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ARTICLE

Alleviating neuropathy of diabetic foot ulcer by co-delivery of venlafaxine and matrix metalloproteinase drug-loaded cellulose nanofiber sheets: production, in vitro characterization and clinical trial

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Abstract

Background The objective of the present study was co-delivery of venlafaxin (VEN) and doxycycline (DOX), a matrix metalloproteinase inhibitor drug, for alleviating inflammation and neuropathy in diabetic foot ulcer (DFU).

Methods Bacterial cellulose nanofiber sheets (BCNS) were loaded with DOX and VEN and categorized by their loading efficiency, release profiles and ex vivo permeation throughrat skin. The optimized nanofibers were used in patients with DFU to compare with the standard wound care regimen during a 12-week trial. Wound area was measured every 2 weeks. Biochemical parameters and microscopic studies of the skin were examined prior and at the end of the treatment. The Michigan Neuropathy Screening Instrument (MNSI) questionnaire was utilized to assess diabetic neuropathy.

Results The optimum formulation showed loading efficiency of $37.8 \pm 1.6\%$ for DOX and $48 \pm 1.9\%$ for VEN. Rat skin permeation was 40% for DOX after 7–29 h and 83% for VEN during 105 h. Patients treated with BCNS showed no significant difference in their biochemical parameters before and after intervention. The ulcer size showed faster reduction after 12 weeks in the treatment group compared to the control group. The abnormal responses in the MNSI questionnaire decreased and pain-free walking distance increased significantly in the treatment group compared with the control group ($p < 0.001$). Microscopic studies of the skin after using nanofibers showed a large number of polymorphonuclear chronic inflammatory cells and formation of new capillary beds.

Conclusions The BCNS loaded with DOX and VEN may expedite healing and reduce neuropathy in the DFU of diabetic patients.

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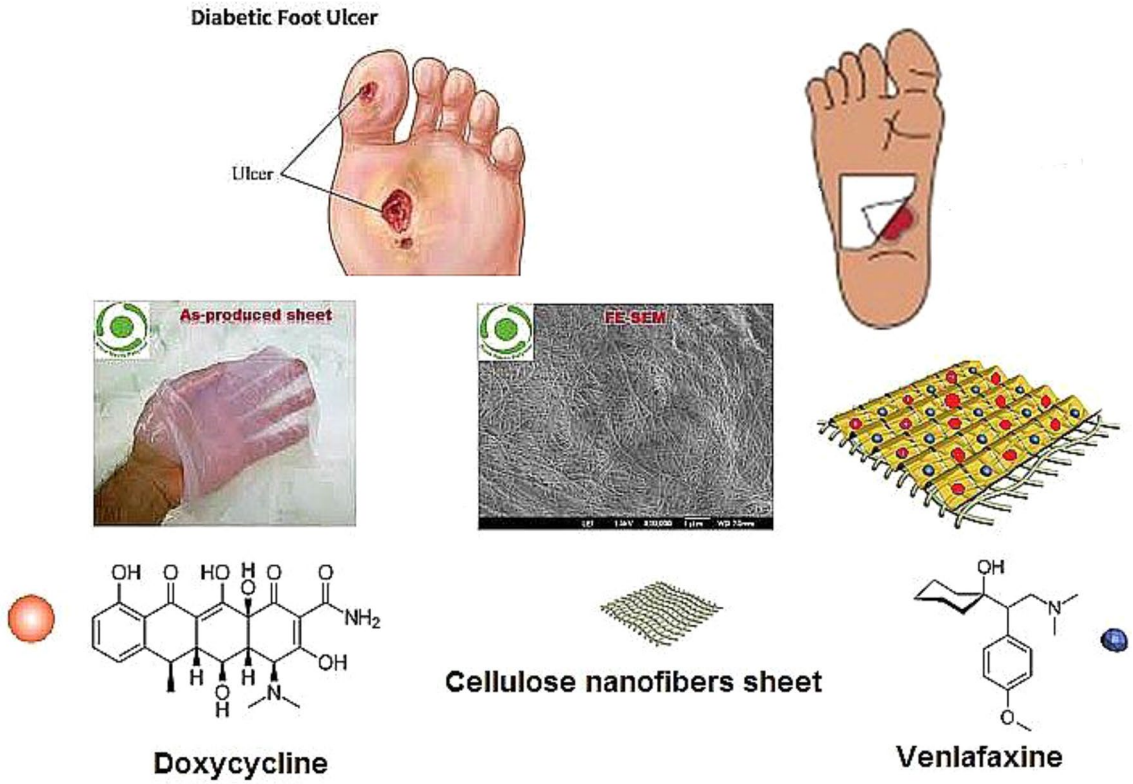
Graphic abstract



Keywords Bacterial cellulose nanofibers sheet · Doxycycline · Venlafaxine · Neuropathy · Diabetic foot ulcer

Introduction

According to data from 2013, an estimated 366 million people live with diabetes and this will increase to 552 by 2030 [1].

Diabetes mellitus (DM) has many morbidities including diabetic foot ulcer (DFU). Diabetic ulcer is a complication of DM and may result in hospitalization and even lib amputation [2]. As many studies have shown, 85% of amputations may be preceded by ulcers [3] and collateral wound site new ulcer formation has an incidence of 50% [4].

Tissue injury triggers a physiological response. In a healthy person, this is a sequence of events that result in the restoration of biological and functional integrity and ultimately results into wound repair and closure [5]. This does not happen in DFU, therefore, it is essential to understand the pathology of the wound in DFU and how to expedite the healing process [2].

There are many approaches to wound management and it depends on individual symptoms and stages of the ailment [6]. Conventional wound dressings have limited value and due to different stages in the process of healing, there is a need for multifactorial ones. Mostly the wound dressings are used to create a suitable environment for the healing process to take place and facilitate healing through additives that allow for a moist environment, removal of exudate, antibacterial effects, and the stimulation and proliferation of fibroblasts and keratinocytes at the site of injury [7, 8]. Patient compliance is essential to facilitate application and removal without aggravating the symptoms [4]. Cost effectiveness is an issue as well.

A suitable dressing must possess the following characteristics (especially in the case of an underlying peripheral neuropathy) possess a multilayered structure, maintain a moderately moist environment, inhibit bacterial growth, manage excess exudates and promote re-epithelialization of the ulcer [4].

Polymers with various characteristics and manufactured from different sources render nanofiber dressing a superior choice for diabetic injury in comparison with the ordinary dressing types [9].

Peripheral neuropathy (PN), deformity, and macrovascular disease are the main causes of preventing the healing process of DFU [6] and a major drawback is the lack of

accord for the main line treatment in patients with peripheral neuropathy [10].

VEN, a serotonin–noradrenaline reuptake inhibitor, is successful in the treatment of different categories of pain. Its unfavorable impact profile is essentially superior to that of tricyclic antidepressants [11]. In one clinical trial, VEN has been shown to be safe and well tolerated as an analgesic drug for the symptomatic treatment of diabetic PN with minimal adverse effects [12].

Another problem with DFU is its chronic nature that leads to infections, which can in turn be challenging [4]. High levels of bacteria (particularly, *Pseudomonas aeruginosa* and *Staphylococcus aureus*), the presence of non-viable tissues and repetitive mechanical trauma to the wound, cause excessive levels of the matrix metalloproteases (MMPs) [13] which are a specific group of basically related proteolytic enzymes. MMPs have been found in chronic wounds and their persistent elevation retards progression toward wound closure [13].

Drugs with MMPs activity inhibition properties include minocycline and DOX [14]. A dressing containing DOX has been previously reported to have dual effects as a matrix degradation inhibitor and an antibiotic and has the capability to both reduce the infection and MMPs level [15]. A composite membrane of bacterial cellulose (BC) loaded with tetracycline HCl was also designed by Chen et al. [16].

The aim of the present study was the development of a wound dressing based on bacterial cellulose nanofibers loaded with DOX as an antibacterial agent and VEN for alleviating neuropathy of DFU. A randomized controlled trial was conducted to study the effects of the designed wound dressing in diabetic patients suffering from DFU neuropathy.

Materials and methods

Materials

Bacterial cellulose nanofiber sheets with a purity of 99% and prepared by the static culture of *G. xylinus* (ATCC 53582) with an average nanofiber diameter of 45 nm were purchased from Nanonovin Polymer Co. (Iran). Venlafaxin (VEN) was provided by FARABI Pharmaceutical coco (Iran) and doxycycline (DOX) was procuredfrom DUOPHARMA Co (Sdn. Bhd, Malaysia).

Drugs loading in cellulose nanofibers sheets

After 30 days culture of the bacteria in HS medium, the bacterial cellulose sheets were purified using 0.1 N NaOH. Then they were washed thoroughly using distilled water, cut into 2 cm × 2 cm size sand dried at 50 °C for 8 h. Drugs were loaded into the nanofibers by a passive technique. Initially, VEN and DOX were separately loaded onto the nanofibers. In order for this to work, each drug was dissolved in deionized water, then nanofibers were soaked in the drug suspension and stirred for 24 h at 400 rpm at room temperature. After that the nanofibers surface was washed for three times to remove unloaded drugs remaining on the surface of the nanofibers. In the next step, both drugs were loaded simultaneously onto the cellulose nanofibers sheets. The effect of three variables including the pH of the loading medium (2, 4, 6, 7, 8, 9 and 11), different ratios of the two drugs (0:1, 2:1, 4:1 and 1:0 of VEN:DOX) and also the different ratios of the nanofibers to drugs (1:1, 1:2, 1:2.5, 1:3 and 1:4) were studied on the drugs loading efficiency and drug release from the nanofibers.

Measurement of drug loading efficiency in nanofibers

Drug loading efficiency percent (LE%) was determined for each drug by sampling from the residual solution of the loading medium and the amount of free drug in the clear solution was measured by UV spectrophotometer (UV mini 1240, Shimadzu, Japan) at 341 nm and 228 nm for DOX and at 228 nm for VEN. VEN did not have any UV absorption at the wavelength of 341 nm which was the λ_{max} of DOX, but DOX interfered with the λ_{max} of VEN. Therefore, to omit the UV-absorption of DOX at 228 nm which is the λ_{max} of VEN, the UV-absorption of DOX at this wavelength was obtained from the standard curve of DOX at 228 nm. Another standard cure was plotted for DOX at 341 nm to calculate the concentration of DOX in the samples. In the next step with awareness of concentration of DOX in the sample, its absorption at 228 nm was calculated by its standard curve at this wavelength and it was subtracted from the absorption of the samples at the wavelength of 228 nm to obtain the pure absorption of VEN. The standard curves of DOX in water at 228 nm and 341 nm were linear over the concentration range of 1.0–50.0 µg/ml. Also the standard curve of VEN was linear in water at 228 nm. All measurements were carried out three times and the LE% of both drugs were calculated using the following equation:

$$LE\% = \frac{\text{total drug} - \text{feed drug in residual solution}}{\text{total drug}} \times 100. \quad (1)$$

Attenuated total reflectance-fourier transform infrared spectroscopy (ATR—FTIR) and fourier-transform infrared spectroscopy (FTIR) spectroscopy

FTIR spectra of VEN and DOX powders and ATR-FTIR spectra of nanofibers and drug-loaded nanofiber sheets were

taken by FTIR spectrometer (6300 Jasco-Japan) at wave numbers of 650–4000 cm^{-1} using the KBr disc method to find the possible interaction between the drugs and bacterial cellulose nanofibers sheets [17].

In vitro drug release studies from bacterial cellulose nanofiber sheets

To study VEN and DOX release rate from nanofibers, each formulation was placed in a dialysis bag (molecular weight cut-off 12 kDa) and immersed in a beaker containing 50 ml phosphate buffered saline (PBS) (pH 7) at room temperature. By sampling from the medium at different time intervals from 1 to 132 min, the amount of drugs released from the nanofibers was determined by UV spectrophotometer at $\lambda_{max} = 341$ and 229 nm for DOX and $\lambda_{max} = 229$ nm for VEN. The standard curves of DOX in PBS at 229 nm and 341 nm were linear over the concentration range of 1.0–50.0 µg/ml. Also the standard curve of VEN was linear in PBS at 229 nm. All measurements were carried out three times and the results were reported as mean ± SD [18, 19].

Field emission–scanning electron microscopy (FE-SEM)

The morphology and diameter of the nanofibers and drug-loaded nanofibers were assessed using FE-SEM (Hitachi, Japan). Samples were sputter-coated with gold under vacuum before imaging.

The assessment of percutaneous absorption of DOX and VEN using Franz-type diffusion cells

Transdermal diffusion of DOX and VEN through the nanofibers sheets was tested using Franz diffusion cell on a piece of rat skin with surface area of 2.6 cm^2. Sink conditions were achieved in the receptor compartment with PBS at pH7. The volume of the receptor fluid was 30 ml. A full thickness skin excised from adult Wistar rats weighing 150–180 g was cleaned under running water immediately after excision. The hairs of the abdominal skin were removed by shaving and wiped dry. Then the skin fragment was placed horizontally on the Franz diffusion cells, between the donor and receptor compartments and drug-loaded bacterial cellulose sheets ($C_1V_2D_1$, pH 8, freeze-dried) were placed on the skins. During the experiment, the receptor compartments were continuously homogenized using magnetic stirrer such that the dermal side of the skin was exposed to the receptor fluid and the stratum corneum remained in contact with the donor compartment. The temperature was kept at 37 °C using a water circulation system. Serial sampling was performed between 1 and 105 h by taking 1 ml of receptor fluid (PBS) for measurement of DOX and VEN concentration by UV-spectrophotometry method in PBS media at $\lambda_{max} = 341$ and 229 nm for DOX and $\lambda_{max} = 229$ nm for VEN. Equal volumes of fresh PBS were added to the receptor compartmentafter each sampling. The cumulative amount of drug per penetration surface area (Q) was calculated and the curves were plotted as a function of time (t) [20]. The drugs concentration in the receiver cell (C_n) and Franz cell surface area (A) was used for calculation of Q:

$$Q = \left(C_n V + \sum_{i=1}^{n-1} C_i S \right) / A \tag{2}$$

C_n concentration of drug determined at each sampling interval, V volume of individual Franz cell, $\sum_{i=1}^{n-1} C_i$ sum of the concentrations of each drug determined at different sampling intervals from the first sample through the sample of $n-1$, and V_i volume of sampling aliquot. The total percentage of drugs released were calculated by the following eq.:

$$\text{Accumulative release (\%)} = (\text{drug released}/\text{total loaded drug}) \times 100 \tag{3}$$

The test was repeated three times and the results were reported as mean ± SD.

Clinical studies

Patient selection

All clinical studies were conducted according to the guidelines of Ethical Committee of Isfahan University of Medical Sciences with the license code of 9531. An aggregate of 20 subjects were chosen for diabetic foot ulcer examination. In every class, the subjects were randomized into cases and control groups. Eligible volunteers attending the Diabetes Clinic of Isfahan Endocrine and Metabolism Research Center, Isfahan, Iran, between October 2016 and December 2018, were randomly assigned into either the treatment group (10 patients) or the control group (10 patients). Along with receiving nanofibers loaded with drugs, the cases additionally got the routine standard injury care during their follow-up period. The controls received only the standard injury care system and were followed up in the same manner as the nanofibers group.

The patients were thoroughly informed about the risks, benefits, and the trial process itself. Informed consent was taken from the patients, and they were informed of the inclusion and exclusion criteria. The individuals who fit the examination criteria were taken as subjects. The subjects were given a data sheet that illustrated a randomized controlled preliminary test that was being conducted to compare the new treatment with placebo. Patients were allocated to the test or control group using numbers in closed envelopes. The selection relied on clinical history; however,

angiography was also used in a few appropriate cases. The 20 diabetic foot ulcer cases had ulcers without indications of obvious cardiovascular symptoms.

Their blood sugar level was measured at the start of the examination. The patients who met the following requirements were registered after a thorough history and physical examination at the screening visit: (1) age above 21 and below 65 years, (2) non-healing ulcers that do not demonstrate size decrease during a 1-month treatment period with excellent reduction in blood pressure and ulcer therapy, ulcer sizes between 1.7 and 12 cm^2, (3) patient ready and able to read, comprehend and sign an informed consent specific to the study. The exclusion criteria included: (1) septicemia, (2) any hematological disorder, (3) concurrent infection at wound site, (4) serious malnutrition, (5) pregnancy, (6) low-end ulcers owing to other particular causes such as syphilitic foot ulcer, fungal ulcer, and tubercular ulcers, (7) osteomyelitis, (8) ESR > 70 mm/h and (9) ischemia. All standard treatments for the patients including; offloading system, ulcer debridement, infection treatment and revascularization and restoration of tissue perfusion during the 12 weeks of treatment were carried out in line with the principle protocol for treatment of diabetic foot ulcer introduced by the International Working Group on the Diabetic Foot (IWGDF) [21].

Treatment and evaluation of wounds

This clinical trial study with registration code of: (IRCT20160711028878N2) was arranged according to the guidelines of Ethical Committee of Isfahan University of Medical Sciences. When the patients were registered, their wounds were dressed with twice daily moist-to-dry saline gauze dressings. Twice a week, ulcers were managed with drug-loaded nanofibers until the ulcer healed and they were then followed up for up to 12 weeks. If after 12 weeks of therapy the ulcer had not healed, the person could choose to receive extra therapy for a further 12 weeks. Wounds were clinically assessed by the physician and photographed. At every 2 week interval after treatment, wound areas were calculated by multiplying the wound's shortest perpendicular length and width measurements. The percentage of the wound closure was determined by multiplying the longest perpendicular length and width dimensions measuring of the wound at day 0, and the end of treatment. The effect of nanofibers loaded with drugs on healing was compared to routine treatment. Patients were also evaluated for any side effects of therapy at each clinic visit.

The pain-free walking distance (in meters) was also evaluated before treatment and after treatment at every 2-week interval. In addition, when the patients walked, they did so with off loading systems.

Biochemical parameters for the cases and controls were taken at the end of the therapy before and after intervention to verify the body's various physiological responses. The parameters of serum urea, creatinine, bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), cholesterol, triglyceride, and plasma glucose were determined by an auto-analyzer (Siemens, USA).

Tests for diabetic peripheral polyneuropathy

The 15-item self-administered questionnaire of the Michigan Neuropathy Screening Instrument (MNSI questionnaire) was utilized for diabetic neuropathy assessment. The MNSI questionnaire was self-administered. 'Yes' responses to questions 1–3, 5–6, 8–9, 11–12, 14–15 and 'No' responses to questions 7 and 13 were each counted as one point. Questions 4 and 10 were not included in the published scoring panel [22, 23]. This test was validated in Iran [24] and the questionnaire was filled in case and control groups. A score of ≥ 7 was detected as abnormal [18]. In addition, MNSI has two steps including evaluation of neuropathic symptoms and physical examination to assess the appearance and sensation of feet. Patients had neurological examination of both feet according to the MNSI clinical test, involving: foot skin inspection for deformities, dry skin, calluses, infections, fissures, and ulcer, ankle reflex, and vibration sensation tested by a 128 Hz tuning fork placed over great toe. A score of ≥ 2 was considered abnormal. Abnormality in each item gets grades 0.5 to 1 and at least more than 2 abnormal items were required to reach the score of neuropathy [23].

Biopsy

A small mass of tissue was reserved from the edge of the ulcer and sent for slide preparation and staining. A fixative of 10% neutral buffered formalin (4% paraformaldehyde in PBS, pH 7.2) was applied to prevent degradation of the tissue. The tissue was engrossed in ascending grades of alcohol (70%, 90% and absolute alcohol) to eradicate all traces of water and then a clearing agent and xylene was used to remove the alcohol. After the tissues had been dehydrated and infiltrated with paraffin, they were placed into molds along with paraffin, which was then hardened by cooling. These interventions allowed easy cutting of tissue into 5 mm thickness sections. For light microscopy, sections were located on a glass slide and retained in an oven for several minutes to facilitate adherence of the tissue section to the slide. Before staining, the section was de-paraffinized twice with xylene, followed by twice with absolute alcohol. The tissue section was first stained with Hematoxylin for 20 min, washed with running tap water, followed by Eosin staining for 3 min. Water was removed with successive changes of absolute alcohol followed by xylene. Hematoxylin stains the nuclei blue and eosin stains the cytoplasm pink. Biopsy

samples were taken before application of nanofiber loaded with drugs and the end of treatment period.

Statistical analysis

All continuous and categorical data are presented as mean ± standard deviation (SD) and frequency (percentage), respectively. Paired sample *T*-test was used for analysis of differences within groups and independent sample *T*-test and repeated measure analysis of variance (ANOVA) for analysis between groups in each time. Chi-squared test was used to compare categorical data between groups. A value of $p \leq 0.05$ was considered statistically significant. All statistical analyses were conducted using the SPSS software version 15 (SPSS Inc., USA).

Results and discussion

Bacterial cellulose nanofiber sheets are the pure form of cellulose. They have unique properties such as high purity, high capacity, a 3D nano fibrillary network, high mechanical properties, biodegradability, and biocompatibility. Due to the easy adaptation of bacterial cellulose nanofibers for transdermal drug delivery, prevention of skin moisture loss, external infection and contamination, they are very useful in wound healing [26, 28, 29].

The use of these films for the transdermal delivery of a variety of drugs has been reported previously [25–27]. However, their use for co-delivery of DOX and VEN has not been reported so far.

FTIR and ATR-FTIR spectra

FTIR spectra of pure DOX, VEN, drug-loaded bacterial cellulose nanofibers and drug-free nanofibers are represented in Fig. 1. This figure shows a broad peak in the range of 3600–3300 cm^{-1} for drug-free and drug-loaded nanofibers which is identified by the hydrogen bonding of the hydroxyl groups of the cellulose [30–32].

The typical features of cellulosic substrates with intense bands around 3300, 2880, 1100, and 700 cm^{-1}, associated with the vibrations of the –OH, C–H, C–O–C and $–CH_2–$ groups, respectively are also seen in drugs loaded and blank nanofibers [17, 33].

The characteristic peak for VEN at 3348, 1512, 1471, 1245, and 1178 cm^{-1} correspond to the stretching of OH, stretching vibration of the C=C in benzene ring, C–O and amine groups, respectively [34]. In DOX spectrum, some of the characteristic peaks can be observed at 3399, 1670, and 1582 cm^{-1} which are related to hydroxyl group, carbonyl stretching of amide and C=C in DOX rings, respectively [35]. All of the characteristic peaks of VEN with no changes appeared in drug-loaded nanofibers spectrum too [36], which confirms successful loading of VEN in nanofibers. Hydroxyl broad and intense absorption of DOX spectrum was weak when it was loaded in nanofibers, representing the breakage of the intramolecular hydrogen bonds (C=O…HO)

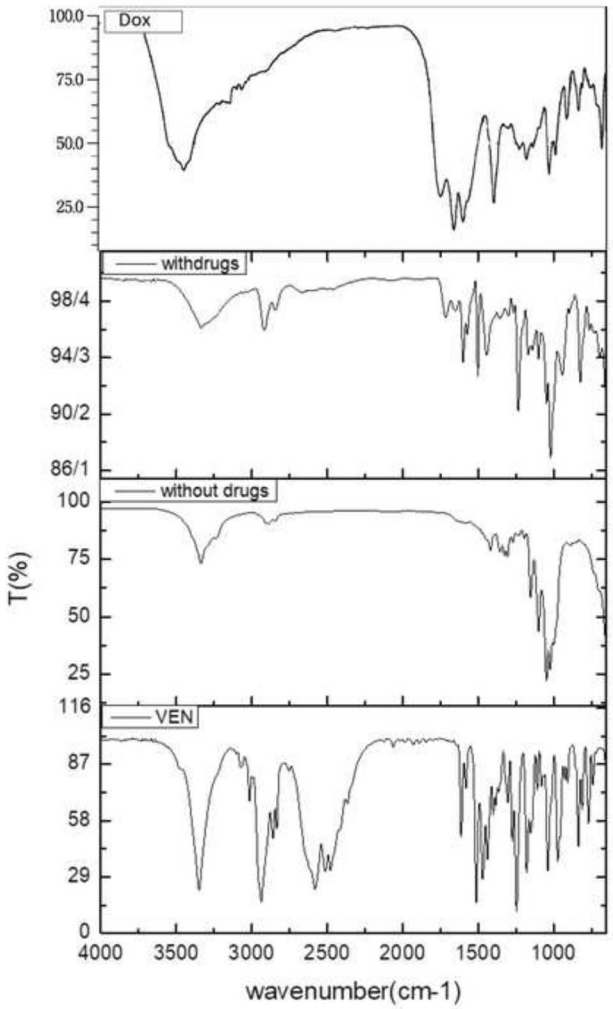


Fig. 1 FTIR spectra of pure DOX, drug-loaded bacterial cellulose nanofibers, drug-free bacterial cellulose nanofibers, and pure VEN

Table 1 Effect of different ratios of DOX and VEN loaded in a 2 cm×2 cm dried bacterial cellulose nanofibers sheet weighing 25 mg at pH 7

Formulation code	Nanofibers: drugs ratio	DOX (mg)	VEN (mg)	LE%	
				DOX	VEN
$C_1D_2V_0$	1:2	50	0	61 ± 6	–
$C_1D_0V_2$	1:2	0	50	–	47 ± 4
$C_1D_1V_1$	1:2	25	25	53 ± 8	20 ± 5
$C_1D_{0.5}V_{0.5}$	1:1	12.5	12.5	55 ± 3	23 ± 2
$C_1D_{1.5}V_{1.5}$	1:4	50	50	53 ± 2	18 ± 4

Table 2 Effect of different ratios of DOX and VEN loaded in a 2 cm × 2 cm dried bacterial cellulose nanofibers sheet weighing 25 mg at different pH

Formulation code	Nanofibers: drugs ratio	DOX (mg)	VEN (mg)	VEN:DOX ratio	pH	LE%	
						DOX	VEN
$C_1V_2D_1$	1:3	25	50	2:1	8	37.8 ± 1.6	48 ± 1.9
$C_1V_4D_1$	1:2.5	12	50	4:1	8	40.0 ± 3.1	50.8 ± 2.0
$C_1V_2D_1$	1:3	25	50	2:1	6	37.1 ± 2.3	47.3 ± 3.4
$C_1V_4D_1$	1:2.5	12	50	4:1	6	36.6 ± 5.9	49.0 ± 1.0

of DOX. Other DOX absorption peaks were exhibited at the same wavelength in drug-loaded nanofibers spectrum with no shift and changes in shape which could be interpreted that the loading process had no effect in DOX structure [37].

Drug loading in nanofibers

In this study, DOX (MMPs inhibitor antibiotic) and VEN (an antidepressant drug) were loaded in bacterial cellulose nanofibers sheets and used for the treatment of diabetic foot ulcer. There are different methods of drug loading in bacterial cellulose nanofibers including; in situ loading and post modification of the fibers which allows the preformed nanofibers swell to equilibrium in the drug solution.

In one research study [38], different methods were used for loading of lidocaine hydrochloride and ibuprofenin bacterial cellulose membranes. The researchers immersed the dry form of the membranes in the concentrated solution of the drug and let the naonofibers absorb all of the drug solution.

In the present study, the drugs were loaded after the preparation of the nanofibers allowing the produced gels to equilibrate in the drug solution and provide mild conditions, avoiding harmful impacts of gel formation and purification on drugs. This method is advantageous over other techniques such as the chemical cross-linking technology used during the chemical creation of the gels network to load hydrogels because it prevents unwanted chemical modification of the drugs.

DOX and VEN (ratio of 1:1) were loaded into the nanofiber. The total weight of the drugs to the weight of the nanofibers was 1:2. Nanofiber sheets were immersed into the drugs solution, stirred for 24 h and the pH was adjusted to 7 in room temperature. Nanofibers' surface was washed to remove the unloaded drugs remaining on the surface and then freeze-dried. Table 1 shows the results of drug loading efficiency in the nanofibers prepared with different ratios of the drugs to each other and drugs to nanofibers.

As shown in Table 1, LE% of DOX and VEN when loaded alone in nanofibers were about 61% and 47%, respectively. The high loaded drug amount obtained could be explained by the fine network structure of bacterial cellulose sheets consisting nanofibers, which caused a large fiber surface area. However, when the two drugs of nanofibers-to-drugs with a ratio of 1–2 were loaded together, LE% for both drugs was decreased although VEN was more affected. The carrier-to-drugs ratio was changed to investigate the effect of this variable on the LE% of VEN and DOX. As shown in Table 1, LE% increased by increasing the carrier to drugs ratio although the changes were not significant ($p > 0.05$). One possible reason may be the higher porosity and free

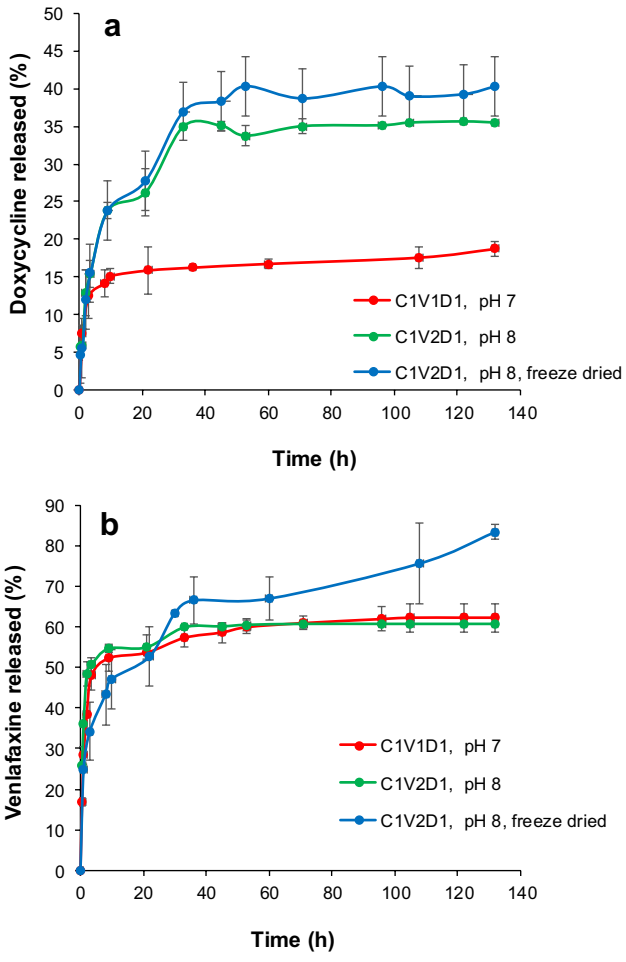


Fig. 2 In vitro release profiles of **a** DOX and **b** VEN from different studied formulation (mean ± SD, $n = 3$)

spaces in higher ratios of carrier which caused more entrapment of the drugs in the nanofibers sheets.

The study showed that changes in the ratio of total drugs to carriers had no significant effect on the low LE% of VEN and it was necessary to find another solution to enhance its low payload. A possible reason for low LE% of VEN may be the competition between VEN and DOX in entrapment in nanofibers. Therefore, an alternative strategy might be changing the ratio of VEN to DOX. On the other hand, considering the negative charge of cellulose fibers sheets and positive charge of VEN and DOX in the pH range lower than 3.5, it was expected that LE% was affected by pH too. So influence of pH on LE% was assessed by drug loading at a different pH.

For this reason, drug loading was studied in the different pHs of 2, 4, 6, 7, 8, 9 and 11 and three different ratios of the two drugs were loaded (Table 2). It was observed that pH greater than 8 and lower than 5 caused instability of the drugs and solution color change. Therefore, pH 6 and 8 were selected to continue the study and their results were compared with pH 7. As shown in Table 2, LE% of VEN increased by increasing the initial concentration of VEN in the loading solution. LE% of VEN increased from 20 to 50% while, LE% of DOX decreased from 50 to 40%. The pH did not have a significant effect on the LE% in the studied range. The optimum formulation was considered to be $C_1V_2D_1$ at pH 8 in which the ratio of VEN to DOX was 2:1, and the ratio of nanofiber carrier to total drugs was 1:3 (Table 2).

Previous studies had reported that the drug loading capacity per unit surface area of bacterial cellulose nanofibers sheets was 0.116 mg/cm^2 while in the present study, the drug loading capacity per unit surface area of the sheets was calculated to be 6.37 mg/cm^2 for VEN and 2.31 mg/cm^2 for DOX i.e., 300-fold and14-fold the previous reports, respectively [17, 39].

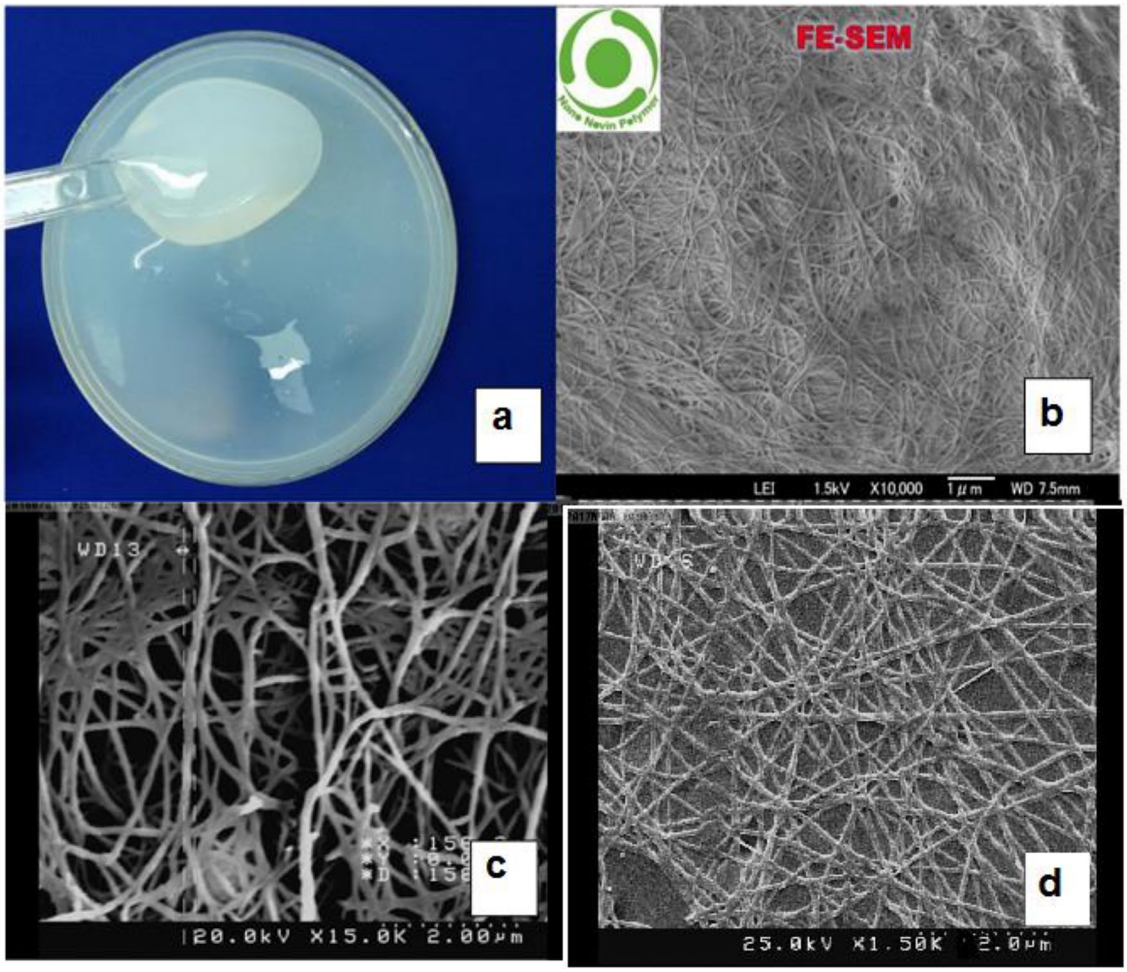


Fig. 3 **a** Physical appearance of bacterial cellulose nanofibers sheets, FE-SEM images of **b** the nanofibers, **c** drug-free freeze-dried nanofibers and **d** freeze-dried nanofibers loaded with the drugs

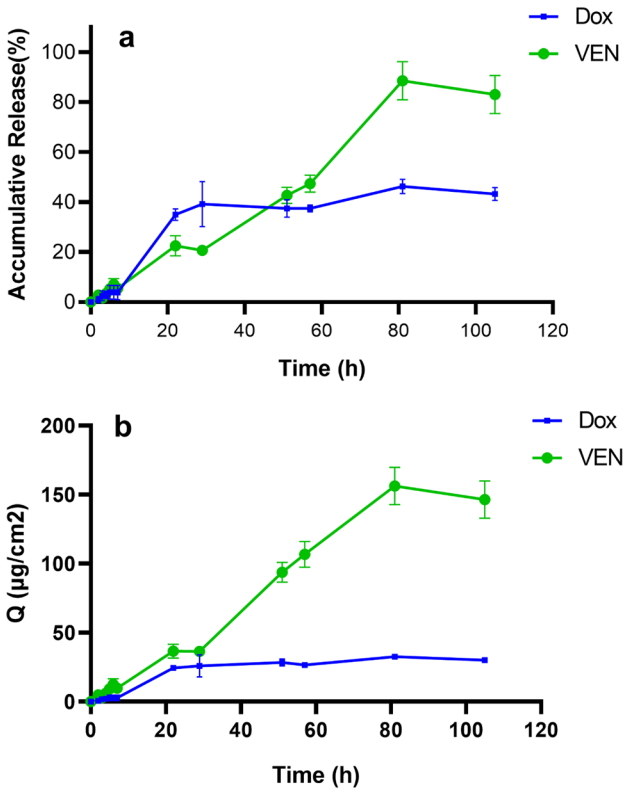


Fig. 4 **a** Accumulative release profile and **b** cumulative amount of drug released per surface area of rat skin (Q) of DOX and VEN from drug-loaded bacterial cellulose nanofibers sheet ($C_1V_2D_1$) in Franz diffusion cell (mean ± SD, $n = 3$)

In vitro drugs release studies

Drug-loaded bacterial cellulose nanofiber sheets were placed in a dialysis bag with a molecular weight cut-off of 12 kDa and immersed in 50 ml of PBS (pH 7, at 37 °C). The release profiles of VEN and DOXfrom $C_1V_2D_1$ prepared at pH 8 and $C_1D_1V_1$ prepared at pH 7 are presented in Fig. 2a, b, respectively. For both drugs, a burst release was seen in the initial hours which could be related to the release of the drugs located near the surface of the nanofibers. In two formulations, VEN showed more burst release and around 50% of VEN was released at the first 20 h. After that release, behavior altered to sustained release and continued up to 130 h. The two formulations did not show significant differences in their release pattern for VEN. The cumulative VEN released from the two formulations after the same times were 61% and 63.5% for $C_1V_2D_1$ prepared at pH 8 and $C_1D_1V_1$ prepared at pH 7, respectively.

DOX release profiles for these formulations were slightly different (Fig. 2b). In $C_1D_1V_1$ prepared at pH 7 about 12% of DOX was released at the first 2 h and after 132 h just 16.9% of the total DOX was released from this formulation. On the other hand, 34.5% of DOX was released slowly during 132 h from $C_1V_2D_1$ prepared at pH 8.

Release of VEN and DOX from $C_1V_2D_1$ prepared at pH 8 after freeze drying was assessed too and the results are shown in Fig. 2a, b, respectively. As shown in Fig. 2a, sudden release of VEN from freeze-dried formulation of $C_1V_2D_1$ prepared at pH 8 after the first hour is less than

Table 3 Clinical characteristics of the patients treated by bacterial cellulose nanofiber sheets dressing loaded with DOX and VEN and control group

	Treated group ($n = 10$)	Control group ($n = 10$)	*df*	*t* or chi-squared value	*P* value
Age (mean ± SD), years	78.0 ± 2.5 (52–86)	76.0 ± 3.1 (48–84)	18	1.53	0.64#
Sex (men/women)	5/5	6/4	1	0	0.46##
Smoking, *n* (%)	2 (20)	1 (10)	1	0	0.92##
Diabetes duration (mean ± SD), years	10.8 ± 7.1	11.1 ± 4.0	18	0.12	0.96#
Hba1c (%), mean (SD)	7.2(0.78)	7.25(0.80)	1	0.88	0.82##
Wound duration (mean ± SD), months	3.2 ± 2.2	4.2 ± 3.2	18	0.81	0.87#
Wound size (mean ± SD), cm^2	12.22 ± 6.53	13.1 ± 5.0	18	0.34	0.91#
Cr (mean ± SD), mg/dL	1.3 ± 0.1	1.2 ± 0.9	18	0.37	0.76#
History of cardiovascular accident, *n* (%)	3 (30)	3 (30)	1	0.81	0.21##
Hyperlipidemia, *n* (%)	6 (60)	3 (30)	1	0.24	0.12##
Hypertension, *n* (%)	6 (60)	4 (40)	1	0.80	0.98##

#Resulted from independent sample *T*-test

##Resulted from Chi-squared test

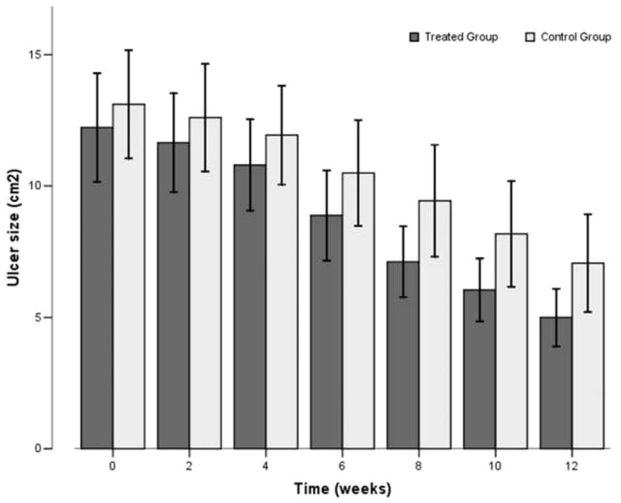


Fig. 5 Ulcer size in diabetic patients after treatment with bacterial cellulose nanofiber sheets dressing loaded with DOX and VEN ($n=10$) compared to the control group ($n=10$). Data are shown as mean ± standard deviation (SD), analyzed by independent sample *T*-test and repeated measure ANOVA test

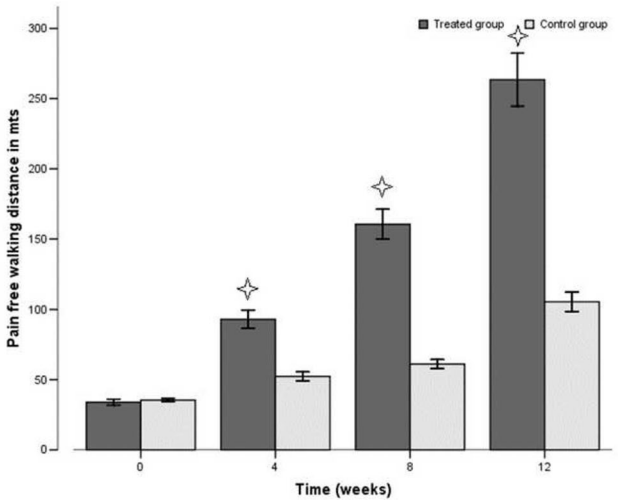


Fig. 6 Pain-free walking distance measured in meters (mts) in the diabetic patients after treatment with bacterial cellulose nanofiber sheets dressing loaded with DOX and VEN compared to the control group. Data are shown as mean ± standard derivation (SD). *Shows significant difference with the control group ($P<0.001$), analyzed by independent sample *T*-test and repeated measure ANOVA test

the wet sheets and was about 10% that could be because of wetting time and beginning of the drug release. After the first hour and until the end of the 8th hour, the release rate was relatively high and about 40% of the drug was released during this period. After that, the release rate decreased and continued slowly until about 83% of VEN was released at the end of 132 h. DOX release profile from $C_1V_2D_1$ prepared at pH 8 after freeze drying was similar to the wet formulation and after 132 h 40% of total DOX was released.

These results were not concordant with previous literature. In the Müller et al. [40] study, bovine serum albumin (BSA) was released from bacterial cellulose nanofibers with a sharp burst release in the initial 8 h and followed by a lower release rate up to 48 h at room temperature in PBS and at pH 7. They showed the burst release of BSA was more pronounced with higher protein concentrations. The higher BSA loaded onto the nanofibers caused the higher BSA release. They observed direct loading of BSA into the dry nanofibers was difficult because the dried fibers sheets were floating on the surface of the loading medium until complete wetting. They pre-swelled the dry nanofibers for 2, 12, and 24 h in purified water before use in loading experiments. The period of the pre-swelling stage had no effect on the total amount of BSA released or its release pattern. This result shows the reduced swelling ability of dried nanofibers sheets due to the structural changes during freeze-drying caused an inhibition of protein diffusion into the inner core of the nanofibers sheets network. Although freeze-drying is accepted as a mild drying method for the bacterial cellulose sheets, minor structural changes leading to an incomplete re-swelling is widely reported in the literature [40].

Shi and his colleagues [41] produced hybrid hydrogels made up of sheets of bacterial cellulose nanofibers and sodium alginate as a dual-stimuli responsive delivery system for ibuprofen but 90% of ibuprofen was released very quickly in 30 h.

In a similar study, Valo et al. [42] synthesized a highly porous nano-cellulose aerogels by freeze-drying from bacterial cellulose for oral delivery of beclomethasone dipropionate and observed 10–40% of the immobilized drug was released after the first 10 min and about 80% of it was released during the 11 h [42].

Silva et al. [25, 33] investigated the in vitro dissolution of diclofenac loaded in bacterial cellulose sheets as transdermal systems and observed about 60% of the total drug was released in the first 5 minutes and more than 90% after 10 min. They explained this phenomena by diclofenac diffusion through the porous tri-dimensional network of the bacterial cellulose sheets. They believed drug release profile also depended on the physicochemical properties of the drug and on their possible interactions with the bacterial cellulose nanofibrils [25, 43].

Morphology and size of bacterial cellulose nanofibers

Morphology and diameter of bacterial cellulose nanofibers were studied by the FE-SEM. As shown in Fig. 3, the diameter of these nanofibers was 100–150 nm. FE-SEM imaging were taken after drug loading and freeze drying of the sheets to investigate the effect of these processes on the

Table 4 Summary of analysis of repeated variance for the average of wound size and walking without pain in treated and control group

	Main effect interaction									
	Week 0	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	P1 time	P2 group	P3 time*group
Wound size (cm^2)										
Treated group	12.22 ± 6.53	11.64 ± 5.93	10.79 ± 5.49	8.87 ± 5.41	7.12 ± 4.23	6.04 ± 3.78	5.0 ± 3.46	0.001	0.53	0.83
Control group	13.10 ± 5.04	12.60 ± 5.01	11.93 ± 4.61	10.49 ± 4.94	9.43 ± 5.21	8.17 ± 4.90	7.06 ± 4.55			
P4	0.91	0.74	0.67	0.56	0.34	0.34	0.32			
Walking without pain (m)										
Treated group	34 ± 6.86		93 ± 20.67		160.5 ± 33.83		263.5 ± 59.6	0.001	0.001	0.001
Control group	35.50 ± 3.27		52.33 ± 7.89		61.16 ± 7.83		105.5 ± 17.0			
P4	0.62		< 0.001		< 0.001		< 0.001			

P1, P2 and P3 based on repeated measure ANOVA and P4 based on sample *T*-test, all variables are presented as mean ± SD

structure and shape of the fibers structures. Figure 3 shows that the shape and size of the fibers were unchanged after the preparation process. While drug loading caused no change in size and structure of the nanofibers, it caused changing the nanofibers sheet texture and morphology as compared to the drug-free nanofibers [40].

Franz diffusion cell study

In vitro diffusion of DOX and VEN from skin of adult Wistar rats were studied on Franz diffusion cells. Data of the drug release evaluation and the relationship between Q (cumulative amount of drugs released per surface area of membrane; μg/cm^2) versus time are shown in Fig. 4a, b, respectively.

As shown in Fig. 4, sudden release of VEN and DOX from nanofibers sheet at the first hours which were observed in drugs release test (Fig. 3) were eliminated in the diffusion study from skin for both drugs. In the first hours, drugs permeated slowly from the skin and until 7 h both permeated VEN and DOX through skin were very little, after 7–29 h DOX release rate was relatively high and about 40% of total DOX loaded in formulation, passed from the skin. After that time the release rate decreased again and continued slowly at the end of the study and only an additional 5% was released over 105 h. On the other hand, 83% of VEN was passed after 7 h gradually during 105 h from the skin.

In general, drugs release from the optimal formulation ($C_1V_2D_1$, pH 8, freeze-dried) in Franz diffusion cell study was slower and more homogenized (Fig. 4) compared to the bag dialysis study (Fig. 3). These results were quite predictable considering the multi-layers of the skin and the presence of these barriers in the passage of the drug. However, in general, the total released amount of drugs during the study for both drugs were similar in the two methods. Although in some studies, up to 100% of the loaded drug in the formulation has passed through the skin, but in the present study, neither DOXnor VEN released completely in the dialysis bag and Franz diffusion cell methods. The reason for this phenomenon may be due to the very porous and tangled structure of cellulose nanofibers sheet that did not allow drugs to leave this structure and trapped them in its complex structure [44].

Clinical studies of nanofibers in diabetic patients

Clinical characteristics of the patients in the treated and control groups are presented in Table 3. In the treated ($n = 10$) and control group ($n = 10$), in the first week of treatment the ulcer size was 12.22 ± 6.53 cm^2 and 13.1 ± 5.05 cm^2, respectively. Obvious reduction in the ulcer size was observed within 12 weeks so; in the treated and control group, it

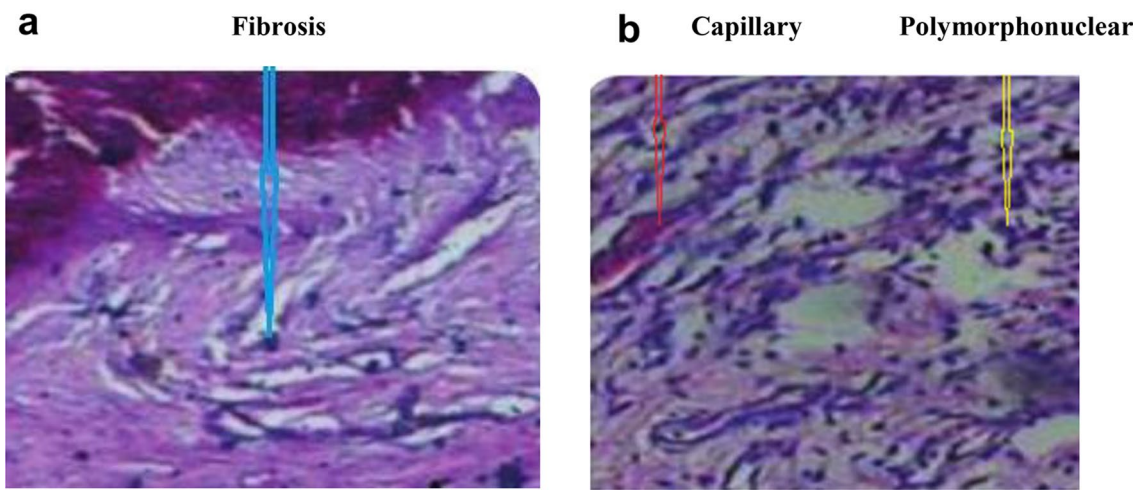


Fig. 7 Micro section of tissues from the wound site **a** before treatment by nanofibers dressing loaded with DOX and VEN showing fibrosis and **b** obtained 12 weeks after treatment by nanofibers dressing loaded with drugs showing polymorphonuclear cells along with new capillary bed formation

Table 5 Biochemical characteristics of the patients treated ($n=10$) by bacterial cellulose nanofiber sheets dressing loaded with DOX and VEN

Biochemical parameter	Before treatment Mean ± SD	12 weeks after treatment Mean ± SD	*df*	*t*-value	*P* value
FBS (mg/dl)	120.1 ± 16.6	117.6 ± 8.6	9	1.76	0.67
Urea (mg/dl)	39.0 ± 8.8	39.2 ± 9.6	9	0.27	0.96
Creatinine (mg/dl)	1.3 ± 0.1	1.39 ± 0.2	9	1.35	0.21
Serum total bilirubin (IU/L)	0.78 ± 0.3	0.70 ± 0.1	9	3.32	0.39
SGOT (IU/L)	30.8 ± 5.3	32.5 ± 5.0	9	3.31	0.46
SGPT (IU/L)	36.5 ± 2.5	35.2 ± 7.0	9	1.34	0.47
ALP (IU/L)	79.9 ± 12.5	78.8 ± 8.8	9	1.24	0.82
Total cholesterol (mg/dl)	145.6 ± 22.1	145.4 ± 22.4	9	0.11	0.98
Triglyceride (mg/dL)	100.8 ± 1.3	102.8 ± 2.8	9	9.57	0.17

Resulted from paired *T* test

reduced to 5.00 ± 3.46 cm^2 and 7.06 ± 4.55 cm^2, respectively ($p<0.001$). Although there was no significant difference between the treated group and the control group in the ulcer size during 12 weeks treatment, this difference was not observed even in week 12 ($p=0.32$); however, the reduction in the treated group was speedier (Fig. 5).

The general reduction in the ulcer size was more evident in the treated group at the end of the follow-up period. The mean ± standard deviation (SD) wound recovery (%) was 63.6 ± 20.5 and 51.3 ± 25.4 in the treated group and the control group, respectively. This difference in wound recovery percentile was not significant ($p=0.3$) during the 12-week but an increase in the recovery was observed in the treated group, showing the therapeutic efficacy of the nanofibers loaded with DOX and VEN. In Fig. 5, ANOVA test for repeated measures showed no statistically significant difference between groups ($p=0.537$) and when groups were compared during study period ($p=0.836$), but ANOVA test for repeated measures in study the time effect showed statistically significant difference in times ($p=0.001$).

In the treated group ($n=10$), the pain-free walking distance increased from 34.00 ± 6.86 m to 263.50 ± 59.63 m ($p<0.001$), whereas in the control group, it increased from 35.5 ± 3.27 m to 105.5 ± 17.01 m at 12 weeks follow-up ($p<0.001$) (Fig. 6). There was an increase in pain-free walking distance in both groups, and a significant difference in the pain-free walking distance was seen between the treated and the control groups ($p<0.001$). In Fig. 6 according to ANOVA analysis for repeated measures, a statistically significant difference was seen between groups ($p=0.001$), when groups were compared during the study period ($p=0.001$) and in different times ($p=0.001$).

The mean ± SD MNSI questionnaire pre-treatment were 6.8 ± 4.2, 7.1 ± 6.8 in treatment and control groups,

respectively. After treatment, these abnormal responses in the MNSI questionnaire decreased significantly in the DOX and VEN group (4.2 ± 0.98) when compared with the control group (8.3 ± 4.1) ($p < 0.05$). In this study, the effect of venlafaxine on improving neuropathic pain scale and free walking distance has been observed, suggesting that it could alleviate neuropathic pain. This drug has been used as a common choice to treat neuropathic pain in practice. Kadiroglu et al. [12] used the McGill pain questionnaire in their study and showed a reduction rate of 53% in pain with venlafaxine as compared to 22% in control group. Finally, Razazian etal. [45] showed that with the visual analog scale (VAS) scores for venlafaxine treatment groups at the baseline and end point revealed significant reduction with treatment.

The laboratory data indicating safety of administration, were collected and compared with pretreatment levels. Serum urea, creatinine, total bilirubin, glutamic oxaloacetic transaminase (SGOT), glutamate pyruvate transaminase (SGPT), alkaline phosphate (ALP), cholesterol, triglyceride, and plasma glucose were measured before treatment and 12 weeks after treatment in the treated group. There was no significant difference observed in the parameters before and after treatment with bacterial cellulose sheets, indicating no change in parameters (Table 4).

Slides of tissue were obtained from the border of the ulcer for microscopic pathology studies. The sections that were studied before using nanofibers dressing loaded with DOX and VEN showed few dermal cells and extensive fibrosis, consistent with dermal scar formation. Dermal scar formation is a consistent feature of a non-healing wound (Fig. 7a). However, tissue slides obtained 12 weeks after using nanofibers dressing loaded with the drugs showed abundant chronic inflammatory cells, mostly polymorphonuclear cells along with new capillary bed formation (Fig. 7b).

Conclusions

In this study, bacterial cellulose nanofiber sheets were loaded with DOX and VEN and categorized by their loading efficiency and release profiles with an optimum formulation deciding the ratio of 1:3 of nanofibers to total drugs, loaded at pH of 8 and with a 2:1 ratio of VEN to DOX. This compound was used in patients with diabetic foot ulcer in a controlled clinical trial (Table 5).

Treatment with bacterial cellulose sheets did not result in any significant difference in the biochemical parameters of subjects.

The ulcers showed faster reduction in size after 12 weeks in the treated group compared to the control group but this was not clinically significant.

After treatment, the abnormal responses in the MNSI questionnaire decreased significantly in the group using drugs loaded with nanofibers compared with the control group and in the microscopic slides of the skin, obtained 12 weeks after use, an improvement was noticed with new capillary bed formation. The results show that bacterial cellulose nanofibers sheet loaded with DOX and VEN may be an option in the management of diabetic foot ulcer neuropathy and may expedite reduction of the ulcer size.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals All procedures performed in this study involving human participants were in accordance with the ethical standards of the Isfahan University of Medical Sciences ethics committee (license code of 9531) and have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki.

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