden to patients (2).

Wound healing is a complex and dynamic process in which the skin and the tissues under it repair after injury. Thus, different materials are employed as dressings to improve wound healing and prevent bulk loss of tissue (3). Although the conventional cotton-gauge dressings help wound healing normally, some problems occur in these dressings. Modern wound dressing does not only cover wounds but they are also designed to facilitate wound healing. The two essential characteristics of modern wound dressing are rapid hemostasis and good antibacterial properties (4).

Nowadays, different types of new wound dressings such as films (5), hydrogels (6), and nanofibers (7) have been developed to enhance the wound healing process. Novel dressings have good permeability for moisture and oxygen, provide a moist environment, and promoted cell adhesion and proliferation (8). A wide range of biocompatible polymers such as polylactic acid (PLA), polycaprolactone (PCL), and polyethylene glycol (PEG) are used for wound treatment (9, 10). Polylactic acid is a biodegradable, biocompatible, and non-toxic polymer widely used in biological and medical applications (11). Electrospinning has been extensively explored as a beneficial and effective technique to produce nanofibers of polymers. Electrospun nanofibers can be used in many industrial and biological fields, especially in wound dressing (12, 13). Wound dressings, composed of electrospun nanofibers, display favorable properties for improving the wound healing process. Their three-dimensional construction mimics the structure of the skin extracellular matrix of normal skin, which plays a vital role in supporting cell adhesion and proliferation (14, 15). Further, the porous structure of these nanofibers is suitable for absorption of wound exudates and nutrients exchanges, while also preventing bacterial invasion and wound infection (16, 17). Polylactic acid nanofibers are an attractive candidate in wound dressing due to their high specific surface area, high porosity, and good permeability (18).

Antibacterial nanofibers are usually fabricated by incorporating drugs or antibacterial agents in polymers (19, 20). Zinc oxide nanoparticles (ZnO-NPs), as a bio-safe material with fascinating antibacterial properties, have attracted great interest worldwide (21). They are one of the best appropriate sources for wound healing applications by enhancing re-epithelialization while reducing inflammation and bacterial growth (22). Recently, some researchers have incorporated ZnO-NPs into polymeric nanofibers as multifunctional, antibacterial, and self-cleaning agents revealing a desired performance for the hospital gown and band-aids (23, 24).

Tranexamic acid (TXA) is a synthetic derivative of the amino acid lysine used as a medicine to treat or prevent excessive blood loss, and might contribute to better wound healing (25). This compound exerts antifibrinolytic effects through the reversible blockade of lysine binding sites on plasminogen molecules (26). Some researchers relied on TXA incorporation into nanofibers as a drug delivery system for hemorrhage control applications (27). Recently, many researchers have investigated the effect of antimicrobial agents or antifibrinolytic drugs loaded in nanofibers on wound healing (28). However, no report has been released on the incorporation of both ZnO-NPs and antifibrinolytic drugs in wound dressings. Following our previous research in wound treatment (29-31), here we report our results on fabrication and characterization of novel electrospun polylactic acid / zinc oxide nanocomposites loaded with tranexamic acid (PLA/ZnO/TXA NC) as an effective dressing for wound treatment. Also, *in vitro* cytotoxicity and *in vivo* skin wound healing of this dressing in mice have been examined.

2. Objectives

The major goal of the present study was to prepare PLA/ZnO/TXA nanofibrous nanocomposites as a novel and effective dressing for wound treatment. The antibacterial effect of this substance against some pathogenic bacteria was also evaluated. Finally, the coagulant assay, *in vitro* cytotoxicity, and *in vivo* skin wound healing model of this nanofibrous mat were assessed in the mice.

3. Materials and Methods

3.1. Materials

Polylactic acid (PLA, Mn = 70,000–90,000), tranexamic acid, zinc nitrate ($Zn(NO_3)_2.6H_2O$), sodium hydroxide (NaOH), and dichloromethane were obtained from Sigma–Aldrich Chemicals. All reagents were of analytical grade and they were used without further purification. Synthesis of zinc oxide nanoparticles was carried out through a direct precipitation technique using zinc nitrate and sodium hydroxide as precursors according to the literature (32).

The bacterial strain used for the antibacterial activity was Gram-positive bacterial strains including *Staphylococcus aureus (S. aureus*: ATCC 25023 (and Gram-negative bacterial strains including *Escherichia coli (E. coli:* PTCC 1399) prepared from the Persian Type Culture Collection (PTCC) Tehran, Iran. Human

skin fibroblast (HSF) and mesenchymal stem cell (MSC) were prepared from Skin Research Center, Tehran University of Medical Sciences. Also, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle Medium (DMEM), and phosphate buffer saline (PBS) were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

3.2. Preparation of PLA/ZnO/TXA Nanofibrous Nanocomposite

PLA solution (10 wt.%) was prepared by dissolving 1 g PLA in 10 mL dichloromethane at room temperature. Zinc oxide nanoparticles (0.5, 1, and 2 wt.%) and TXA (2 wt.%) were added to PLA solution. The concentration of the drug-loaded nanofibers was determined according to the literature (33). The products were sonicated for 30 min until a homogeneous blend was obtained.

The sample was filled into a 5 mL plastic syringe (inner diameter 0.8 mm) and spun using an electrospinning machine (NANOAZMA, model side by side, IRAN). The parameters were adjusted at high voltage: 20 kV, flow rate:0.6 $mL.h^{-1}$, tip-to-collector distance: 10 cm, and collector speed: 100 RPM. After 1 hour, the nanofibers were collected on aluminum foil and peeled off for characterization.

3.3. Characterization

The surface morphology of the structure was determined via field emission scanning electron microscope (FESEM, model: Mira 3-TESCAN). The high-resolution SEM images of samples were quantitatively analyzed by Image J software (National Institutes of Health version 1.48v).

The elemental analysis was surveyed by energy-dispersive X-ray spectroscopy (EDS, model SAMx) at an accelerating voltage of 100 KV. Fourier-transform infrared spectroscopy (FTIR) was carried out using a spectrophotometer (Shimadzu, IRTracer-100) at 4000- 400 cm^{-1} wavenumber.

Fluorescence images were recorded via a fluorescence Stereomicroscope, model: Leica M165 FC.

The contact angle and wettability of electrospun PLA/ZnO/TXA nanocomposites mat was determined using the sessile drop method. The contact angle is defined as the angle made by the interaction between the water interface and the nanocomposites interface. It was performed by an especially arranged microscope equipped with a camera (Canon EOS 700D) and PCTV vision software.

3.4. Antibacterial Activity

The antibacterial activity of electrospun PLA/ZnO/TXA nanocomposites was investigated against *Staphylococcus aureus* (Gram-positive, ATCC 25023) and *Escherichia coli* (Gram-negative, PTCC 1399) by colony count method.

For this purpose, the samples of the nanofibrous mat were sterilized by exposing them to UV- radiation for 15 min. Then, 1 mL of bacterial suspensions with a concentration of 1.5×10^{8} CFU. mL^{-1} was added to the nutrient broth. Next, circular discs of nanofibrous mats (2.5×2.5cm) were kept inside a bacterial solution which was followed by incubation at 37 °C for 72 h. Finally, the bacterial reduction was calculated by (Equation 1):

Bacterial reduction (%) = ×100

It is based on the number of colonies formed in Petri dishes before (B) and after (A) treatment with the sample respectively.

3.5. Coagulation Assay

The coagulant properties of the electrospun PLA/ZnO/TXA nanocomposite samples were determined via the blood clotting test described in previous papers (34). Briefly, the nanofibrous mat sample at a dimension of 20× 20 mm was cut and placed into a single well of the 12-well tissue culture plate. Coverslip without nanofibers was selected as the control. Then, 0.2 mL of human blood was dropped onto the surface of the samples. Finally, 1mL of $CaCl_2$ solution (0.5 M) was added to each blood drop and incubated at 37 °C for 1 h. The absorbance values of hemoglobin were monitored at 540 nm using a Jenway Model 7315 UV-visible spectrophotometer.

3.6. In vitro Cytotoxicity

In vitro cytotoxicity of the synthesized nanocomposite was determined using MTT assay. The nanofibrous nanocomposite samples (1x1 cm pieces) were punched, sterilized with UV light for 45 min, and placed into 96-well plates. The human skin fibroblast cells were seeded on the nanofibers samples into plates containing DMEM, 10% PBS, and 1% penicillin-streptomycin at the density of 5×10^{3} cells/well and incubated in a humidified incubator at 37 °C. Then, 10 µL of MTT reagent was added to each well and was further incubated for 4 hours. The fibroblast was kept in culture for 72 hours after which the nanofibers were separated from the solution.

Plates with complete medium, nanofibrous mat, and MTT reagent, without cells were used as blanks. Formazan crystals formed after 4 hours in each well

were dissolved in 150 µL of DMSO. The amount of formazan was then determined from the optical density at 570 nm by a microscan spectrum (Electro Thermo, Milford, USA) and used for MTT assay for cell viability determination. The results obtained in this study were statistically analyzed by KRUSKAL–WALLIS TEST and the results were significant. Dunnett and Tukey tests were used to examine the significant differences between the control group and other groups which both yielded the same result.
The control group is significantly different from the MSc and HSF groups.

3.7. In vivo studies

In vivo wound healing activity assessed in mice was obtained from the Central Animal House, Tehran University of Medical Sciences. There were 10 mice in total, which were divided into two groups of five each. Initially, the back of each mouse was shaved well and then the animal was anesthetized with xylazine/ ketamine at the ratio of 1:1 through biliary injection. The dorsal area of the mouse was depilated and the operative area of skin was cleaned with alcohol. Then, approximately 2 ×2.5 cm and 0.5 mm-deep incisions were made on the dorsal skin using a pair of sterilized surgical scissors and forceps. The mice were divided into control and treated groups. Treated groups were dressed up with electrospun PLA/ZnO/TXA nanocomposites, while the control group mice with bare wounds were covered using cotton gauze. The mice were housed individually in cages at room temperature with the wound healing process monitored over 7 and 14 days. The biopsies of the wound area were obtained at different times (7 and 14 days) using a 0.6 mm biopsy punch and fixed in neutral buffered formalin. The samples were then stained with a hematoxylin-eosin (H&E) reagent for histological observations.

4. Results

4.1. Characterization of PLA/ZnO/TXA Nanofibrous Nanocomposite

The SEM image and EDS analysis of electrospun PLA/ZnO/TXA nanofibrous nanocomposite with ZnO nanoparticles (1 wt. %) and tranexamic acid (2 wt.%) are displayed in **Figure 1**. It is obvious that the sample has uniform and smooth nanofibers with an average size of 90 nm diameter without any beads. ZnO nanoparticles were dispersed in the nanofibrous matrix in an average particle size distribution of 45 nm with spherical morphology. Further, the elemental analysis via energy-dispersive X-ray spectroscopy showed three main peaks for Zn, O, and C, confirming the successful synthesis of the PLA/ZnO/TXA nanocomposite. When tranexamic acid is incorporated into nanofibers, their existence and distribution can also be described through Fourier transform infrared spectroscopy. FTIR spectrum analysis of PLA/ZnO loaded with tranexamic acid had characteristic absorption bands at 1270, 1455, 1635, and 3501 cm^{-1}, representing the (C-O), (C-H), and (NH) stretch bend respectively. Also, the characteristic frequency of PLA was located at 1759 cm^{-1} assigned to carbonyl group (**Fig.S1**, in supplementary file). The contact angle measurement of ZnO/PLA/TXA nanofibrous nanocomposite showed a hydrophilic nature with a contact angle around 85◦ (**Fig. S2**, in supplementary file).

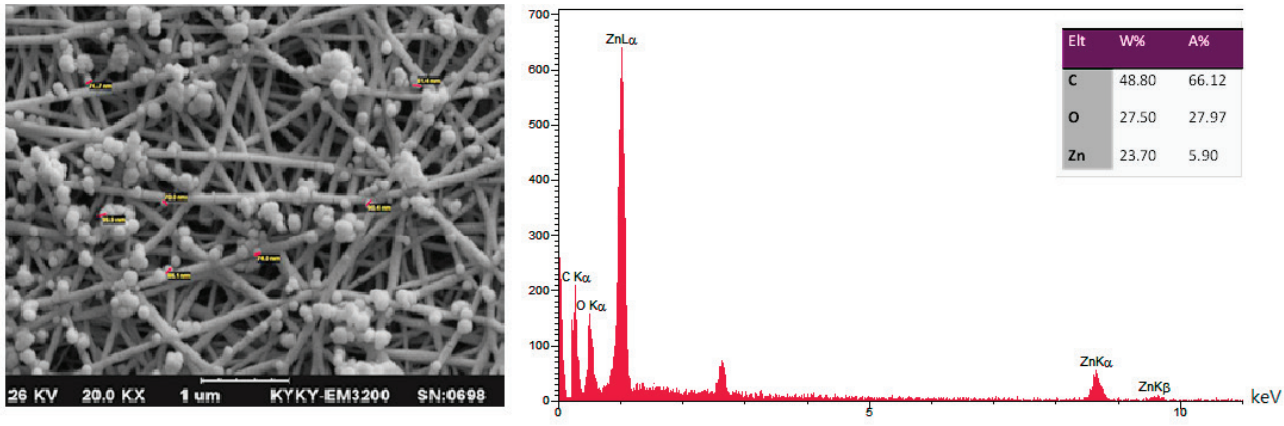

Figure 1. FESEM image and EDS analysis of PLA/ZnO/TXA nanocomposites

4.2. Antibacterial Activities

The antibacterial activity of PLA/ZnO/TXA nanocomposites was calculated using percentage reduction in bacterial count in the sample. PLA nanofibers loaded with ZnO nanoparticles 2 wt.% showed 75 and 98 % reduction in CFU/mL against *E. coli* and *S. aureus,* respectively (**Fig. 2**).

4.3. Coagulation Assay

The optical density (OD) values of hemoglobin at 540 nm of the sample after incubation on different materials under fixed time intervals displays in **Figure 3.** In this study, there was no significant difference in the OD values between the pure PLA nanofibers and the control sample at different time points. In contrast, under similar experimental conditions, PLA/ZnO nanofibrous nanocomposite mat loaded with TXA revealed lower OD values and significant clotting behavior after incubation for 60 min.

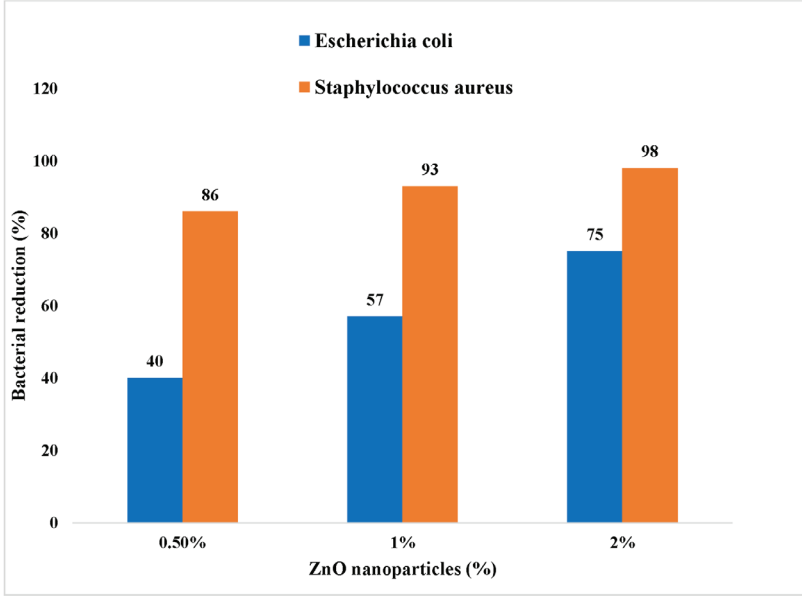

Figure 2. Antibacterial activity graph of PLA/ZnO/TXA nanocomposites against two bacteria

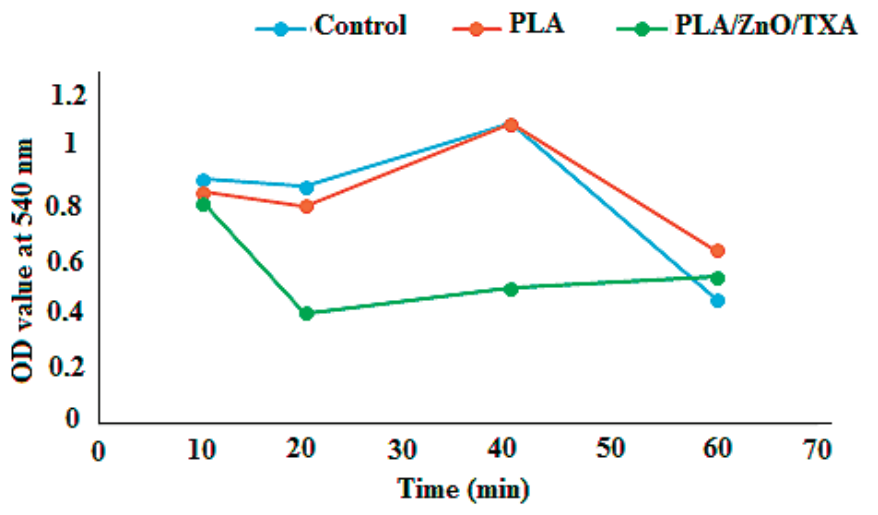

Figure3. Coagulation assay of PLA/ZnO/TXA nanocomposites at different time intervals

4.4. In vitro Cytotoxicity

The cytotoxicity of the nanocomposite was tested via quantitative analysis using the MTT assay. The fluorescence microscopy images of human fibroblast cell viability of the samples after exposure to the extraction medium for 72 showed that the metabolic activity of human skin fibroblast cells was not inhibited by the sample medium (**Fig. 4**). Further, our results indicated that PLA/ZnO/TXA nanofibrous nanocomposite displayed weak cytotoxicity on human skin fibroblast (HSF) and mesenchymal stem cell (MSC) with an 11.7% and 20.8% loss of cell viability respectively when incubated for 3 days.

4.5. In vivo Wound Healing

The results of in vivo wound healing performance of PLA/ZnO/TXA nanocomposites were investigated after 7 and 14 days. As shown in **Figure 5A**, the wound areas diminished progressively, and fewer wound leakages or infections were observed after coating with the PLA/ZnO/TXA nanofibrous nanocomposite. A significantly higher wound contraction was observed in PLA/ZnO/TXA nanofibrous membranes group on day 7 as compared to the control group. On day 14, 90% wound closure was observed in PLA/ZnO/TXA nanofibrous membranes treated group, while the control group showed a far lower percentage of contraction i.e, 65% wound closure only.

The hematoxylin and eosin (H&E) stain of the skin tissues surrounding wound sites is shown in **Figure 5B**. The control group without treatment after 14 revealed random deposition of collagen bundles in both papillary and reticular dermis. PLA/ZnO/TXA nanocomposite coating after 14 days illustrated complete re-epithelialization and deposition collagen bundles parallel to surface epidermis.

5. Discussion

In the present study, we report the preparation of PLA/ZnO nanofibrous nanocomposite loaded with tranexamic acid as a novel wound dressing. We try to survey the tissue property, antibacterial effect, cytotoxicity, and biocompatibility of the nanocomposite, as well as the subsequent effects in wound healing.

5.1. Characterization of PLA/ZnO Nanofibrous Nanocomposite Loaded with Tranexamic Acid

The morphological structure of the nanocomposite assessed by the FESEM image exhibited a bead-free and smooth surface of electrospun nanofibers with almost uniform diameters within the range of 90 nm. Also, the ZnO nanoparticles were dispersed uniformly in the nanofibrous matrix.

Fourier Transform Infrared (FTIR) spectroscopy indicated successful incorporation of TXA in PLA/ZnO nanocomposites fibers. In the PLA/ZnO/TXA spectrum, there were peaks at 1635 and 3501 cm^{-1} for the presence of NH group assessed by IR spectrum of TXA. The FTIR spectrum of the PLA/ZnO/TXA nanofibers exhibited vibrations of the carbonyl group in PLA at 1759 $cm^{-1.}$ These results are similar to the results of other articles (35).

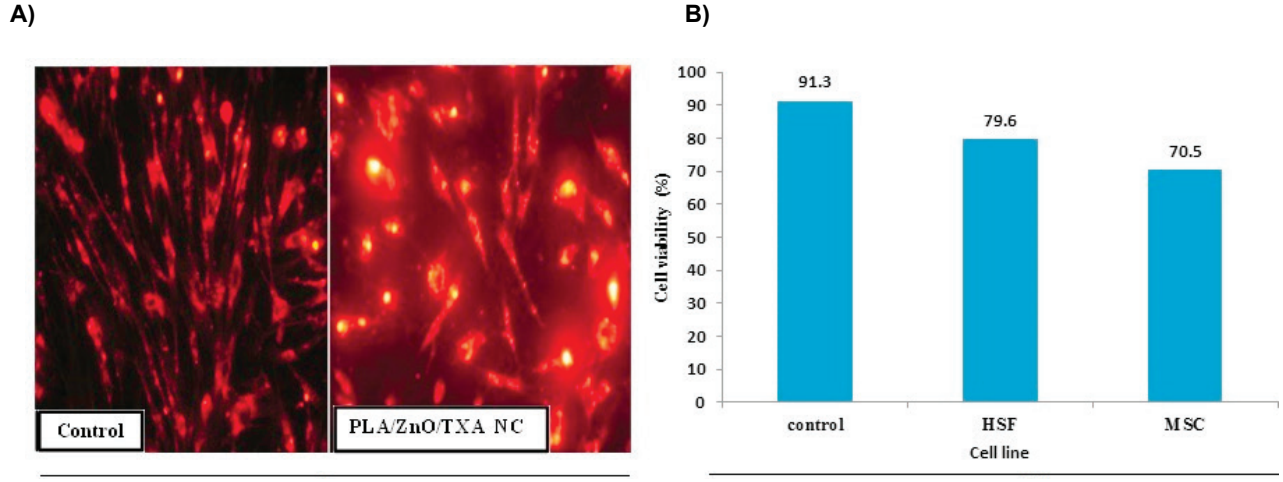

Figure 4. A) Fluorescence microscopy images of human fibroblast cell viability for control group and in the presence of PLA/ZnO/TXA nanocomposites. **B)** Cell viability analysis (MTT assay) of human skin fibroblast (HSF) and MSC (Mesenchymal Stem Cell)

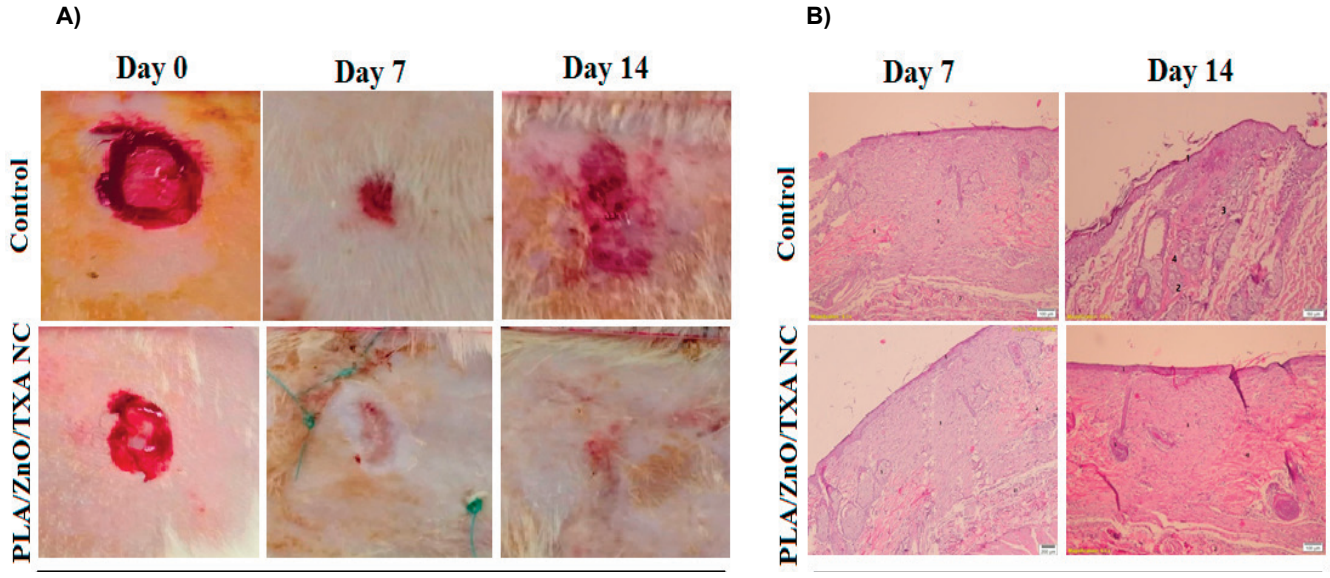

Figure 5. **A)** The representative images of skin wound healing in mice after treatment with the PLA/ZnO/TXA nanocomposites. **B)** H&E staining images of wound healing histology for the control group and PLA/ZnO/TXA nanocomposites coating at days 7

The wettability of mat surfaces is a very important property of wound dressing, which improves cell attachment and accelerates the process of wound healing. The hydrophilicity of nanofibers mat is controlled based on the chemical and percentage composition of their ingredients. A previous report determined that zinc oxide nanoparticle is basically a highly hydrophilic material (36). Herein, the addition of ZnO-NP modified the contact angle of nanocomposites to 85◦, showing the hydrophilic behavior of composite nanofibers.

5.2. Antibacterial Activities

One of the main challenges in chronic wounds is an infection, which occurs when germs grow within the damaged skin of a wound, resulting in notable patient morbidity and mortality. Thus, infection prevention strategies are necessary for wound treatment. ZnO-NPs have a broad spectrum of antibacterial activities against many pathogenic microbes. They participate in the formation of reactive oxygen species (ROS), which penetrate the bacterial cell membrane producing oxidant injury and exhibiting antibacterial activity. Also, zinc is an essential cofactor for many metalloenzymes required for cell proliferation and re-epithelialization of skin wounds. In this study, PLA/ZnO/TXA nanofibrous nanocomposites exhibited strong antibacterial activity against both *Escherichia coli* as gram-negative and *Staphylococcus aureus* as gram-positive bacteria. Comparing both types of bacteria, *E. coli* exhibited low sensitivity to the mats containing different concentrations of ZnO nanoparticles. These results agree with those reported by Emami-Karvani (37) confirming that Gram-negative bacteria are more resistant to ZnO nanoparticles than Gram-positive bacteria.

5.3. Wound Healing Assays

Wound healing is a complex process requiring the collaborative efforts of many different cell types and molecules. Healing of acute wounds involves the cooperation of four distinct phases included: hemostasis, inflammation, proliferation, and remodeling. Many factors are effective in improving and reducing the duration of wound healing. The *in vitro* cytotoxicity MTT assay of PLA/ZnO/TXA nanofibrous nanocomposites was evaluated as an effective therapeutic agent for chronic wound healing. The biosafety of polylactic acid has been approved by the FDA for use in the human body and had previously been studied by *in vivo* tests (38). The results indicated that PLA nanofibers loaded with ZnO-NP and TXA have no cytotoxic effect on human skin fibroblast. Regarding nanocomposites properties, a coagulant assay was also conducted to evaluate the antifibrinolytic effect of PLA/ZnO/TXA

nanofibrous nanocomposites. Tranexamic acid is one of the antifibrinolytic drugs that reduce postoperative blood losses in emergency surgery. Loading TXA in wound dressing has been used successfully to control bleeding in chronic wounds. As seen in **Figure 3**, the rate of blood coagulation is measured by the optical density values of hemoglobin at different times. A higher optical density value represents a higher hemoglobin concentration and shows less coagulation of blood. For PLA/ZnO/TXA nanofibrous nanocomposites, the lowest optical density value was obtained in 0.4 at 20 min, which is far lower than that of the PLA fibrous samples. These results imply that the PLA/ZnO nanofibrous nanocomposites mats loaded with TXA possess good blood clotting effects. In vivo wound healing activity refers to the regeneration of dermal and epidermal tissues. In the present study, the course of wound healing in mice was observed over 7 and 14 days. The PLA/ ZnO/ TXA nanofibrous nanocomposites showed a faster rate of healing, while the control wound showed slow healing even after 14 days. At the end of the treatment, the wound was esthetically healed and fewer wound leakages or infections were observed. The results show that the infiltration of fibroblasts to the wound area is facilitated by PLA/ ZnO/TXA nanofibrous nanocomposites.

Histological analysis further confirmed that PLA/ ZnO/TXA nanofibrous membranes were well tolerated in the mice, with mild inflammatory responses. Also, no cell necrosis and apoptosis were observed on days 7 and 14, besides an epithelial layer formed at the wound site of mice coated with nanofibrous mat. These results are consistent with the results obtained from other articles in which ZnO nanoparticles were used for wound treatment (39). By loading tranexamic acid in nanofibers, the hemostatic performance of the mats was obviously improved and prevented bleeding wounds. PLA nanofiber mats offer definite operational advantages in wound treatment such as high porosity and specific surface areas, which help the easy release of TXA drug to the wounds over a prolonged period. Porous nanofibers with good permeability are suitable mats for the volatilization of tissue fluid to adjust the humidity of skin tissue. The acceleration of wound healing is likely due to the micro/nanoporous structure of the nanofibrous matrices, which are advantageous in promoting cell attachment and proliferation. These results confirm previous findings in wound treatment using polylactic acid or other polymeric nanofibrous scaffolds (40).

6. Conclusion

Nanofibrous dressings are efficient polymeric membranes in wound healing compared to other traditional bandages due to their very high surface area to volume ratio, adjustable porosity, and ability to load various drugs. In the present study, novel PLA nanofibrous mats containing ZnO-NPs as an antibacterial agent and TXA as an antifibrinolytics factor were fabricated by the simple, efficient, and controllable method of electrospinning. The morphology of the nanocomposite showed successful bead-free smooth nanofibers with ZnO-NPs dispersed uniformly. FTIR spectroscopy confirmed the successful incorporation of the TXA used in the produced nanofibrous mats. These electrospun nanofibers showed a small pore size, allows appropriate permeation of atmospheric oxygen to the wound. Also, the extraordinary abilities of this membrane such as high antibacterial and antifibrinolytic activity led to accelerating wound healing. Further, *In vitro* cytotoxicity evaluation of the nanofibrous mats demonstrated the wound dressing material did not induce any adverse effect on the human dermal fibroblast cells. Antibacterial studies against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* revealed 98% and 75% reduction in colony-forming units, respectively. *In vivo* studies on the mice model clearly demonstrated that the PLA/ZnO/TXA nanofibrous nanocomposites dressing enhanced the wound healing process. The results suggest that this nanocomposite can be used as a helpful wound dressing where rapid wound healing and proliferation of skin cells are required.

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