Preparation of PLA / Chitosan Nanoscaffolds Containing Cod Liver Oil and Experimental Diabetic Wound Healing in Male Rats Study

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Research

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Abstract

Background

Diabetes mellitus is one of the most common metabolic disorders. One of the important metabolic complications in diabetes is diabetic foot ulcer syndrome, which causes delayed and abnormal healing of the wound. The formulation of nanoscaffolds containing cod liver oil by altering the hemodynamic balance toward the vasodilators state, increasing wound blood supply, and altering plasma membrane properties, namely altering the membrane phospholipids composition can be effective in wound healing. In this study electrospinning method was used to produce poly lactic acid/chitosan nanoscaffolds as a suitable bio substitute.

Results

The results showed that the optimum formulation of cod liver oil was 30%, which formed a very thin fiber that rapidly absorbed to the wound and produced significant healing effects. According to the results poly lactic acid / chitosan nanoscaffolds containing cod liver oil can be a suitable bio product to use in the treatment of diabetic foot ulcer syndrome.

Conclusions

The poly lactic acid / chitosan nanoscaffolds containing 30% cod liver oil show more healing and less wound area on the fourteenth day which this can be due to permeability and sufficient oxygen for tissue repair. Moisture retention of the wound medium to accelerate its healing, color change and pH changes to keep the wound site safe from bacteria, contamination and non-contamination are among the characteristics of the nanoscaffold produced.

Research Highlights

a. Poly lactic acid / chitosan nanoscaffolds were synthesized with microwave-assisted electrospinning process.

b. Nanoscaffolds show high potential in wound healing recovery after fourteen days.

c. PLA/Chitosan nanoscaffolds containing 30% cod liver oil were synthesized with size about 50-150nm.

d. Wound area indicated that on the fourteenth day there was significant improvement in wound surface.

1. Introduction
The global prevalence of diabetes has increased dramatically over the past two decades(1). Diabetes mellitus is the most common heterogeneous metabolic disorder(2), which is associated with a disorder in the metabolism of sugars, lipids and proteins, and is characterized by elevated blood glucose or insulin response to tissues(3, 4). Diabetic patients have limited ability to stimulate the immune response and are very susceptible to infection and at the risk of terminal limb amputation and recurrence of the wound(5). Fatty acids have physiological and pathological roles in diseases such as atherosclerosis, inflammation, or normal wound healing(6, 7). The effect of fatty acids on wound healing is through alterations in plasma membrane properties(8), such as changes in membrane phospholipids composition(9), increased growth factor activity (10), cell differentiation, decreased eicosanoids production, and lipid mediators of inflammation, followed by reduction inflammation, production of interleukin-1 and collagen(11). The fish liver oil as a rich source of omega-3 fatty acids has many potential effects in modulating various diseases, especially diabetes mellitus(12, 13). Many scientific researches have shown which fish liver oil accelerate many of the potential mechanisms involved in wound healing(14–16). In recent years, new drug delivery systems such as nanofibers(17), nanoparticles(18), cell therapy and stem cell(19) use as alternative therapies to common pharmaceutical methods have received much attention which reduce the need for continuous follow-up of the disease and increase the quality of treatment(20). Chitosan structures have a good crosslink structure for encapsulating drugs(21), and polylactic acid possesses properties such as the ability to form hydrogels in physiological conditions(22), mild gel degradation for a wound to heal successfully, and the growth and movement of nutrients(23).

In recent years, the science of nanotechnology has attracted particular attention from researchers in various fields of medicine and pharmaceutical field(24). Nanofibers and nanoparticles can release the drug in a controlled approach for a long time (25). These structures can act as an appropriate topical drug delivery system that can provide the appropriate drug concentration, and other advantages of this system include the ability to transport hydrophilic and lipophilic drugs simultaneously depending on their structure(26). Examples of natural polymers which used in the fabrication of nanofibers include creatine(27), gelatin(28), cellulose(29), and polysaccharides such as chitosan and alginate. Microwave irradiation as a cost-effective, low environmental pollution (eco-friendly) and high efficiency method is used in the preparation of nanoparticles for various applications(30), electrospinning process using a high-voltage power generate polymer fibers in nanometer dimensions which exhibit unique physical and chemical properties(31). In this study new developments in the fabrication of nanoscaffold materials such as the microwave-assisted electrospinning process were applied to preparation and formulation of poly lactic acid / chitosan containing cod liver oil as a suitable cost effective methods. Summary of researches about nanoscaffolds applications in wound healing recovery have been displayed in Table 1. The results also indicated that the synergistic effect quantity of the poly lactic acid / chitosan containing hydrogel is the key factor in obtaining a suitable biological wound for wound dressing.
| Types of Nanocomposites                  | Particle Size (nm) | Synthesis method          | Characterization             | References |
|----------------------------------------|-------------------|----------------------------|------------------------------|------------|
| PVA–clay nanocomposite                 | 60–100            | cyclic freezing–thawing    | SEM, XRD, Tensile modulus    | (33)       |
| Chitosan                               | 20–40             | coprecipitation            | FTIR, TGA, DSC, SEM and TEM  | (34)       |
| Cerium oxide nanoparticle-containing poly (ε-caprolactone) | 50                | electrospinning            | SEM, DLS, MTT                | (35)       |
| Halloysite and chitosan oligosaccharide | 200               | composition                | SEM, EDX, UHRTEM, Zeta potential In vivo | (36) |
| Montmorillonite/chitosan               | -                 | Solid state                | HRTEM, XRD, XEDS, TGA        | (37)       |

2. Experimental

2.1 Preparation of PLA/ Chitosan nanocomposites

To prepare the polymer phase at the first 0.2 g of PLA dissolved in 18:3 ml ratio of deionized water and ethanol after heating and stirred under 50 ° C and RPM 400 for 45 minutes, then 5 ml of NaOH (2 mol / Lit) added to the above solution and this solution was heated at 60 °C and stirred for 30 min. In the next step, 0.05 g of chitosan was dissolved in 2:1 ml ratio of deionized water and dimethylformamide, after 110 minutes, Subsequently, the solutions transferred into a beaker and exposed to microwave irradiation oven under the power of 450 watts for 5 minutes. Regular cycles of the microwave irradiation were set to 30 seconds off and 60 seconds on. Finally, the solutions were placed in an environment free of contamination for 24 hours to complete the crystallization process.

2.2 Cod liver oil loading

2 ml of polymeric solution was added to 100 µl of the drug with concentrations of 15% and 30% by weight in the present of 350 µl tween as surfactant agent and place on reflux system for 30 minutes at 50 ° C and 500 rpm on the shaker for 15 minutes. PLA/ Chitosan nanoscaffolds containing cod liver oil were formed with an electrospinning device. Nanoscaffolds containing 30% w/w and 15% w/w fish liver oil were prepared at a speed of 2 ml/h, 12.1V and jet rotation speed of 100 rpm were used to form nanofibers.

2.3 In vivo study
For in vivo study male rats were divided into four groups (each group containing 6 mice weighing approximately 200 g). Animals were diabetic by intraperitoneal injection of 60 mg / kg and their diabetes was confirmed after 3 days by measuring glucose using a glucometer. Then, after anesthetize the rats with ketamine / xylazine, an ulcer about 1.5 cm in area between the two scapula was created by punch biopsy and then drug was positioned topically on each group. For this study, four groups of mice were divided into the following groups, Group 1 mices which treated with nanofiber alone, group 2 mices which treated with fish oil only, group 3 mices which treated with nanofiber delivery system containing fish liver oil for wound healing and group 4 mices which not receiving any treatment (negative control).

3. Result And Discussion

3.1. Physicochemical characteristics

Morphological properties and surface features of the nanofibers were observed through scanning electron microscopy (SEM) images. SEM images with approximate scaffold size of poly lactic acid / chitosan nanofibers containing 30% and 15% cod liver oil displayed in Fig. 1a and 1b respectively. As can be seen from the SEM images of the nanoscaffold structures are uniform and without any cracking along the formation. The nanoscaffold structures encapsulated different concentration of cod liver oil without any systemic defects and nanofibers containing 30% cod liver oil show less diameter than 15% cod liver oil which this may be due to the higher solubility of the polymer phase at higher concentrations of oil phase. According to SEM images average size of the diameter scaffold tube estimated between about 50–150 nm. To investigation of the three-dimensional (3D) images of the fibers we did transmission electron microscopy (TEM). From the TEM images it can be seen that cod liver oil trapped uniformly in micelles during in poly lactic acid / chitosan scaffolds. The oil phase ranges in the polymer phase are well defined. The TEM image of 30% w/w cod liver oil cod liver oil distributed in poly lactic acid / chitosan nanoscaffolds is shown in Fig. 1c.

Here Fig. 1a, b and c.

Energy dispersive spectroscopy (EDAX) as a suitable technique used for the semi-quantitative analysis of elements. This method is mainly used to obtain point chemical composition and quantitative investigation of the poly lactic acid / chitosan nanoscaffolds containing cod liver oil. About 37.17 percent of the total atomic weight of the final products is related to C atoms whish this could be related to carbon atoms in poly lactic acid, chitosan, omega3 in cod liver oil and Tween structures. The existence of the O, Na and N atoms to the amount about 31.63, 5.31 and 15.36 percent could be related to the existence of these atoms in poly lactic acid, chitosan, cod liver oil, tween, NaOH and dimethylformamide structures. The small amounts of the Ti, Ca atoms could be related to the unpredictable impurities in the final products. The EDAX analysis of the poly lactic acid / chitosan nanoscaffolds containing 30% cod liver oil is shown in Fig. 2a. The size distribution obtained of the nanoscaffolds is as a plot of the relative intensity of light scattered by nanoscaffolds in various size classes and introduced as an intensity size distribution. Results related to size distribution of the nanoscaffolds obtained from dynamic light
scattering analysis show well match with SEM images and estimates the size of the poly lactic acid / chitosan nanoscaffolds containing 30% cod liver after 15 min ultrasonic irradiation at 60 W fibers about 50–150 nm in Fig. 2b.

Here Fig. 2a and 2b.

UV-Vis as a general qualitative technique can be used to identify and confirm functional groups in a compound by matching the absorbance spectrum. Absorption in UV-Vis spectroscopy follows Beer's Law:

$$A = \varepsilon \times b \times C \text{ Eq. 1}$$

where $\varepsilon$ is the molar attenuation coefficient, $b$ is path length, and $C$ is concentration. UV-Vis absorption spectra of poly lactic acid / chitosan nanoscaffolds containing cod liver oil shows that with increasing concentration from 15% w/w to 30% w/w absorbent peaks become noticeably more intense, however cod liver oil not absorbed alone and it can be concluded that more loading of cod liver oil take place in poly lactic acid / chitosan nanoscaffolds. Figure 3a demonstrated UV–vis absorption spectra of poly lactic acid / chitosan nanofibers containing cod liver oil 30% w/w, 15% w/w compared with cod liver oil(32).

Fourier Transform infrared spectroscopy (FT-IR) as an analytical technique used to identify functional groups in materials. Figure 3b and 3c show FT-IR spectrum of the prepared poly lactic acid / chitosan nanoscaffolds containing 30% w/w and 15% w/w cod liver oil in the region 400–4000 cm$^{-1}$ respectively. The absorption peaks at 3454 cm$^{-1}$ and 1630 cm$^{-1}$ region are attributed to the stretching and bending vibration of O–H groups from chitosan and omega3 structure in cod liver oil. The obtained peaks at 2884 cm$^{-1}$, 1650 cm$^{-1}$, 1600 cm$^{-1}$ that expressed existence of stretching mode C-H, CH$_2$ groups, C = O, C = C and C-N region in chitosan omega3 in cod liver oil and poly lactic acid structures. The reflectance at 3093 cm$^{-1}$ shows N-H band in chitosan. In general, it can be said that cod liver oil structures with forming chemical bonds are located in nanobio polymeric nanoscaffold structures.

Here Fig. 3a, 3b and 3c

3.2. In vivo Study

Diabetic rats were evaluated in two ways; a) blood glucose measurement by glucometer, rats with blood glucose above 200 mg / dl were considered diabetic and selected for further study, b) the selected rats were examined for the appearance of diabetic symptoms including polyuria, overeating and thirst and were assured of their diabetic status. The wound healing process in diabetic rats in the four study groups was evaluated on days 0, 3, 7 and 14 by measuring the wound area and were presented in Table 2. As shown in the results and tables, in the group using nanofibers mixed with cod liver oil, the wound healing process (assessed by measuring the wound area) was investigated. Significantly, it has shown better results than the group that used nanofiber alone or cod liver oil alone. It also appears that 30% cod liver oil supplementation with polyelactic acid / chitosan nanoscaffolds heals 94.5% wound healing on day 14, whereas 15% cod liver oil can heal wounds about 86% on day 14 which were presented in Table 3. Macroscopic changes of the wound were evaluated for treatment progress on days 0, 3, 7 and 14 after
treatment and recorded with a photograph. The percentage of wound healing was calculated using the following formula:

$$\text{Percentage of recovery} = \frac{(\text{Surface wound on the first day} - \text{Surface wound in day } X)}{(\text{Surface wound on the first day})} \times 100$$

**Table 2**

| Day                      | Zero  | Three | Seven  | Fourteenth |
|--------------------------|-------|-------|--------|------------|
| Control group            | 152.67| 133.88| 116.85 | 87.05      |
| Nanofiber + cod liver oil 30% | 154.15| 96.60 | 54.31  | 8.43       |
| Nanofiber + cod liver oil 15% | 154.15| 102.83| 71.61  | 20.07      |
| Poly Lactic Acid / Chitosan Nanofibers | 154.15| 133.88| 100.81 | 61.53      |
| Drug soluble group       | 154.15| 110.30| 93.18  | 61.81      |

**Table 3**

| Percentage of recovery per day | Seven | Fourteenth |
|-------------------------------|-------|------------|
| Control group                 | 23.5  | 42.0       |
| Nanofiber + cod liver oil 30% | 64.8  | 94.5       |
| Nanofiber + cod liver oil 15% | 53.5  | 86.0       |
| Poly Lactic Acid / Chitosan Nanofibers | 34.1  | 60.0       |
| Drug soluble group            | 39.5  | 59.9       |

Figure 4 demonstrated a 14-day course examination of the wound surface and a photograph of the wound healing process. At day zero, third, seventh, and fourteenth, the rate of wound healing in the nanoscaffold saline-treated mice with 30% cod oil was significantly different from that of the untreated control group and also from the other groups. This prospect which poly lactic acid / chitosan nanoscaffolds as biocompatible and biodegradable polymers able to interact with skin cells and their environment and accelerate the healing process. Due to the high porosity of the nanoscaffold coating and also due to the hydrogel like properties of the polymers used, the coating swells after absorption of moisture and creates a very small gap between the coating and the wound surface. On the 14th day, these characteristics are well observed and the nanoscaffold porosity property permits oxygen to pass through the wound while keeping its surface moist.

Here Fig. 4
Wound area in five groups of animals studied on days 0, 3, 7 and 14 (Mean ± sd; N = 3) is shown in Fig. 5. As can be seen from the diagram, the area of the wound in the group with 30% cod oil was significantly less than the other groups, this indicates a greater improvement in this group than in the other groups. The presence of biocompatible nanofibers not only induces immune and allergic responses but also induces the body to resemble the original tissue located in the wound. As a result, biochemical signals are needed to accelerate recovery and eventually wound healing will occur faster.

Here Fig. 5

4. Conclusion

In this research work, the nanofibers scaffold produced with electrospinning method were used to repair wounds on the skin of the rats. Macroscopic and microscopic studies were performed on the wounds to determine the efficacy of the nanoscaffolds produced and after the desired time. The poly lactic acid / chitosan as biocompatible and biodegradable polymers scaffold were designed for wound healing to be able to control drug (cod liver oil) release over a long period of time by using improve in chemical structure. The poly lactic acid / chitosan nanoscaffolds containing 30% cod liver oil show more healing and less wound area on the fourteenth day which this can be due to permeability and sufficient oxygen for tissue repair. Moisture retention of the wound medium to accelerate its healing, color change and pH changes to keep the wound site safe from bacteria, contamination and non-contamination are among the characteristics of the nanoscaffold produced.

5. Materials And Instrumentations

All materials and precursors used in this research work were pure without any impurities and were purchased directly from reputable commercial centers. Chitosan (CAS: 9012-76-4, MW Mol wt: 50,000 daltons based on viscosity, 99.90%), Polylactic acid (C(CH₃) HC(= O) O–) and Dimethylformamide (DMF, MW; 73.095 g·mol⁻¹) were purchased from Sigma Aldrich agents in IRAN.) Polysorbate 80 (tween 80, C₆₄H₁₂₄O₂₆, MW: 1.310 g/mol) was purchased from FLOKA company in Switzerland. NaOH (d: 2.13 g/ml, MW: 39.9971 g/mol, 99.99%) was purchased from Dr. Abidi company in IRAN. Xylazine and Ketamine for anesthesia and intraperitoneal tolerance in rats were purchased from Alfasan group of companies in Netherlands. Male rats were obtained from Kerman university of medical animal's farm. Also this study received ethical approval from the local ethical committee of the kerman university of medical sciences as a thesis research at the faculty of pharmacy kerman university of medical sciences with number 1124. Male rat weighing 150–200 g was fed with standard diet and kept under 12:12 hr light/dark cycles, at 20°C and relative humidity of 25–30%. XRD patterns for crystalline phase detection were recorded by a Rigaku D-max C III, X-ray diffractometer using Ni-filtered Cu Ka radiation. Microscopic morphology and investigation of surface properties of the products were characterized by SEM (LEO 1455VP). The energy dispersive spectrometry (EDS) analysis to determine the elements in the samples was studied by XL30. Transmission electron microscopy (TEM) images were obtained with a Philips EM208 transmission electron microscope with an accelerating voltage of 200 kV. Fourier transform infrared (FT-
IR) spectra were recorded on Shimadzu Varian 4300 spectrophotometer in KBr pellets. To absorption evaluate samples Ultraviolet–visible spectroscopy analysis was carried out using Shimadzu UV-2600 UV–Vis spectrophotometer.

**Declarations**

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**Authors' contributions**

Payam Khazaeli, conceived of the presented idea, writing, evaluation

Maryam Alaei, helped supervise the project, contributed to sample preparation, evaluation, writing, evaluation

Mohammad Khaksarihadad, designed and performed the experiments, writing

Mehdi Ranjbar, evaluation, writing

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