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Ethosomal Insulin Loaded Biodegradable Electrospun Fibrous Mats: A Novel and Smart Delivery System for Effective Treatment of Diabetic Wounds

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Abstract

Delayed wound healing associated with diabetes mellitus remains a critical challenge that still needs to be targeted with novel and smart approaches. A novel ethosomal insulin loaded polyvinyl alcohol (PVA) electrospun fibrous mat was designed and prepared as a local delivery platform with sustained and efficient insulin release for mechanistic treatment of diabetic wounds. Insulin loaded and insulin free (blank) ethosomes were both prepared through thin layer hydration method with an average hydrodynamic particles size of 263.8 nm and 221.9 nm, respectively. Insulin loaded ethosomes were nearly spherical in shape as imaged by transmission electron microscopy (TEM) with a reported insulin encapsulation efficiency of 64.5%±4.98. All PVA elesctrospun fibrous mats fabricated with either pure insulin, blank ethosomes or insulin loaded ethosomes were nearly uniform in size as indicated by scan electron microscopy (SEM). Insulin loaded ethosomes released 84.8% of loaded insulin during 8 hours whereas insulin loaded ethosomal PVA mats released only about 41.3% of insulin at the same time. I*n-vivo* study proved the potential medical effect of fibrous mats loaded with ethosomal insulin in healing diabetic wounds successfully and efficiently by fourth day. The prepared PVA/insulin loaded ethosomes embedded into polymeric electrospun fiber could be a novel, effective therapeutic alternative for diabetic wound treatment. Further investigation should be performed on diabetic individuals to validate the potential wound healing effect of the generated ethosomal insulin loaded PVA patches.

Keywords: PVA electropsun nanofiber, ethosomes, ethosomal insulin, wound dressing, diabetic rat

Introduction:

Diabetes mellitus is a chronic metabolic disorder characterized by high blood glucose levels due to either inadequate insulin production or insulin resistance. (1-3). Over time, poorly controlled diabetes can lead to a variety of complications, including neuropathy, nephropathy, retinopathy, cardiovascular disease, kidney failure and diabetic foot ulcers (**DFUs)**. The latter is one of the major complications of diabetes and is commonly located on the bottom of the foot (1-3). Despite the development of successful therapeutic interventions against various diseases and disorders, DFUs remain challenging and notoriously difficult to treat due to the underlying conditions that accompany them, such as neuropathy, poor circulation, and impaired immune responses. All those factors combine to delay wound healing process leading to increased infection risk. (3-6). It was estimated that the lifetime risk for developing such devastating disorder among population is 25%. In 2005, the average frequency of non-traumatic lower extremity amputations in diabetic patients was one every 30 seconds. These amputations have far-reaching adverse implications for quality of life, mortality, and health resource (1, 3, 6).

Relative or absolute lack of either insulin quantity or insulin action is one of the major hallmarks that would lead ultimately and over prolonged period to impaired glucose metabolism and homeostasis leading to development of diabetes mellitus complications such as neuropathy, nephrorpathy, retinopathy and delayed wound healing capability. Other factors that specifically contribute to delayed wound healing in diabetes mellitus include angiogenic response, decreased growth factor production, angiogenic response, collagen accumulation, macrophage function, fibroblast and keratinocyte proliferation/migration and epidermal barrier function (6-8).

Over the past few decades, several studies have indicated that topical application of insulin on skin had significantly contributed in enhancing wound healing capability (6-20). It was extensively reported with plethora of published research studies that topical insulin accelerated healing of skin wounds in diabetic humans and rats as well without induction of systemic hypoglycemic effect It was reported that insulin stimulated development and growth of different types of cells which in turn affected migration, proliferation and secretions by fibroblasts, endothelial cells and keratinocytes (21-24). Topical treatment that contained low concentration of insulin doses stimulated local development and growth of different types of

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keratinocytes and affected migration, proliferation and secretions by fibroblasts and endothelial cells for faster recovery of wounds. Topical insulin application led to significant modulation of inflammatory response which in turn initiated wound recovery process by enhancing endothelial permeability, macrophage polarization and infiltration, neutrophils apoptosis, secretion of various growth factors such as vascular endothelial growth factor and chemokines such as vascular endothelial growth factor (VEGF) and stromal cell derived factor-1 (SDF-1α) which in particular promoted skin regeneration through stimulating epidermal stem cell migration (25-36). Deep skin layers do not respond generally to conventional topical therapeutic approaches against diabetic foot ulcer due to lack of efficient drug permeation into deep skin layers and sub-dermal tissues. Therefore, such conditions are usually treated by high drug doses of parentral or oral routes due to low blood supply, ischemia, infections, and inflammation of the affected areas (22-24).

In contrast to liposomes, ethosomes with their colloidal dispersion system were found to exhibit superb physicochemical properties over the past two decades with the capability for effective entrapment and encapsulation of both hydrophilic and hyrdrophobic drugs in addition to their ability to smoothly and more efficiently penetrate and disturb skin stratum corneum towards deep skin layers far behind the traditional liposomes (37). Ethosomes are soft malleable phospholipid bilayers vesicles that contain higher concentrations of lipids and ethanol which enable drug solubilization and formation of deformable lipid structures not only for skin penetration but also for skin retention through intercellular lipid extraction at deeper layers (38). Extensive research studies along with several recent publications demonstrated deep skin penetration capability of ethosomes for the treatment of various diseases not only through in-vitro models but also and more importantly through in vivo models as well (39-45). Owing to their enhanced skin penetration properties, ethosomes might be a potential target to act as promising carriers for insulin delivery towards deep skin layers with subsequent controlled release for effective and prolonged management of delayed wound healing associated with chronic complications of diabetes.

Another crucial factor that should be considered to effectively facilitate and accelerate efficient wound closure is to cover the wound site with a sterile dressing material in order to prevent microbial infection. However, the conventional bandage requires frequent exchange and cleaning/monitoring of wound site which is problematic for patients having chromic disorders such as venous ulcer or diabetes which delay wound healing capability. Several gels and creams were proposed for this purpose, however they were expensive and had low to moderate therapeutic efficacy in addition to the need for frequent change of wound dressing which was painful for many patients. With this regard, our aim was to revolutionize an efficient wound healing approach that would accelerate and enhance the rate and quality of wound healing without being painful or harmful for patients. With the advancement of therapeutic fabrics, a new generation of effective and efficient wound dressing materials could be designed and prepared not only to maximize wound healing process but also to alleviate much of the pain associated with repetitive and frequent applications of dressing materials.

Herein, a novel polyvinyl alcohol (PVA) dermal patch was developed to deliver insulin towards deep skin layers through ethosomal vesicles loaded with insulin. It was hypothesized that, embedding ethosomal insulin into biodegradable and biocompatible fibrous mats would enhance their pharmaceutical use and boost their efficacy in wound ulcer treatments for longer period. Biodegradable and electrospun wound fibrous mats would be applied without the subsequent need of removal or exchange for the efficient and long term treatment of wound ulcer. Electrospinning is an effective and a simple fabrication process that could generate fiber mats in nano-to micro-scale (1, 2, 4, 46-48). Electrospun mats comprise of ultra-fine nano-fibers with large surface area-to-volume ratio and also possess high degree of porosity owing to fiber random deposition (46-48). Therefore, such porous network could mimic the normal structure of extracellular matrix (ECM) strucure, and could have great applications in various biomedical fields such as drug delivery carriers, tissue engineering scaffolds and wound dressing (4, 5).

Various types of polymers, such as polyvinyl alcohol (PVA), carboxymethylchitosan (CMCS) and polyethylene oxide (PEO) are electrospun into nanofibers membranes. PVA, CMCS & PEO are soluble in hot water and have high mechanical strength. PEO is semi-crystalline polymer that contains crystalline phases and amorphous regions at room temperature. It is highly soluble in cold water and is used widely owing to its high strength and thermal stability. The latter enable PVA, CMCS and EPO to be ideal materials to act as the matrix of composited electrospun nanofibers (23-24,46-52). The extreme high solubility of those polymers in aqueous medium renders them very safe for both patients and environment.

According to latest literature review and to the best of our knowledge, this would be the first attempt to rationally develop and formulate a novel insulin loaded ethosomes uniformly dispersed in an electrospun biodegradable fibrous mat for effective treatments of wounds in diabetic foot ulcer**.** The novel system was designed to compose of PVA mats enriched with ethosomal insulin vesicles for local and sustained treatment of diabetic wounds. The novel system is expected to control the rate of insulin release through dual mechanisms. The first beneficial mechanism would be granted through the excellent flexibility of ethosomal vesicles to deeply cross various skin layers which would in turn enable gradual and effective release of drug in several skin layers. The second mechanism would be related to the use of fibrous mat as novel, accurate and efficient drug delivery system. The latter could provide a new generation of wound dressing materials capable of alleviating pain without the need for frequent changing of wound dressing materials. The nanofibers would be formed by an electrospinning technique and will be subjected *to in vitro* investigations and will be tested *in vivo* on experimental streptozotocin induced diabetic rats with skin wounds.

In this particular study, we developed, for the first time, a novel PVA electrospun nanofibrous mat loaded with ethosomal insulin for local and sustained release of insulin towards deep skin layers in an attempt to treat wounds of streptozotocin induced diabetic animal models. We tested their ability to heal diabetic wounds efficiently. The in vivo model is more reliable and convincing than the in vitro model as it challenges the novel delivery system not only at tiny cellular level but rather at more complex and sophisticated level with involvement of all tissues and organs.

Material and Methods:

Material

Polyvinyl alcohol (PVA) with average molecular weight 1,15,000 and degree of polymerization 1700-1800 was purchased from lobahemie PVT.LTD. Cholesterol, phospholipid (lecithin), and streptozotocin (STZ) were purchased from Sigma Aldrich (USA). The remaining materials and chemicals used were of high laboratory grade.

Methods:

Preparation of insulin loaded and insulin free (blank) ethosomes

Insulin loaded ethosomal vesicles were prepared by thin film hydration method. The calculated amount of lipid, surfactants and cholesterol were dissolved in a mixture of chloroform and ethanol. The organic solvent was then removed under vacuum using rotary evaporator. This step led to formation of a thin layer of the lipid inside the flask wall which was kept overnight for complete evaporation of the solvent. Using phosphate buffer (pH 7.4) which contained the required amount of insulin (15 IU/mL), the organic solvent free film was then hydrated and shook gently for 15 minutes to produce ethosomal colloidal suspensions. Varying concentration of lipids, surfactants and edge activators were used to study their effect on the physicochemical properties of the obtained dispersions. The final dispersion of ethosomes was kept at room temperature for 30 min under continuous stirring. Formulations were stored in the refrigerator and evaluated. Similarly, insulin free ethosomes were prepared applying the same steps except for the hydration of the thin layer that was carried out using insulin free buffer.

Preparation of PVA mats loaded with pure insulin

Ten grams of PVA polymer was dissolved in 100 mL deionized water and heated at 90°C with stirring in a water bath to obtain homogenous 10 % (w/v) PVA solution. To 10 mL of this solution, 0.008 mg of insulin was added and stirred in ice water bath for 10 hrs. Electrospinning technique was performed to fabricate the nanofibers mats loaded with pure insulin. The electrospinning process was carried out by applying 30 kV voltage on polymeric solution with 0.5 mL/hr feed rate using 5 mm diameter syringe. The electrospun samples were generated on aluminum foil covered plate placed 15 cm from the spinneret tip.

Preparation of PVA mats loaded with blank ethosomes

PVA mats loaded with blank ethosomes were prepared by adding 5 mL of the prepared blank ethosomal formulation to 15 mL of 10% PVA solution (with equivalent percentage of 33.3% of blank ethosomes in PVA solution). The resulting dispersion was stirred for 2 hrs to obtain homogeneous solution then the electrospinning process was performed as mentioned above.

Preparation PVA fibrous mats loaded with ethosomal insulin

PVA fibrous mats loaded with insulin containing ethosomes were prepared by adding 5 mL of insulin ethosomal insulin formulation to 15 mL of 10% PVA (with equivalent percentage of 33.3% of ethosomal insulin in PVA solution)., stirred for 2 hrs, then the electrospinning process was carried out as mentioned above.

Characterization of the prepared blank/insulin loaded ethosomes

Particle Size and Zeta Potential

The particle size and zeta potential of insulin-loaded and insulin-free vesicles were determined by dynamic light scattering (DLS) (Zetasizer, Malvern ZS 90, UK). Samples were properly diluted and the hydrodynamic size was obtained by measuring the scattered light at scattering angle equal to 90 at 25°C.

Transmission Electron Microscope (TEM)

Surface morphology of the prepared vesicles was imaged and evaluated by the transmission electron microscope (TEM). A droplet of diluted ethosomal suspension placed on a copper grid with carbon film for few minutes, then residual fluid was removed with a filter paper. Afterwards, a single drop of 1% (w/v) uranyl acetate was added and kept for few minutes and then excess solution was removed. The grid was then dried for 2 days before imaging by Transmission electron microscopy (TEM; JEM 1011, JEOL-Japan) using 80 kV acceleration voltage.

Encapsulation Efficiency

The ultrafiltration method was used to evaluate the encapsulation efficiency (EE) of vesicles. The EE value was identified as the percentage of the loaded insulin over the original added amount of insulin. The insulin loaded vesicles were ultra-filtered with 30 kD cutoff filtration membrane (Milipore Co. Ltd., USA), and the content of insulin in the ultra-filtrate was determined using UV methods. The EE will be calculated as follows:

$$\% \text{ EE} = \frac{\text{the feeding amount of insulin} - \text{the amount of insulin in the filtrate}}{\text{the feeding amount of inuslin}} X\ 100$$

Insulin is a polypeptide with aromatic amino acids (phenylalanine, tyrosine and tryptophan) that possess aromatic ring with conjugated diene that has characteristic UV absorbance at the 276 nm wavelength. UV-Vis spectrophotometer (Perkin Elmer Lambda 45 spectrometer, USA) was used to determine insulin concentration from a standard calibration curve/plot for pure insulin measured at (276 nm λ_{max}) wavelength.

Characterization of the polymeric solution

Viscosity and conductivity

The viscosity of the PVA polymeric solutions was determined at room temperature using a rotation viscometer (Brookfield-DVBT). The electrical conductivity of polymer solutions prepared for electro-spinning was measured using Myron L Ultrameter II, Model 6P.

Characterization of the prepared fibrous mats

Scanning Electron Microscopy (SEM)

The surface morphology of the resulting nanofibers mats was investigated using scanning electron microscopy (SEM) (Quanta 250 Field emission Gun, USA). Using sputter coater (S150A Edwards-England), electrospun mats were deposited in vacuum on aluminum sheet that was coated with a gold layer as a conductive surface. Features of sample morphology were obtained in 5–10 kV.

***In vitro* release of insulin from ethosomes**

The *in vitro* release of insulin from the prepared vesicles was compared to the release of the pure drug solution. Amount of the ethosomes containing drug equivalent to 0.5 IU were placed in a clean dialysis bag secured with 2 clamps without affecting release rate. The USP dissolution tester (Erweka Apparatebau GmbH, Germany) was applied for all samples using 500 mL of phosphate-buffered saline (pH 7.4) at 37 ^{o}C and 60 rpm for 8 hrs. Aliquot samples (2 ml) was taken, at each appropriate time point, and replenished immediately with 2 ml of fresh buffer. The amount of insulin released was spectrophotometrically determined at 276 nm and percentage of the released insulin was calculated against the standard calibration curve/plot as indicated above.

***In vitro* release of insulin from ethosomes embedded in the fibrous mat compared to insulin directly loaded in fibrous mat**

A piece of specific dimension (2cm X 2cm) either of the insulin loaded ethosomes embedded into the polymeric electrospun mat or insulin loaded electrospun mat was dissolved in water and the released insulin drug was investigated the same UV spectrophotometer at 276 nm wavelength. The latter was compared to the release of the pure drug solution and drug loaded ethosomes. A mat dimension containing drug equivalent to 0.5 IU was placed in a in a clean dialysis bag secured with 2 clamps without affecting release rate. The same USP dissolution tester was applied for all samples using the same conditions and sample aliquot volume mentioned above. The percentage of released insulin was spectrophotometically determined at 276 nm.

It is important to indicate that PVA structure itself consists mainly of saturated carbon-carbon bonds and hydroxyl groups which lack any chromophores or unconjugated diene system and does not have UV absorbance in the UV-Vis range. Likewise, blank ethosomes are composed primarily of phospholipids and ethanol that lack chromophoric groups and therefore have no intrinsic **UV absorbance**. On the contrary, Insulin is a polypeptide with aromatic amino acids (phenylalanine, tyrosine and tryptophan) that possess aromatic ring with conjugated diene that has characteristic UV absorbance at the 276 nm wavelength. UV-Vis spectrophotometer (Perkin Elmer Lambda 45 spectrometer, USA) was used to determine insulin concentration from a standard calibration curve/plot for pure insulin measured at (276 nm λ_{max}) wavelength.

***In vivo* study**

Animals

Adult male Wistar rats weighing 180-200 gm were purchased from the Egyptian organization for biological products and vaccines (Cairo, Egypt). The animals were allowed for acclimatization for one week before experimentation under routine lab conditions throughout the study (55±10% humidity and room temperature 24–27°C) with alternating 12 hrs dark and light cycles. The animals were permitted free access to water and food (El-Nasr Pharmaceutical Chemicals Co., Cairo, Egypt). All the procedures conducted for the animal study and the care of animals were performed according to the standard ethical guidelines. The experimental protocol was approved by the ethical research committee at the National Research Centre (NRC).

Induction of diabetes mellitus

Streptozotocin (STZ) was used to induce diabetes in rats. Firstly, rats were fasted for 10-12 hrs and then they were injected intraperitoneal with a freshly prepared single dose (50 mg/kg) of streptozotocin dissolved in 0.1M sodium citrate buffer (pH 4.5). After 72 hours of streptozotocin injection, blood samples were taken to measure blood glucose level (BGL) using Glucometer® apparatus (USA). Rats were considered diabetic when their BGL reached more than 250 mg/dl in three separate occasions over three days.

Experimental design

Diabetic rats were distributed randomly into four groups (6 rats per group) and then subjected to skin excision. The animal groups were categorized into:

Group I: Diabetic rats with pure insulin loaded PVA electrospun fibers (Patch 1) covering the skin excision.

Group II: Diabetic rats with ethosomal insulin loaded PVA electrospun fibers (patch 2) covering the skin excision.

Group III: Diabetic rats with PVA (10%) electrospun fibers (patch 3) covering the skin excision.

Group IV: Diabetic rats with skin excision allowed to heal without PVA mat intervention (natural healing).

Additionally, two groups of healthy rats (6 rats each) were subjected to the same skin excision, the skin excision of the first group was allowed to heal naturally, whereas the skin excision of the second group was covered with PVA (10%) electrospun fibers (patch 3).

Wound excision

For wound excision, animals were anesthetized using intraperitoneal xylazine (7 mg/kg) and ketamine (70 mg/ kg). Dorsal hair was removed, skin was sterilized with 70% alcohol, then wounds were created down to the fascia of the panniculus back of the rat. The animals were individually housed after wound excision procedure, inspected daily, the wounds were pictured on day 2, 4 and 8 to observe efficiency of wound closure rate.

Wound healing measurement

The wound size was measured quantitatively in day 2, 4 and 8 using Image J 1.52 software through a scaled picture from each wound. Healing percentage was measured using the following equation (100 – ((wound area at day n/wound area at day 0)*100)), where n = 2, 5, 7 or 9.

Determination of serum insulin level

Blood samples were withdrawn from the retro-orbital plexus on day 8th of patches application and allowed to clot. Blood samples were then centrifuged, and the serum was collected for determination of serum insulin level using the insulin ELISA (enzyme linked immunosorbent assay) kit obtained from Cloud-Clone Corp, USA.

Determination of blood glucose level (BGL)

Blood glucose level (BGL) was measured on day 8 of patches application using Glucometer® device.

Histopathological examination

At the end of the treatment period, rats were sacrificed under ether anesthesia, wound area and skin were rinsed with saline and preserved in 10% neutral formalin buffer. Tissues were embedded in paraffin blocks, and 4 µm thickness sections were sliced and stained with eosin and hematoxylin for histopathological examination.

Skin irritation studies

For skin irritation study, the skins of normal rats treated with different patches were examined for any undesirable skin changes, i.e., changes in color and/or changes in skin morphology for a period of 8 days.

Statistical analysis

Data from experimental animal work was represented as mean ± standard error (SEM). One-way analysis of variance (ANOVA) was conducted to compare between different groups and was then followed by Tukey-Kramer test. Results obtained were considered significant with *P* values less then 0.05. Graphpad software instat (version 2) was utilized for statistical analysis and comparison.

Results and Discussion

Characterization of the prepared blank/insulin loaded ethosomes

Particle Size and Zeta Potential

The average hydrodynamic diameter, determined by light scattering, for the prepared blank ethosomes replicates was found to be 221.9±9.16 nm (Mean±SEM) with PDI equal to 0.305±0.001 (Mean±SEM) with and surface charge (zeta potential) was found to be -60.9±3.3 mV (Mean±SEM) **Figure 1A and Figure 1B**. These results indicated that the blank ethosomes were in the nanometer size range with mono-size distribution as indicated by the low value of PDI as shown in **Figure 1A**. The magnitude of the zeta potential is a key parameter in colloidal science as it refers to the electric potential at the slipping plane of a particle in a suspension. It reflects the overall charge a particle acquires in a specific medium and influences how the particles interact with each other. It therefore gives a good indication of the potential stability of the colloidal system. A negative value of zeta potential indicates that the particles in the suspension are negatively charged with repulsive forces between particles, helping to prevent them from clumping together and aggregation. A high negative zeta potential (-30 mV or more negative values – 60 mv, -70 mv or more negative values…) generally implies that the particles are more stable with strong repel for each other, maintaining a dispersed state with no tendency for aggregation. A low negative zeta potential (closer to 0 mV) suggests weaker repulsion, which can initiate particle flocculation and aggregation leading to instability in the colloidal suspension. In our case, the five replicates of the blank ethosomes exhibited highly negative zeta potential average values (-60.9 mv) indicating that the blank ethosomes were highly stable with no or least tendency towards self-aggregation and hence would ensure maintaining their particle diameter in nano size with no subsequent increase in the particle diameter with time.

The average hydrodynamic diameter for the prepared insulin loaded ethosomes replicates was found to be 263.8±8.22 nm (Mean±SEM) with a PDI 0.347±0.002 (Mean±SEM) **(Figure 1C)** while the zeta potential was found to be -68.5±2.1 mv (Mean±SEM) **(Figure 1D)**, indicating that the insulin loaded ethosomes were in the nanosized range and of great stability with no liabilities for self aggregation with no subsequent increase in the particle diameter with time.

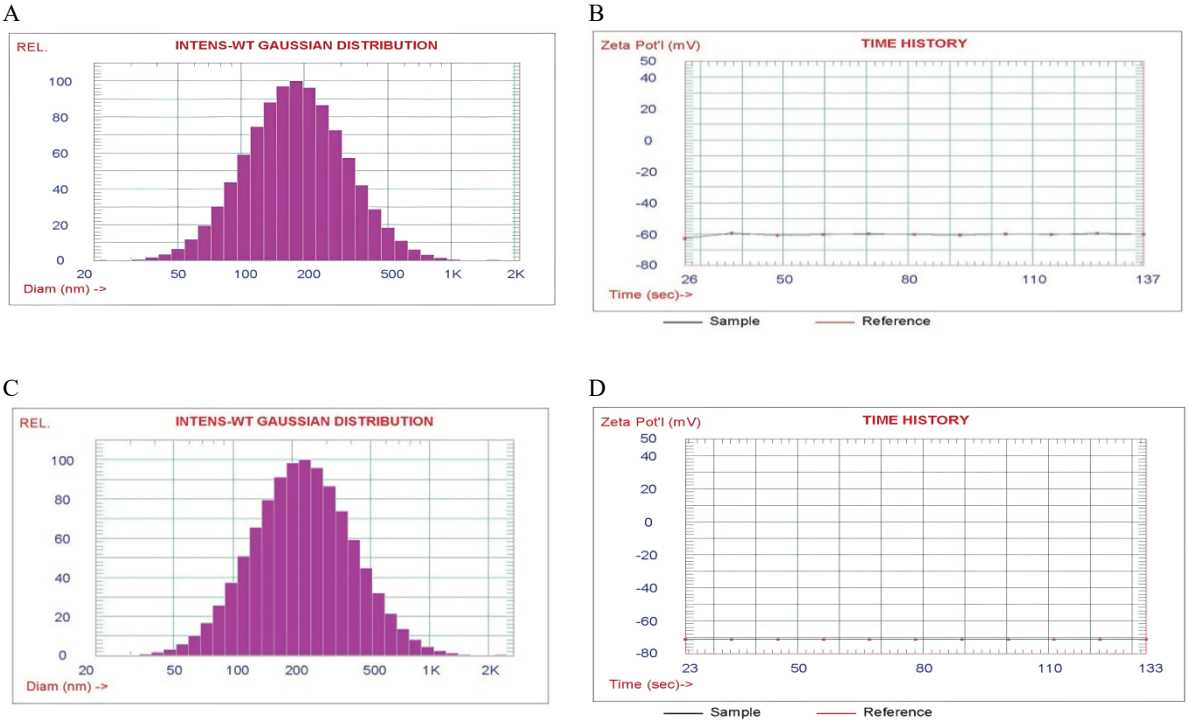


Figure (1): Particles size distribution (A & C) and Zeta potential (B & D) of prepared ethosomes. Blank ethosomes (A & B); insulin loaded ethosomes (C & D).

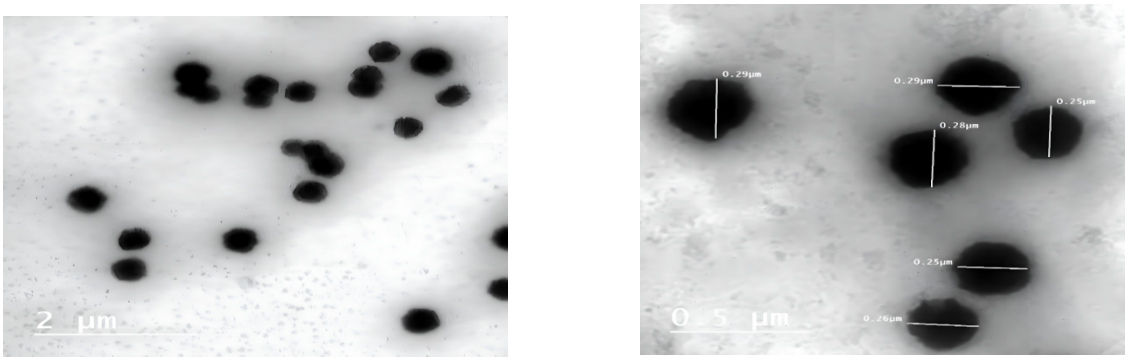
Transmission Electron Microscope

Transmission Electron Microscope (TEM) photograph of the prepared particles was performed to get detailed information about the morphology and the size of the prepared ethosomes. TEM results showed that the prepared blank and insulin loaded ethosomal formulations were uniform in size with nearly spherical shape **(Figure 2A and Figure 2B)**. The core of each vesicle appeared denser indicating insulin encapsulation meanwhile the craters of each vesicle were lighter representing the lipid bilayers. Particle size detected from the TEM shots were slightly less small than those reported by the zetasizer. The smaller particle size observed by TEM than that obtained by zeta sizer could be attributed to the fact that TEM determines the actual particles size of the vesicles while zeta sizer determines the hydrodynamic size of the particles, the actual particle size and the surrounding moving layers of the solvents.

As indicated previously ethosomes are soft malleable phospholipid bilayers vesicles that contain high concentrations of ethanol and lipids. Ethosomes with their colloidal dispersion system have the ability to solubilize both hydrophilic and hydrophobic molecules. Ethosomal lipid bilayers have high electron dense capability which interact with the electron beam used in TEM imaging technique leading to electron transmission blockage creating contrast that make them appear darker compared to the surrounding medium especially in thin sections. Moreover, liposomal appearance in TEM images could look even darker especially if a staining technique (like negative staining with heavy metals such as uranyl acetate or phosphotungstic acid) is used. In the current study, ethosomal suspension was placed on a copper grid with carbon film for few minutes, then a single drop of 1% (w/v) uranyl acetate was added for staining purposes. The staining step would lead to appearance of darker ethosomes on TEM imaging. Unlike other imaging techniques, TEM provides high contrast, and since ethosomes are composed of lipid bilayers, they typically show up as dark rings or spheres depending on the staining technique used.

Drug loading and the percentage of encapsulation efficiency are considered key parameters to evaluate the potentiality of delivery of any vesicular formulation. The percentage of encapsulation efficiency of ethosomal formulations showed an average of 64.5%±4.98. Form the obtained results it was observed that encapsulation efficiency of the prepared ethosomal insulin was relatively high and convenient for further continuation of the study.

A



B

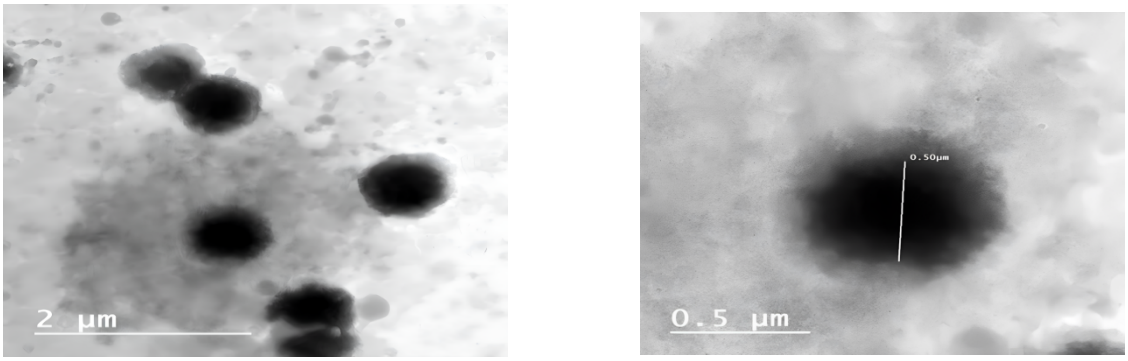


Figure 2: TEM images of blank ethosomes (A) and insulin loaded ethosomes (B) Encapsulation Efficiency

Characterization of the polymeric solution

It is known that diameter, density and morphology of the electrospun fibers depend on many conditions such as polymer viscosity, conductivity, concentration, surface tension and dipole moment. The latter are the main parameters that have great impact on the electro-spinnability of a polymeric solution. For instance, a low viscous polymeric solution would not generate nanofibers. On the other side, solutions with high viscosity could not also be ejected in the electrospinning process itself. Moreover, electrical conductivity is another parameter which is essential to initiate the electrospinning process via counteracting viscoelastic force. Increased electrical conductivity, for instance, could generate non-continuous fibers. Furthermore, other factors such as electric field, temperature, flow rate, air flow, collector composition, needle shape and tip-to-collector distance would significantly affect electrospinning process and mat construction. Therefore, is essential to optimize all those conditions to obtain desirable fiber morphology and diameter. Herein, the optimal electrospinning conditions were as follows; 0.5ml/hr flow rate through 5 mm syringe using 30 kV DC voltage and samples were collected on aluminum covered plate that was placed 15 cm from the spinneret tip.

As shown from table 1, the conductivity of PVA containing pure insulin solution was 140 (mS/cm) and the viscosity was 769.9 **cP**. The addition of blank ethosomes to PVA solution led to significant reduction in polymer viscosity to 275.8 cP with noticeable increase in polymer conductivity to 172.2 mS/cm. On the other side, addition of insulin loaded ethosomes to PVA solution decreased polymer viscosity remarkably to 349.3 cP with increase in polymer conductivity to 152.5 mS/cm **(Table 1)**.

Table (1): Viscosity and conductivity of different PVA solutions

Preparation	Viscosity (cP)	Conductivity (mS/cm)
PVA/Insulin	769.9±35.2	140±5.8
PVA/Blank ethosomes	275.8±11.4	172.2±5.6
PVA/Insulin loaded ethosome	349.3±16.2	152.5±6.4

Scanning Electron Microscopy (SEM) the prepared fibrous PVA/Insulin mat

It was confirmed by the scan electron microscopy (SEM) **(Figure 3)** that all the prepared PVA electrospun nanofibers surface morphology had homogenous and uniform size diameter. SEM images indicated that all the prepared electrospun nanofibers were continuous, uniform, straight with almost smooth and flexible interconnected porous mats with no noticeable deformation. The average diameter for pure insulin loaded PVA mat replicates was 0.41±0.026 µm. Meanwhile the average diameter for blank ethosomes and ethosomal insulin loaded PVA fibrous mats replicates were 0.34±0.032 µm and 0.33±0.033

μm, respectively. Furthermore, the incorporation of either blank or insulin loaded ethosomes to the PVA polymeric solution was proven not to have negative effect neither on the electrospinning efficiency nor on the morphology of the fabricated PVA nanofiber mats. The successful and the smooth incorporation of the small particle size of the prepared ethosomes within the porous structure of the polymeric solution is worthy of mentioning.

It was also observed that the increased conductivity recorded in both PVA/blank ethosomes and the PVA/insulin loaded ethosomes had led to generation of the most finest and spinnable nanofiber diameter which might be attributed to increased charge density of the polymeric solution with enhanced stretching forces and improved self-repulsion of the jet. Subsequently, it was clear that all the prepared electrospun nanofibers were in nanometer size and could therefore be convenient for application in wound healing.

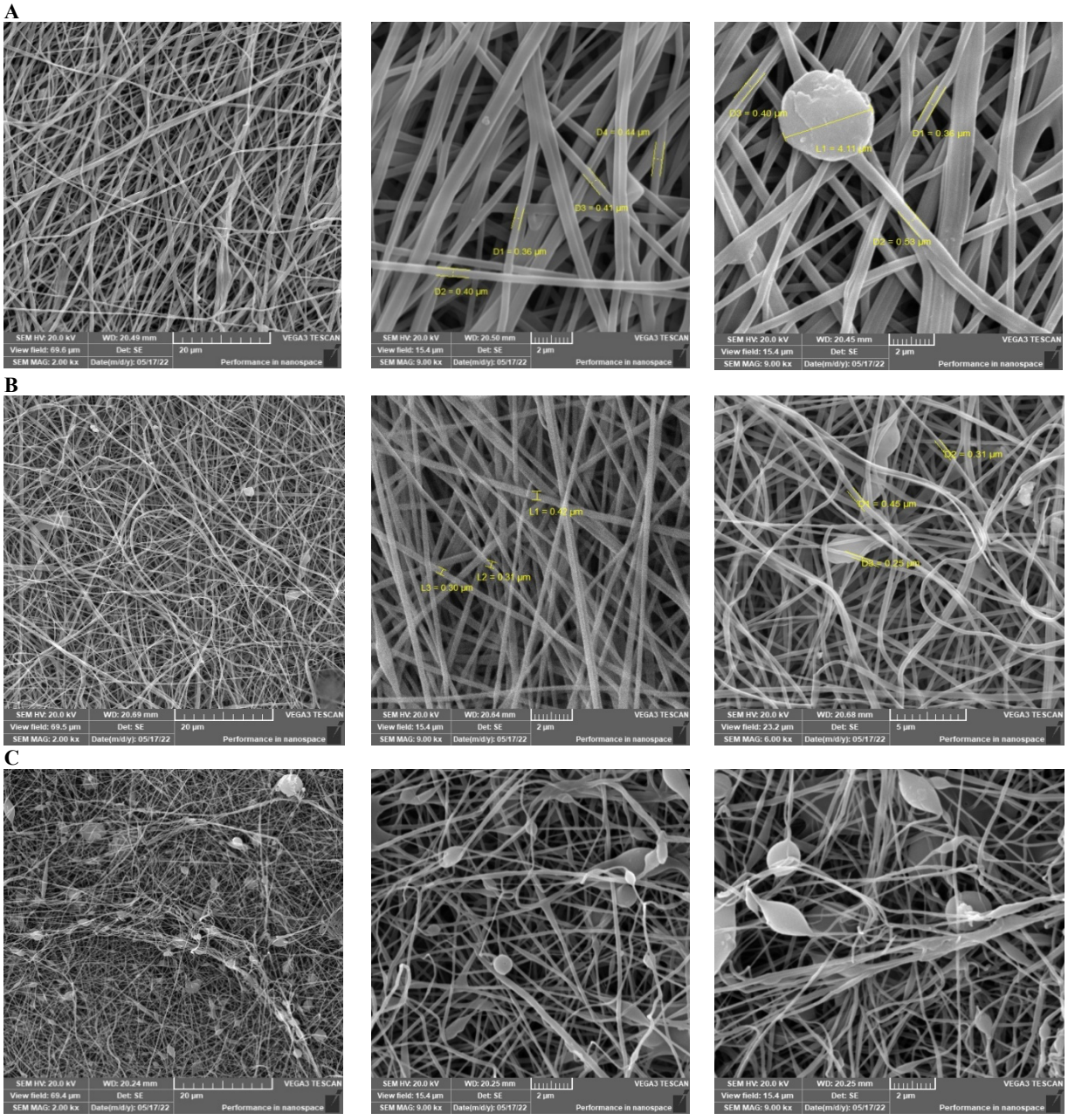


Figure (3): SEM images for prepared PVA mats. PVA/ Insulin mat (A); PVA/ Blank ethosomes mat (B); PVA/ insulin loaded ethosomes mat (C)

***In vitro* insulin release studies**

The *In vitro* insulin release from the prepared insulin loaded ethosomal formulations was found to be higher than that released from the same formulation embedded into the electrospun PVA mats as indicated in **Figure 4**. Insulin loaded ethosomes released 84.8% of loaded insulin during 8 hours whereas insulin loaded ethosomal PVA mats released only about 41.3% of insulin at the same time **(Figure 4)**. *In vitro* release kinetics indicated that the mechanism of insulin release from the prepared ethosomal insulin vesicles was through diffusion and this proves that insulin release had most probably occurred through the vesicular bilayer wall. In details, insulin release occurred firstly from entrapped drug solution through the vesicle wall into the surrounding adjacent solution and then released drug diffused to the bulk of the release medium through the release membrane. Whereas the release from the insulin loaded ethosomal PVA mats showed a biphasic release pattern which might highlight the dual mechanisms involved in the release of insulin from the ethosomal insulin loaded mats. Initially, Insulin is released by diffusion from the ethosomal structure then followed by gradual and slower release from the mat structure by erosion. This also might explain the lower cumulative insulin percentage released from the mats loaded with ethosomal insulin compared to that from the ethosomes in 8 hrs time 41.3% vs 84.8%, respectively **(Figure 4)**. Based on the *in vitro* insulin release profile from the designed mat it is expected that the prepared mat would be able to deliver the loaded insulin in a sustained release manner over a prolonged period of time which may reach 24 hrs. The prolonged release of insulin form mats is very advantageous because the dressing (insulin loaded ethosome fibrous mat) would be effective over 24 hrs and will be applied to the diabetic foot wound once daily. Additionally the sustained released and prolonged delivery of insulin will not lower the blood glucose sharply and consequently protect the diabetic patients from hypoglycemic induced the rapid and immediate release of insulin.

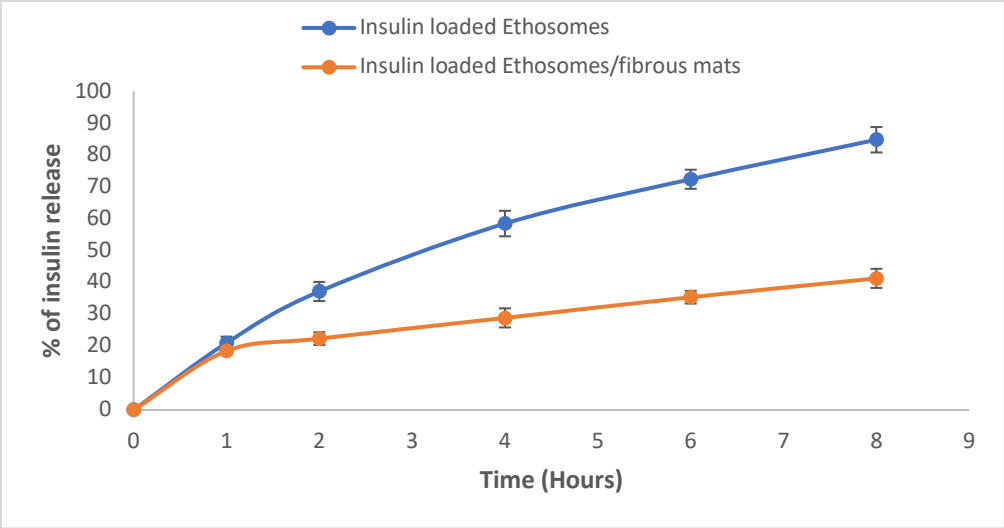


Figure 4: In vitro insulin release from insulin loaded ethosomes and fibrous mats containing insulin loaded ethosomes

***In-vivo* wound healing**

As shown in **Figure 5**, the wound area for the untreated diabetic rats had increased over days from day 2 to day 8 with no wound closure at all. On Days 2, 4 and 8, untreated diabetic rats had wound areas of 0.75, 0.85 and 1.19 cm^2, respectively. Unlike untreated diabetic rats, normal untreated rats initiated wound closure slowly with minor reduction in wound area from 0.36 cm^2 by Day 2 to 0.21 cm^2 by Day 4. On Day 8, although they did not show complete wound closure, they had a minimal wound area of 0.18 cm^2 as compared to untreated diabetic rats. Although diabetic rats treated with patch 3 exhibited smaller wound area of 0.46 cm^2 on Day 2 as compared to diabetic untreated rats, they had larger wound area when compared to untreated normal rats on the same day. On Day 4, more wound closure was noticed for diabetic rats treated with patch 1 with a wound area of 0.1cm^2 which was even better and smaller than that of untreated normal rats. By Day 8, the wound area was kept at 0.05 cm^2 which was still far better than that reported for the untreated normal group itself **(Figure 5)**. On the other side, diabetic rat treated with patch 1 and patch 2 showed initial improvement and acceleration of wound healing following Day 2. The wound areas left for patch 1 and patch 2 treated diabetic rats on Day 2 were 0.33 cm^2 and 0.2cm^2, respectively. Ethosomal insulin loaded PVA fibrous mat (patch 2) showed more efficient and faster wound closure than PVA insulin mat (patch 1) on Day 2. By Day 4, there was almost complete recovery of wound without formation of any abscess or necrosis or exudative lesions by both patches with wound areas of nearly 0.05 cm^2 and 0.03 cm^2 for patch 1 and patch 2 treated diabetic rats, respectively. By day 8, both groups had complete wound healing with no visible wounds at all with recovery of hair surround the induced wound **(Figure 5)**. It was obvious that addition of ethosomal insulin had an effective and a promising wound closure activity in streptozotoci induced diabetic rats. The rate of wound healing in rats treated with patch 2 was faster compared to wound covered with patch 1 or
patch 3 at all-time intervals **(Figure 5)**.

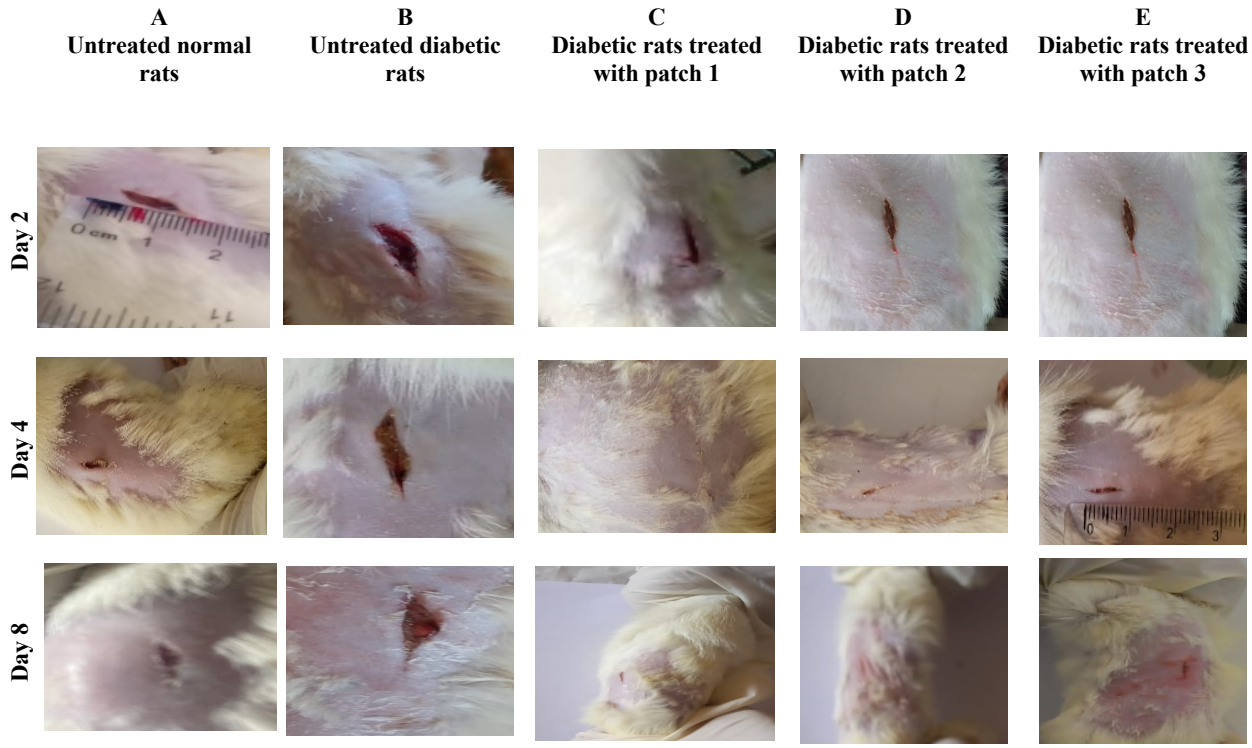


Figure 5: Pictures of rats wound area at the 2nd, 4th and 8th day post wounding. A: untreated normal rats, B: untreated diabetic rats; C: diabetic rats treated with patch 1; D: diabetic rats treated with patch 2; E: diabetic rats treated with patch 3.

Biochemical parameters

Following treatment of diabetic rats for 8 days with different patches, blood glucose levels were determined for all rat groups. Both untreated diabetic rats and patch 3-(PVA mat only) treated diabetic rats had high blood glucose levels reaching 307±36.9 mg/dl and 288.3±1.2 mg/dl, respectively on Day 8. There was no significant difference between the two rat groups. On the other side, diabetic rats treated with patch 1 (PVA/insulin) and patch 2 (ethosomal insulin loaded PVA mat) had better blood glucose levels of 71.2 ± 1.3 mg/dl and 74.4 ± 1.5 mg/dl, respectively than those of untreated and patch 3 treated diabetic rats. The hypoglycemic threshold in rats starts at blood glucose levels below 50 mg/dl with appearance of clinical symptoms such as seizures, muscle tremor, weakness, ataxia, piloerection, hypothermia, hunched posture and irregular breathing. It is crucial to indicate that both patch 1 and patch 2 treated diabetic rats did not exhibit systemic hypoglycemic effect following treatments for 8 consecutive days without appearance of any clinical signs for hypoglycemia at all. These findings prove that the ethosomal insulin loaded PVA nanofibrous mat accelerated wound healing efficiently and the slow release of insulin did not induce systemic hypoglycemia in treated rats. The application of medicated patches to wounded areas enhanced and promoted the transdermal delivery of insulin due to the lack of skin barriers. For this reason, we used a vesicular system (ethosomes) to sustain insulin release as proved by the *in vitro* insulin releases **(Figure 4)**. Moreover, the alcoholic contents of ethosomes could acquire nanofibrous mat with antiseptic properties to facilitate bacterial growth inhibition and to minimize the bacterial contamination of wounds. Accordingly, our designed insulin delivery system is considered a very smart alternative which overcomes the drawbacks and problems associated with the use of traditional dressing for treating the wounded diabetic rats.

Skin irritation studies

Neither allergic symptoms like inflammation, redness, irritation nor morphological changes appeared on rats' skin over the 8 days of patches treatment (Data not shown). In addition, histological examination of the shaved skin of the rats showed no histopathological alteration in the skin compared to normal features **(Figure 6)**.

Histopathological Findings

Normal unwounded rat skin exhibited no histopathological alteration with normal histological structure of the hair follicles, dermis with sebaceous glands and epidermis as well as the underlying subcutaneous tissue and musculature **(Figure 6)**. For wounded non-diabetic rats, there were mild degeneration, necrosis and inflammatory cells infiltration in the hair

follicles **(Figure 6)**. On the other side, untreated diabetic rats showed severe focal area of ulceration and necrosis in the epidermis and dermis with loss of hair follicles and sebaceous glands. The cellular examination of ulcers before and after treatments with different patches indicated that diabetic wounded rats treated by patch 3 showed focal acanthosis in the epidermis with underlying fibrosis as well as loss of hair follicles and sebaceous glands detected in the dermis **(Figure 6)**. Group I treated with patch 1 exhibited mild focal acanthosis in the epidermis with mild loss of hair follicles and sebaceous glands from the dermis **(Figure 6)**. On the contrary, the cellular examination of group 2 treated with patch 2 showed no histopathological alteration in the epidermis, dermis with hair follicles and sebaceous gland as well as the subcutaneous tissue and musculature **(Figure 6)**. Table 2 also summarizes the inflammatory, degenerative and histopathological changes/severity. Wounded diabetic rats treated with patch 2 (ethosomal insulin loaded PVA mat) had similar histopathological score (- inflammatory and degenerative score) as compared to normal unwounded non-diabetic rats. On the other side wounded diabetic rats treated with patch 1 (PVA insulin) more mild inflammatory score (+ inflammatory and degenerative score) than same group treated with patch 2 but with less than inflammatory score than the same group treated with patch 3 (++ inflammatory and degenerative score). The latter had a similar inflammatory and degenerative score similar to wounded non-diabetic rats that were untreated (++ inflammatory and degenerative score score). This indicated that PVA mat had no wound healing effect on rats. The wounded diabetic rats that were not treated had the highest inflammatory and degenerative score (+++ inflammatory and degenerative score).

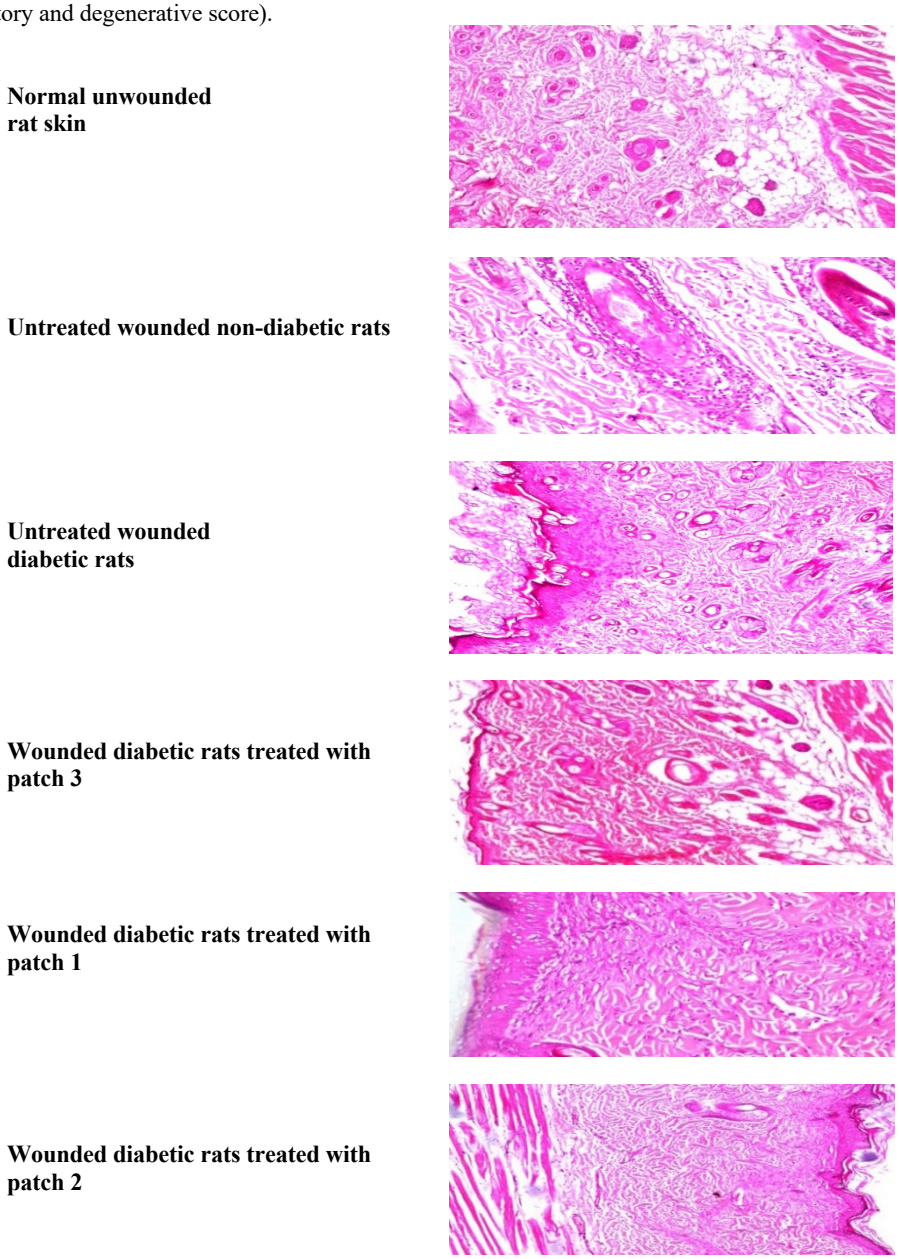


Figure 6: Histopathological examination of normal rat skin, untreated injured non-diabetic, untreated diabetic, patch1 treated diabetic, patch 2 treated diabetic and patch 3 treated diabetic groups.

Table 2: The Severity of Histopathological Alterations in skin of different Experimental Groups

Group	1	2	3	4	5	6
Inflammatory and degenerative changes/score	+	-	++	+++	++	-

Where group 1 indicates diabetic wounded rats treated by patch (I), group 2 indicates diabetic wounded rats treated by patch (II), group 3 indicates diabetic wounded rats treated by patch III, group 4 indicates wounded diabetic rats without treatment, group 5indicates wound rats wounded non-diabetic rats without treatment and group 6 indicates normal unwounded rats(unwounded healthy group).
(+++) indicates severe inflammation, (++) indicates moderate inflammation, (+) indicates mild inflammation and (-) indicates no inflammation (Nil)

Conclusion

The significant improvements in rats treated by patch 2 (PVA/insulin loaded ethosomes embedded into the polymeric electrospun fiber) could be attributed to the slower and sustaining release of insulin form ethosomes loaded patches. The sustained release of insulin is beneficial for the diabetic rats because it avoids the rapid hypoglycemic effect of rapidly released insulin which may subject the treated rats to hypoglycemic coma due to sharp and rapid decreased of blood glucose level. As it well known, lowered blood glucose level, hypoglycemia, is harmful to many body organs especially the brain. Compared to patch 1 (PVA/insulin loaded electrospun fibers) which is characterized by rapid insulin release, patch 2 is considered an ideal treatment alternative for diabetic rats.

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References

1. Boulton AJ, Vileikyte L, Ragnarson-Tennvall G, Apelqvist J. The global burden of diabetic foot disease. The Lancet. 2005;366(9498):1719-24.
2. Papanas N, Maltezos E. The diabetic foot: established and emerging treatments. Acta Clinica Belgica. 2007;62(4):230-8.
3. Bakker K, Foster A, van Houtoum W, Riley P. International Diabetes Federation and International Working Group of the Diabetic Foot. Time to act The Netherlands. 2005.
4. Reiber GE, Ledoux WR. Epidemiology of diabetic foot ulcers and amputations: evidence for prevention. The evidence base for diabetes care. 2002:641-65.
5. Papanas, Maltezos, Edmonds. St. Vincent declaration after 15 years or who cleft the devil's foot? Vasa. 2006;35(1):3-4.
6. Falanga V. Wound healing and its impairment in the diabetic foot. The Lancet. 2005;366(9498):1736-43.
7. Galkowska H, Wojewodzka U, Olszewski WL. Chemokines, cytokines, and growth factors in keratinocytes and dermal endothelial cells in the margin of chronic diabetic foot ulcers. Wound Repair and Regeneration. 2006;14(5):558-65.
8. Maruyama K, Asai J, Ii M, Thorne T, Losordo DW, D'Amore PA. Decreased macrophage number and activation lead to reduced lymphatic vessel formation and contribute to impaired diabetic wound healing. The American journal of pathology. 2007;170(4):1178-91.
9. Sharaf S, El-Naggar ME. Wound dressing properties of cationized cotton fabric treated with carrageenan/cyclodextrin hydrogel loaded with honey bee propolis extract. International journal of biological macromolecules. 2019;133:583-91.
10. El-Feky GS, Sharaf SS, El Shafei A, Hegazy AA. Using chitosan nanoparticles as drug carriers for the development of a silver sulfadiazine wound dressing. Carbohydrate polymers. 2017;158:11-9.
11. Sharaf S, Higazy A, Hebeish A. Propolis induced antibacterial activity and other technical properties of cotton textiles. International Journal of Biological Macromolecules. 2013;59:408-16.
12. Hanam S, Singleton C, Rudek W. The effect of topical insulin on infected cutaneous ulcerations in diabetic and nondiabetic mice. The Journal of foot surgery. 1983;22(4):298-301.
13. Apolinário PP, Zanchetta FC, Breder JSC, Adams G, Consonni SR, Gillis R, Saad MJA, Lima MHM. Anti-inflammatory, procollagen, and wound repair properties of topical insulin gel. Braz J Med Biol Res. 2023 May 15:56:e12640.
14. Apikoglu-Rabus S, Izzettin F, Turan P, Ercan F. Effect of topical insulin on cutaneous wound healing in rats with or without acute diabetes. Clinical and Experimental Dermatology. 2010;35(2):180-5.
15. Belfield W, Golinsky S, Compton M. The use of insulin in open-wound healing. Veterinary medicine, small animal clinician: VM, SAC. 1970;65(5):455-60.
16. Weringer EJ, Kelso JM, Tamai IY, Arquilla ER. Effects of insulin on wound healing in diabetic mice. European Journal of Endocrinology. 1982;99(1):101-8.

17. Madibally SV, Solomon V, Mitchell RN, Van De Water L, Yarmush ML, Toner M. Influence of insulin therapy on burn wound healing in rats. Journal of surgical research. 2003;109(2):92-100.
18. Greenway S, Filler L, Greenway F. Topical insulin in wound healing: a randomised, double-blind, placebo-controlled trial. Journal of wound care. 1999;8(10):526-8.
19. Wilson J, Baines R, Babu E, Kelley C. A role for topical insulin in the management problematic surgical wounds. The Annals of The Royal College of Surgeons of England. 2008;90(2):160-.
20. Rezvani O, Shabbak E, Aslani A, Bidar R, Jafari M, Safarnezhad S. A randomized, double-blind, placebo-controlled trial to determine the effects of topical insulin on wound healing. Ostomy/wound management. 2009;55(8):22.
21. Benoliel A-M, Kahn-Perles B, Imbert J, Verrando P. Insulin stimulates haptotactic migration of human epidermal keratinocytes through activation of NF-κB transcription factor. Journal of cell science. 1997;110(17):2089-97.
22. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. Nature. 2008;453(7193):314-21.
23. Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. The Journal of clinical investigation. 2007;117(5):1219-22.
24. Gallagher KA, Liu Z-J, Xiao M, Chen H, Goldstein LJ, Buerk DG, et al. Diabetic impairments in NO-mediated endothelial progenitor cell mobilization and homing are reversed by hyperoxia and SDF-1α. The Journal of clinical investigation. 2007;117(5):1249-59.
25- Silveira Jr VF, Silva JLG, Lima MHM, Saad MJA. Effects of topical insulin on second-degree burn wound healing: brief report. Wound Manag Prev. 2024 Jun;70(2).
26- Rajalekshmy GP, Ramesan RM, Geetha CS, Pratheesh KV, Shenoy SJ, Anilkumar TV. *In Vivo* Wound-Healing Efficacy of Insulin-Loaded Strontium-Cross-Linked Alginate-Based Hydrogels in Diabetic Rats. ACS Biomater Sci Eng. 2024 Apr 8;10(4):2552-2566.
27- Carreira LM, Silva R, Alves J, Inácio F, Pires G, Azevedo P. The Use of Fast-Acting Insulin Topical Solution on Skin to Promote Surgical Wound Healing in Cats. Animals (Basel). 2024 Apr 30;14(9):1358.
28- Liu H , Wang J, Deng Y, Zou G , Xu J. Effects of topical insulin on wound healing: a meta-analysis of animal and clinical studies. Endocr J. 2021 Aug 28;68(8):969-979.
29-Macedo AS, Mendes F , Filipe P, Reis S, Fonte P. Nanocarrier-Mediated Topical Insulin Delivery for Wound Healing. Materials (Basel) 2021 Jul 30;14(15):4257.
30- Yang P, Wang X , Wang D , Shi Y, Zhang M, Yu T, Liu D, Gao M, Zhang X , Liu Y. Topical insulin application accelerates diabetic wound healing by promoting anti-inflammatory macrophage polarization. J Cell Sci. 2020 Oct 12;133(19):jcs235838.
31- Wang J, Xu J. Effects of Topical Insulin on Wound Healing: A Review of Animal and Human Evidences. Diabetes Metab Syndr Obes. 2020 Mar 13;13:719-727.
32-Azevedo FF, Moreira GV, Teixeira CJ, Pessoa AFM, Alves MJ, Liberti EA, Carvalho CRO, AraújoEP, Abdala Saad MJ, Lima MHM. Topical Insulin Modulates Inflammatory and Proliferative Phases of Burn-Wound Healing in Diabetes-Induced Rats. Biol Res Nurs. 2019 Oct;21(5):473-484.
33- Yu T, Gao M, Yang P, Pei Q, Liu D, Wang D, Zhang X, Liu Y. Topical insulin accelerates cutaneous wound healing in insulin-resistant diabetic rats. Am J Transl Res 2017 Oct 15;9(10):4682-4693.
34-Sridharan K, Sivaramakrishnan G. Efficacy of topical insulin in wound healing: A preliminary systematic review and meta-analysis of randomized controlled trials. Wound Repair Regen. 2017 Apr;25(2):279-287.
35- Chen X, Liu Y, Zhang X. Topical insulin application improves healing by regulating the wound inflammatory response. Wound Repair Regen. 2012 May-Jun;20(3):425-34.
36- Lima MHM, Caricilli AM, Abreu LL, Araújo EP, Pelegrinelli FF, Thirone ACP, Tsukumo DM, Pessoa AFM, Santos MF, Moraes MA, Carvalheira JB, Velloso LA, Saad MJA. Topical insulin accelerates wound healing in diabetes by enhancing the AKT and ERK pathways: a double-blind placebo-controlled clinical trial. PLoS One. 2012;7(5):e36974.
37- Touitou E, Dayan N, Bergelson L, Godin B, Eliaz M. Ethosomes-novel vesicular carriers for enhanced delivery: characterization and skin penetration properties. J. Control. Release., 65 (2000), pp. 403-418
38- Goindi S, Dhatt B, Kaur A. Ethosomes-based topical delivery system of antihistaminic drug for treatment of skin allergies. J. Microencapsul., 31 (2014), pp. 716-724
39-Huanbutta K, Rattanachitthawat N, Luangpraditkun K, Sriamornsak P, Puri V, Singh I, Sangnim T. Development and Evaluation of Ethosomes Loaded with *Zingiber zerumbet* Linn Rhizome Extract for Antifungal Skin Infection in Deep Layer Skin. Pharmaceutics. 2022 Dec 9;14(12):2765.
40-Li Y, Xu F, Li X, Chen SY, Huang LY, Bian YY, Wang J, Shu YT, Yan GJ, Dong J, Yin SP, Gu W, Chen J. Development of curcumin-loaded composite phospholipid ethosomes for enhanced skin permeability and vesicle stability. Int J Pharmaceutics. 2021. Volume 592, 5 January 2021, 119936
41- Niu, X.P., Zhang, D.P., Bian, Q, Feng, X.F., Li, H., Rao, Y.F., Shen, Y.M., Geng, F.N., Yuan, A.R., Ying, X.Y., Gao, J.Q., 2019. Mechanism investigation of ethosomes transdermal permeation. Int. J. Pharm. Doi: https// doi.org/10.1016/j.ijpx.2019. 100027.
42-Chen ZX, Li B, Liu T, Wang X, Zhu Y, Wang L, Wang XH, Niu X, Xiao Y, Sun Q. Evaluation of paeonol-loaded transethosomes as transdermal delivery carriers. Eur J Pharm Sci. 2017 Mar 1:99:240-245.
43-Yang L, Wu L, Wu D, Shi D, Wang T, Zhu X. Mechanism of transdermal permeation promotion of lipophilic drugs by ethosomes. Int. J. Nanomed., 12 (2017), pp. 3357-3364
44-RobertsS, Mohammed Y, Pastore MN, Namjoshi S , Yousef S, Alinaghi A, Haridass IN, Abd E, Leite-Silva VR, Benson HAE, Grice JE. Topical and cutaneous delivery using nanosystems. J. Controlled Release, 247 (2017), pp. 86-105

45- Marto J, Vitor C, Guerreiro A, Severino C, Eleuterio C, Ascenso A, Simoes S. Ethosomes for enhanced skin delivery of griseofulvin Colloids Surf. B Biointerfaces, 146 (2016), pp. 616-623
46. El-Naggar ME, Abdelgawad AM, Abdel-Sattar R, Gibriel AA, Hemdan BA. Potential antimicrobial and antibiofilm efficacy of essential oil nanoemulsion loaded polycaprolactone nanofibrous dermal patches. European Polymer Journal. 2023;184:111782.
47. Hashem M, El-Shafei A, Sharaf S, Abdel-Sattar R, Mohamed A. Development and evaluation of novel multifunction hybrid containing cationic softener/TiO2/herbal oil for cotton based fabrics. Egyptian Journal of Chemistry. 2017;60(Conference Issue (The 8th International Conference of The Textile Research Division (ICTRD 2017), National Research Centre, Cairo 12622, Egypt.)):171-83.
48. Sharaf SS, El-Shafei AM, Refaie R, Gibriel AA, Abdel-Sattar R. Antibacterial and wound healing properties of cellulose acetate electrospun nanofibers loaded with bioactive glass nanoparticles; in-vivo study. Cellulose. 2022;29(8):4565-77.
49. Fung HB, Chang JY, Kuczynskf S. A practical guide to the treatment of complicated skin and soft tissue infections. Drugs. 2003;63:1459-80.
50. Ibrahim HM , El- Zairy EMR. Carboxymethylchitosan nanofibers containing silver nanoparticles: Preparation, Characterization and Antibacterial activity. Journal of Applied Pharmaceutical Science. 2016;6 (07), 043-048.
51. Abou-Okeil A, Refaei R, Khalil EM, Moustafa Sh.E, Ibrahim HM. Fabrication of novel green magnetic electrospun nanofibers based on Fe_3O_4 nanoparticles/PVA/chitosan/collagen. Egyptian Journal of Chemistry.2022;65 (8), 285-298.
52. Ali MA, Bydoon E, Ibrahim HM. Bioactive Composite Nonwoven Surgical Dressing based on Cellulose Coated with Nanofiber Membrane using the layer-by-layer technique. Egyptian Journal of Chemistry.2022;65 (4), 525-542.