

Original Research Article

In-vitro evaluation of dexpanthenol-loaded nanofiber mats for wound healing

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Abstract

Purpose: To prepare a novel dexpanthenol (DEX)-loaded nanofiber mats for wound healing.

Methods: A novel bioactive wound dressing formulation with dexpanthenol was developed by electrospinning method. Poly (lactic-co-glycolic acid), poly (ethylene oxide) and poly (caprolactone) were used as polymers. Morphological features, swelling properties, *in-vitro* release behavior, and cell viability properties of the formulations were evaluated.

Results: Morphological examination of mats confirmed successful formation of the fibers. Swelling of nanofiber mats results was 34.44 ± 1.05 , 18.59 ± 2.11 , 86.06 ± 3.25 and 44.62 ± 1.75 % for polycaprolactone (PCL), PCL + DEX, poly lactic-co-glycolic acid (PLGA) and PLGA + DEX, respectively, and occurred in a controlled manner. PLGA + DEX formulation has advantage over PCL + DEX and poly (ethylene oxide) (PEO) + DEX due to controlled *in-vitro* release of DEX. The highest cell viability was afforded by PLGA+DEX formulation.

Conclusion: DEX-loaded PLGA nanofiber formulation may be useful as an alternative wound dressing due its suitable mechanical and biological properties.

Keywords: Dexpanthenol, Electrospinning, Wound dressing, Polycaprolactone, Poly lactic-co-glycolic acid, Poly (ethylene oxide)

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INTRODUCTION

Electrospinning has been taken into consideration for many years by scientist; however its application to pharmaceutical field has been delayed. There has been increasing interest in polymeric nanofiber mats for biomedical applications [1]. One of the areas that this kind of products can be used is wound care especially in the form of wound dressings. Wound healing is

known as a natural body response when the skin is damaged [2]. It is achieved through four programmed phases which are hemostasis, inflammation, proliferation, and remodeling. To be success in healing all four phases must occur at the right time and in the right. Healing process could be improved by application of pharmaceuticals. Nanofibers can accelerate the wound healing process due to their large specific surface area. The nanofiber mat covers the

wounded area, protects the damaged skin and stimulates wound healing process by active pharmaceutical agents [3].

There are several techniques used for the production of polymeric nanofibers [4]. Electrospinning technology overcomes the restrictions of conventional spinning. The electrospinning technic has a high rate of nanofiber production, simple set up and low production cost [5]. Polyvinyl alcohol (PVA), PCL, PEO, PLGA, gelatin, collagen, chitosan, Polyglycolic acid (PGA), fibrinogen and silk can be used for producing nanofiber mats. PLGA is a random biodegradable polymer and have been used for several drug-delivery preparations, surgical implants and tissue engineering scaffolds [6]. PEO is water soluble and has a linear structure [7]. It has been widely used for medical applications as electrospun nanofibers [8]. PCL is a semi-crystalline polymer which has non-immunogenicity, shows slow biodegradability and high biocompatibility [9].

DEX is the stable alcoholic analog of pantothenic acid (vitamin B5). It is readily oxidized to pantothenic acid, a building block of coenzyme A (CoA) [10]. It has been reported by clinical observations that topically applied DEX is an aid in superficial wound healing with minimal risk of skin irritancy [11].

The aim of this work to prepare and characterize DEX-loaded PLGA, PEO and PCL nanofiber mats by electrospinning method for wound healing. The formulations were characterized by microscopic observations, thickness measurement, FT-IR analyses, mechanical properties, swelling ability and loading capacity. *In-vitro* release behavior was and cell viabilities were also tested with sterilized formulations.

EXPERIMENTAL

Materials used include DEX produced by BASF which was a gift from ASSOS Pharmaceuticals (Turkey). PLGA, Hexafluoroisopropanol (HFIP)

Table 1: Composition of prepared formulations

Code	PLGA (%)	PEO (%)	PCL (%)	DEX (mg/mL)	Chitosan (%)	Solvent
F1	15					THF/DMF (1:1)
F2	15					HFIP
F3	15					DMF
F4	15			100		HFIP
F5		6				Distilled water
F6		1			1	Acetic acid solution (0.05%)
F7		1			1	Acetone/ DCM (1:1)
F8		1.4			0.6	Acetone/ DCM (1:1)
F9		6		100		Distilled water
F10			12			DCM/DMF (1:1)
F11			12	100		DCM/DMF (1:1)

was purchased from Sigma (USA), Dimethylformamide (DMF) from Dasit, and Tetrahydrofuran (THF) from Merck. PEO and PCL were obtained from Sigma-Aldrich (USA). The other substances obtained commercially were analytical grades or higher.

Preparation of nanofiber mats

The polymer or polymer mixtures and DEX were dissolved in solvent or solvent mixtures to fabricate electrospun nonwoven nanofiber mats. Final volume of mixture was set to 100 ml. The solvents or solvents mixture were chosen in accordance to solubility of polymers. THF/DMF, HFIP and DMF were chosen for PLGA due to its solubility. Since PEO can be dissolved in water, distilled water is chosen as solvent. For the formulations F6, F7 and F8 chitosan was mixed with PEO and in this case different solvents were used. The best results were obtained with DCM/DMF mixture for PCL. The polymer solution with and without drug was loaded to a 5 mL plastic syringe which was fitted with a metallic needle. The flow rate was set on the syringe pump that was mounted horizontally, and the pump forced the piston of the syringe carrying the solution to move (12). The compositions of formulations were given in Table 1 and the conditions of electrospinning operation were summarized in Table 2.

Table 2: The parameters of electrospinning procedure

Formulation code	Solution feed rate (mL/h)	Spinneret-to-collector distance (cm)	Operating Voltage (kV)
F1	2	15	13
F2	2	15	10
F3	4	15	16.4
F4	2	15	10
F5	1	20	15.5
F6	0.5	15	17.5
F7	2	26	24
F8	0.3	26	13
F9	1	20	15.5
F10	2	15	17.5
F11	2	15	18

Characterization of nanofiber mats

The prepared mats were examined with an optical microscope (Olympus BX51, Japan) to confirm the formation of nanofibers. For further observations, the morphology of nanofibers was investigated by scanning electron microscopy (SEM) (XL 30 ESEM with EDAX, Philips, Netherlands).

The excipients and nanofibers were analyzed over 400 - 4000 cm^{-1} wavelength by the Fourier Transform Infrared Spectroscopy (FT-IR, Perkin Elmer Spectrum 100 Spectrometer, USA).

Tensile strength and percentage of elongation at break of mats was determined using TA-XT Plus Texture Analyzer (Stable Micro Systems UK) equipped with 5 kg load cell. 1 x 2 cm mat specimens were mounted on a film holder (ATG tensile grips) of texture analyzer [13].

The thickness of the nanofiber mat samples was measured using a digital micrometer (Digimatic caliper, Mitutoyo, USA) with a measurement resolution of 1 μm . Thickness measurement was made from three different points of nanofibers and the mean of measurement and standard deviations were calculated.

Swelling capacity of the nanofiber mat samples was investigated by direct immersion of formulations in PBS (pH 7.4) to simulate medium conditions [14]. The swelling degree (S) was calculated according to Eq 1 [15].

$$S (\%) = \{(w_t - w_d)/w_d\}100 \dots\dots\dots (1)$$

where W_t is the weight of the swollen sample at time t and W_d is the initial weight of the dry sample.

Determination of dexpanthenol

The high performance liquid chromatography (HPLC) system (Hewlett Packard-1100) consisted of C18 reverse phase column (ACE 5-C18 250 mm x 4.6 mm) with 20 μL sample loop was used to determine DEX. The mobile phase was a mixture of 10:90 (v:v) acetonitrile and 0.01 M ortho-Phosphoric acid. The flow rate, injection volume and UV wavelength were set at 1 mL/min at 25 $^{\circ}\text{C}$, 10 μL and 206 nm, respectively. The HPLC method was validated via linearity, accuracy and recovery, precision, specificity, limit of detection (LOD) and limit of quantification (LOQ) and stability.

Drug-loading capacity

The weighed formulation samples were dissolved with PBS (pH 7.4). Then, the mixture was shaken at 200 rpm for 24 h. Finally, the drug concentrations were determined by analytical method that mentioned above. Drug loading capacity was calculated by dividing practical DEX concentration with theoretical DEX concentration.

Evaluation of *in-vitro* release of dexpanthenol

In-vitro release of DEX through nanofiber mats were performed by dialysis bag diffusion technique. The weighed samples of nanofiber mats were placed in cellulose dialysis bag membranes with molecular weight cut off 12-14,000 D, the membranes were put into 7 mL of PBS (pH 7.4). The study was performed in water bath at 37 $^{\circ}\text{C}$ and the system mixed magnetically at 600 rpm. The amount of DEX released was determined by validated HPLC method.

Sterilization and evaluation of sterility

DEX loaded and unloaded nanofiber mats were sterilized by UV (UVP-CL 1000, Ultraviolet Crosslinker, UK) cabinet at 254 nm to give a total energy of 100 joule/ cm^2 for 4 h [16]. The sterility was controlled by microbiological observation.

Cell culture

Primary human dermal fibroblast–adult (HDFa, Invitrogen C-013-5C) cells were cultured in Medium-106 (Cascade Biologics) supplemented with low serum growth supplement (LSGS, Cascade Biology). Cells were maintained at 37 $^{\circ}\text{C}$ in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 $^{\circ}\text{C}$. Cells obtained from early passages (passages 4 – 5) to eliminate the alterations in morphology, response to stimuli, growth rates of cells were used for the following experiments.

Coverslips (35 mm in diameter) were pre-coated with freshly prepared nanofibers, dried by air and were sterilized by UV light for 24 h, placed in a 12-well culture plate. The cells were seeded on the membrane-coated coverslips at a density of 5000 cells/ cm^2 . After 1, 3 and 5 days of culture, samples were rinsed twice with phosphate-buffered saline (PBS) to remove the non-adherent cells. Subsequently, membrane-coated coverslips were incubated with 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (Invitrogen; MTT) (5 mg/mL) to determine the cell viability. After 4 h of incubation at 37 $^{\circ}\text{C}$, dimethyl sulfoxide was added to

dissolve the red-colored formazan crystals. The absorbance of the formazan solution was measured in a microplate reader (Varioscan Multimode Flash Reader) at 540 nm. Results were the Mean \pm Standard Error of the Mean (Mean \pm SEM) from at least three different experiments.

Statistical analysis

Data were analyzed using unpaired t-test and analysis of variance (one-way ANOVA) followed by a Tukey's multiple comparison test (GraphPad Prism, version 6.02 GraphPad Software Inc). Differences were considered significant at $p < 0.05$.

RESULTS

Microscopic features of nanofibers

Small beads were observed in formulations prepared with PLGA in THF/DMF (F1) and PEO/Chitosan in Acetone/DCM (F7 and F8) Figure 1). The electrospun materials, PLGA in DMF (F3) and PEO/Chitosan in acetic acid solution (F6), could not collect on the collector covered with aluminum foil. Finally; the solutions, PLGA in HFIP, PEO in distilled water and PCL in DCM/DMF were successfully prepared with and without DEX (F2, F4, F5, F9, F10 and F11) and the following studies were performed with these formulations (Figure 2, Figure 3 and Figure 4).

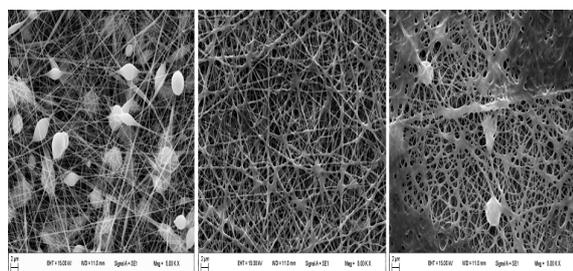


Figure 1: SEM images of F1, F7 and F8 formulation (PLGA in THF-DMF % 15, PEO/chitosan (1:1) in acetone/DCM and PEO/chitosan (1.4:0.6) in acetone/DCM)

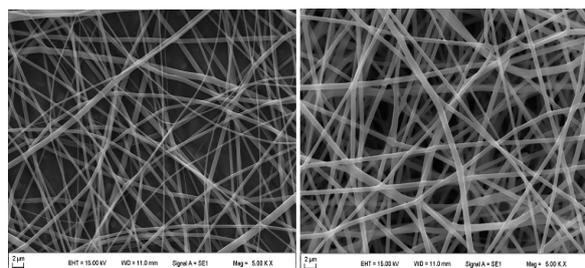


Figure 2: SEM images of F2 and F4 formulations (PLGA in HFIP % 15 and PLGA+DEX in HFIP % 15)

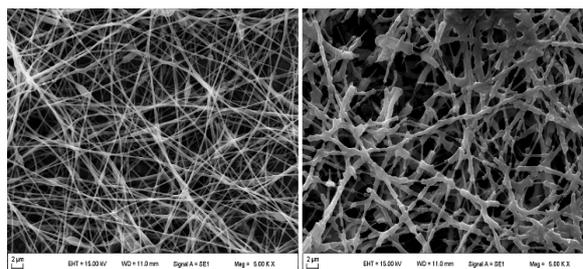


Figure 3: SEM images of F5 and F9 formulations (PEO in distilled water and PEO + DEX in distilled water)

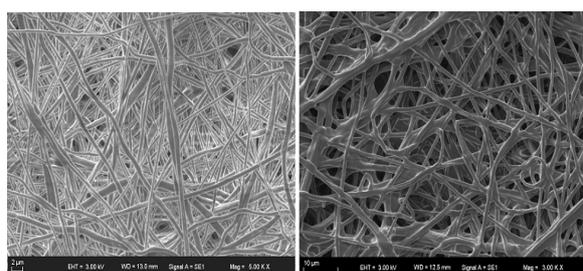


Figure 4: SEM images of F10 and F11 formulation (PCL in DCM/DMF and PCL + DEX in DCM/DMF)

FT-IR spectra of excipients and nanofibers

The FT-IR analysis of pure excipients and drug loaded and unloaded nanofiber mats were shown in Figure 5. The specific bounds for each component could be seen from the figure.

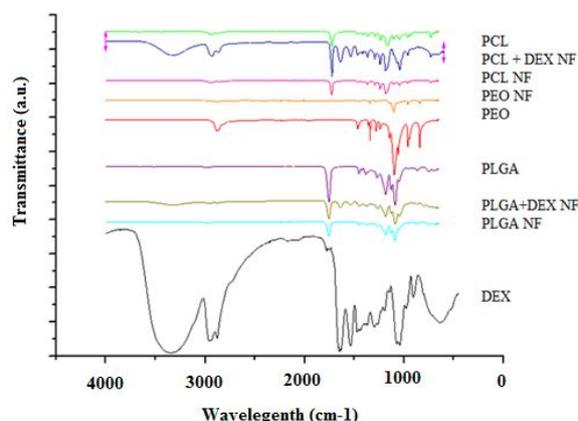


Figure 5: FTIR spectra for DEX loaded and unloaded nanofiber mats and pure substances for the spectral range between 4000 and 400 cm^{-1} (NF; nanofiber)

Tensile strength and elongation of nanofibers

The tensile strength and elongation percentage of formulations are shown in Table 3. The tensile strength and elongation percentage of nanofiber mats decreased with incorporating DEX to formulations.

Table 3: Tensile strength and elongation of the mats

Formulation code	Tensile strength (N/cm ²)	Elongation (%)
F2	0.657±0.001	24.545±0.362
F4	0.0005±0.0000	12.845±0.274
F5	0.0028±0.0001	16.276±0.184
F9	0.0001±0.0000	2.639±0.393
F10	0.817±0.0001	35.81±0.952
F11	0.0038±0.0001	28.285±0.538

Thickness of the nanofiber mats

The highest thickness was observed with PEO and PCL had lowest value (Table 4). DEX loaded formulations had higher thickness than unloaded ones for all formulations.

Table 4: The thickness of nanofiber mats

Formulation code	Polymer/drug	Mean (mm)
F2	PLGA	0.100±0.03
F4	PLGA+Dex	0.163±0.01
F5	PEO	0.127±0.05
F9	PEO+DEX	0.184±0.03
F10	PCL	0.053±0.01
F11	PCL+Dex	0.120±0.03

Swelling of nanofiber mats

The swelling percentages were 34.44 ± 1.05 , 18.59 ± 2.11 , 86.06 ± 3.25 and 44.62 ± 1.75 for PCL, PCL+DEX, PLGA and PLGA+DEX, respectively. The swelling test could not be performed with PEO and PEO + DEX nanofiber mats (F5 and F9) because, after the formulations were submerged in water, they dissolved, immediately

Analytical method for determination of dexpanthenol

A new HPLC method was developed to determine DEX concentration in samples. The method was validated via several parameters according ICH guidance [17]. Coefficient of determination was 0.9986. The relative standard deviations were less than 2.0 % for accuracy, precision/repeatability which confirmed validation of method. LOD and LOQ were 0.122143 and 0.370131 $\mu\text{g/mL}$, respectively. The stability of DEX solution was examined for 48 h and the concentration did not change.

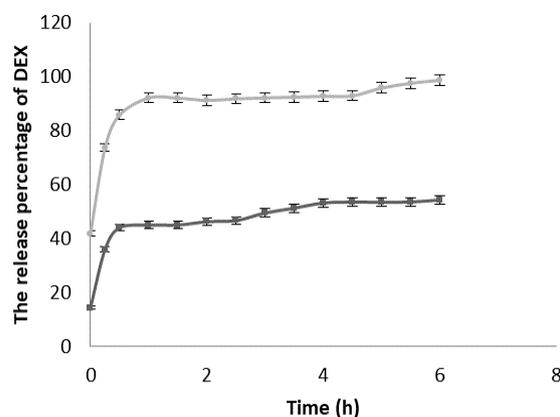
Drug-loading capacity

The drug loaded in the nanofiber mats was determined as mentioned above. The load capacity was 95.42 ± 3.74 , 85.4 ± 0.23 and 98.42 ± 1.93 % for F4, F9 and F11, respectively. The

one-way ANOVA test applied to the results gave f-ratio value of 0.19647, the p-value of 0.831418. The result was not significant at $p < 0.05$.

In-vitro release of dexpanthenol

In-vitro release studies, such as in the swelling test, could not be performed with the PEO + DEX formulation due to high water solubility of PEO. The PEO + DEX mats dissolved immediately and the drug was released after immersing in PBS. The release percentage for F4 and F11 was 54.24 ± 1.15 and 98.66 ± 1.27 , respectively (Figure 6).

**Figure 6:** *In-vitro* release of DEX from the formulations (●PCL+DEX, ■ PLGA+DEX)

Sterility of the mats

Nanofiber mats were sterilized by UV method before cell culture studies. The microbiologic investigation showed that all formulations were sterile after UV irradiation.

Cell proliferation

Figure 7 shows the proliferation rate of HDFa cells on different nanofibers after 1, 3 and 5 days of *in-vitro* cell culture. After 1-day culture, it was determined that cell viability on DEX + PCL, DEX + PEO and DEX + PLGA nanofibers coated slides were significantly higher than and unloaded PCL, PEO, PLGA nanofibers coated slides, respectively ($p < 0.05$). However, only DEX + PEO formulation showed lower cell viability than uncoated control slide for the first day. Moreover, at the 3-day of culture, numbers of cells increased to approximately two times higher than 1-day of culture in all slides. Only DEX loaded PLGA fibers significantly improved the cell viability compared to unloaded PLGA fiber coated slides ($p < 0.05$); while there was no significant increase in DEX loaded PCL and PEO fibers compared to unloaded PCL and PEO formulations ($p > 0.05$). At the 5-day of culture,

DEX loaded PLGA coated fibers significantly enhanced cell proliferation compared to unloaded PLGA coating and control ($p < 0.05$). The graphs show the comparison of HDFa cell viability between Dex-modified and unmodified nanofiber coated cover slides.

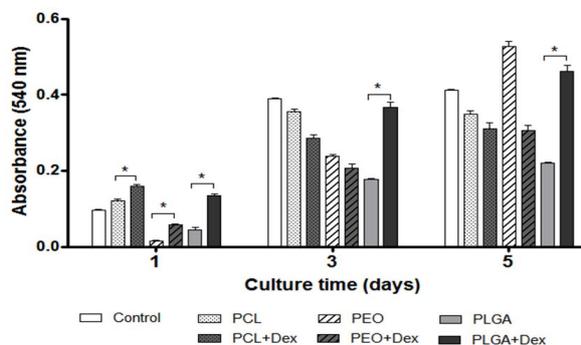


Figure 7: The result of cell viability tests

DISCUSSION

The macroscopic observation results shown that nanofiber mats could prepare successfully with each polymer. But, the best result was obtained with PLGA. There was no fracture and beads on PLGA figures with and without DEX. The scaffold like structure of PLGA mats could provide a suitable space for cell growth.

Infrared spectroscopy has been frequently used to investigate the conformational changes of nanofiber mats. The main characteristic vibration bands of DEX functional groups were observed in the FT-IR spectra [18]. As it was seen in FT-IR spectra, in bulk PLGA polymer, PLGA electrospun nanofibers and PLGA + DEX nanofibers, the C=O stretch and the C–O stretch hovered around 1750 and 1050 cm^{-1} respectively and bands around 3,000 cm^{-1} were observed due to the alkyl groups. There was no change in FT-IR spectra of bulk PLGA and PLGA nanofibers.

Regarding the PCL spectrum, strong bands such as the carbonyl stretching mode around 1723 cm^{-1} could be easily identified for pure PCL, PCL nanofiber and PCL + DEX nanofibers mats. The band around 1290 cm^{-1} which was seen in all spectra was assigned to the backbone C–C and C–O stretching modes in the crystalline PCL.

The FT-IR were used to determine molecular interactions within PEO and DEX loaded PEO nanofibers. The figure showed that there is no significant interactions between drug loaded and unloaded nanofibers. A band around 2900 cm^{-1} was seen in the FT-IR spectra of PEO

nanofibers and pure PEO that is attributed to the symmetric and asymmetric C–H stretching modes. And also the bands at about 1456-1350 cm^{-1} and 1102-962 cm^{-1} were specific for –CH₂– group and C–O group, respectively.

The spectra of all DEX-loaded nanofibers had bounds around 3,800 cm^{-1} that specific for DEX substance. In addition, there was no peak revealed to solvents used for fiber spinning that means there were no residual solvents left after spinning. The absence of residual solvents is an important factor in application of nanofiber mats for wound healing since it may be harmful in biomedical applications.

The presence of DEX weakened the cohesive force between chain molecules of polymer and therefore, the tensile strength of mats reduced. As it was shown with SEM images PEO nanofiber mats with DEX (F9) have some broken areas that indicate lower tensile strength of this formulation. The lowest values were obtained with F9 formulations for tensile strength. Formulations with PEO are more breakable than others. PLGA is a very brittle material, which can easily break if the stress increases while PCL is a flexible polymer [19]. The SEM images also confirmed this data.

The thickness of fiber could vary depending on polymer type, active agents, collection time, etc. The collection time was not changed in this study. But, thickness changed depending on polymer type and the presence of active substance. As mentioned above, presence of DEX increased the thickness due to filling effect of active substance.

The change in weight of the specimens gives an idea of how much water they can hold and swell. The presence of DEX was decrease swelling capacity due to gaps filling effect of active substances. Other formulations showed high swelling capacity due to porous structure of nanofiber mats. High swelling capacity is an important feature in terms of fluid absorption, especially in exudative tissues.

High drug loading was expected due to the passive drug loading technique. The drug incorporated into the polymeric solution and after spinning the solidified drug was entrapped in the fiber mats. Therefore, the risk of drug loss is very low during process. The drug loading was high for all formulations and the differences were not significant.

A rapid release was observed with the F11 formulation while a controlled release was

obtained with the F4 formulation after six hours. Since healing process takes a long time, the goal was to achieve a controlled release of the active and also for the nanofibers not to dissolve easily at the area of application. They should provide a scaffold for growing cells and then the nanofiber mats have to degrade by the time to allow drug release. From this point of view, PLGA + DEX formulation has more advantage than PCL + DEX and PEO + DEX formulations.

The UV radiation is used for disinfection purposes and this sterilization method has advantages such as no need for heating and chemicals [20]. In our study the nanofiber mats sterilized by UV method before cell culture studies.

Recent studies have shown that the properties of a scaffold are very important in regulating the biological properties of cells and in the formation of tissue substitutes (buraya kendi iyi literatürlerinden birini ekleyebilirsiniz). HDFa cells are frequently used in the in vitro studies on tissue scaffold studies [21,22]. These cells capable to synthesize and secrete extracellular matrix proteins and collagen under cell culture conditions. In this study, it was demonstrated that the HDFa cells could adhere and proliferate on all the tested nanofibers which are an essential property for scaffold applications. Furthermore, it was determined that only DEX loaded PLGA coated fibers significantly induced HDFa cell proliferation compared to unloaded PLGA coating and control at the end of the fifth day. Our results suggest that DEX loaded PLGA may use as an effective alternative biocompatible formulation for the scaffold fiber engineering.

CONCLUSION

DEX-loaded PLGA, PCL and PEO nanofibers have been suitably prepared by electrospinning method. Rapid drug release was obtained from PEO mats while PLGA and PEO mats had controlled release. Slower release is an advantage for long wound healing process. PLGA+DEX formulations significantly induced cell proliferation. Furthermore, PLGA + DEX nanofiber formulation is a promising alternative for treatment of skin lesions.

DECLARATIONS

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Disclosure of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. First author is corresponding author and she wrote the manuscript. And also she worked on preparation of formulations, characterization of formulations. Second author is worked on all methods part except cell culture studies. Third author is developed HPLC method and prepared PEO nanofiber mats. Fourth author performed cell culture studies. And last one is consultant of study.

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