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Research Article

Minoxidil and Dexamethasone Eluting Nanofiber Dressing for Cutaneous Wound Healing in Rat

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Abstract

Background: Minoxidil is an antihypertensive agent and vasodilator which may help to promote local blood flow thus hastening the closure of excisional skin wounds. Corticosteroids may down-regulate wound healing. This work aims to develop and characterize nanofiber-eluting dexamethasone and minoxidil and investigate their effect on wound healing in a rat model.

Methods: Minoxidil and dexamethasone-loaded wound dressings were developed and characterized with scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FT-IR). *In vitro*, drug release studies were performed for 12 days. To model the wound-healing activity of the developed formulations, excisional wounds were created on the dorsal section of male rats.

Results: All the electrospun wound dressing nanofibers displayed smooth structures and surfaces without drug crystals. Histological results of Hematoxylin & Eosin and Masson's trichrome staining indicate wound healing suppression in the dexamethasone-treated group and good healing activity in the minoxidil-treated group. Here we identified that the application of topical minoxidil can be effective for wound healing probably driven by the anagen hair growth while dexamethasone suppresses collagen production and prevents scar formation.

Conclusion: The findings suggest that this minoxidil and dexamethasone wound dressing can potentially be developed as a new treatment modality in the clinic to accelerate wound healing while preventing scar formation.

Introduction

An ideal wound dressing should act as a barrier; induce rapid wound closure and reduce scar formation. Various types of wound dressings including electrospun nanofiber and hydrogels have been developed.[1]

Minoxidil is an antihypertensive agent and vasodilator that helps to promote local blood flow thus hastening the closure of excisional skin wounds.[2] This drug has also been shown to induce the proliferation and development of epithelium cells. Contradictory results have also shown its effect on the inhibition of fibroblast proliferation and keratinocyte differentiation which suggest its effect as an antifibrotic agent.[3] *In vitro* activity of minoxidil on human fibroblasts demonstrated an antifibrotic effect with possible application in the treatment of keloids and hypertrophic scars.[4] An attempt was made to establish a relationship between the effect of minoxidil on fibroblast cells and animal models to observe wound contraction. Results suggest a comparable wound healing effect as measured by wound contraction rate and tensile strength measurements relative to the inhibitory *in vitro* findings on human fibroblast cells.[5]

Corticosteroid excess markedly down-regulates wound healing. These drugs reduce re-epithelialization and epidermal cell migration and harm wound healing.[6] Single-dose intraperitoneal dexamethasone administration in rats demonstrated significantly reduced fibroblast and collagen content compared with the control group. On the contrary, vascularity and inflammatory cells were higher in the dexamethasone group compared with the control.[7] A retrospective study on chronic-non healing wound show that topical application of clobetasone butyrate 0.05% induces a faster healing rate in most patients, while some patients experienced slow healing speed.[8]

Electrospinning is a versatile method to manufacture nanofibers with a high-surface-to- volume ratio and excellent properties that mimic the human extracellular matrix (ECM) structure. Nanofiber wound dressings may provide a suitable scaffold for cell growth and proliferation, and have high permeability to absorb exudates.[9]

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The wound healing process involves 4 phases including hemostasis, inflammation, maturation, and proliferation. The initial stage of wound healing is hemostasis which involves vasoconstriction. The second stage of wound healing inflammation occurs concurrently with the first stage and it mainly involves the release of reactive oxygen species (ROS). The proliferation stage consists of granulation, neovascularization, and re-epithelialization. Stage four of wound healing involves remodeling the skin surface and a scar is formed.[10]

This work aims to extend the research on wound healing by employing drug-loaded electrospun wound dressing. Nanofiber eluting dexamethasone and minoxidil were developed and characterized. Their effect on wound healing was investigated in the rat model.

Materials and Methods

Materials

Dexamethasone and minoxidil were obtained from Sepidaj Pharmaceutical, Iran. Acetone was purchased from Merk, *Darmstadt,* Germany. Poly caprolactone (PLC) Mw~ 70000-90000 g/mol was purchased from Sigma-Aldrich.

Electrospinning and fabrication of wound dressing

Nanofibers were fabricated via the electrospinning method. PCL solution was obtained by dissolving the polymer in acetone at 70 °C. After cooling down to room temperature, formulations were prepared as listed in Table 1. Polymeric samples were delivered by a syringe pump (Nanofanavaran, Iran) loaded with a 5-ml syringe with a volumetric flow rate of 1 ml/h with an adjustable DC power supply (Fannavaran, Iran). Electrospinning was carried out at room temperature and the positive voltage applied was 15 kV at room temperature. The as-spun nanofibers were placed in a vacuum before usage.

Scanning electron microscopy (SEM) of nanofiber wound mat

Morphological characteristics of nanofibers were evaluated using a scanning electron microscope (MIRA3, TESCAN, Czech Republic). Samples were fixed on a stud and coated with gold for 10 S before to obtaining the images at an accelerating voltage of 22.0 kV. The distribution of fiber diameter was carried out using image analysis software.

Fourier transform infrared spectroscopy (FT-IR)

FT-IR (Perkin Elmer Spectrum Two) analysis was conducted to observe the interaction between the polymer and drug molecules. Spectras were recorded at room temperature in the spectral region 450–4000 cm^{-1}. Data was collected on Spectrum software version 10.03.02.

Table 1. Composition of the developed wound dressings.

Formulation	Composition w/v		
	PCL	Dexamethasone	Minoxidil
A	10 %	-	-
B	10 %	0.5%	-
C	10 %	-	0.1%
D	10 %	0.5%	0.1%

Drug release studies

In vitro release studies were performed using the total immersion method over 12 days. A known weight sample was placed in a compartment filled with phosphate buffer pH 7.4 (10 ml). Samples were stirred at 800 rpm (shaker-incubator, Behdad, Iran) and drug release was studied at two temperatures including room temperature (25 °C) and skin temperature (32 °C) to study the cumulative drug release of the wound dressing. At fixed time intervals, a 1 ml sample was withdrawn from the container and replaced with a fresh phosphate buffer. Results were presented as % of cumulative release against time in days. Drug quantification was performed on UV-Vis spectrophotometer (Cecil, England). Minoxidil was quantified in water at a maximum wavelength of 288 nm, while dexamethasone was dissolved in ethanol: water (2:1) and was measured at 242 nm.[11,12]

Animal handling and excisional wound model

Male Wistar rats (160-180 g) were obtained from Pasture Institute, Iran. Animals were acclimatized to lab conditions for 10 days and were given food and water Ad libitum. A full-thickness skin incision model was developed on Wistar rats under anesthesia conditions (peritoneal injection of xylazine, 5 mg/kg, and ketamine 40 mg/kg). Hair removal was done using an electric hair clipper and a midline incision was made on the dorsum of each animal with an area of 1 cm2. Animals were divided at random into 4 groups as follows; control group, dexamethasone group, minoxidil group, dexamethasone, and minoxidil group. Each dressing was applied to the wound area every 4 days. Wound contraction was monitored on days 5, 10, and 15. The wound area (mm2) was measured using a ruler. Wound contraction (%) was expressed as follows.[13]

Histomorphological evaluation of wound regeneration

At the end of the two weeks period, all animals were sacrificed and the full-thickness skin tissues were harvested sections were made perpendicular to the wound and placed in 10% formaldehyde, embedded in paraffin. Histopathological changes were assessed microscopically with hematoxylin-eosin (H&E) and Masson's trichrome staining for qualitative analysis and evaluation of re-epithelization, fibroblast immigration, connective tissue synthesis, collagen production, and infiltration of inflammatory cells. Samples were studied under a light microscope (Oxion) at 4, 10, and 40×magnification and photographed with Image Focus Alpha.

Statistical analysis

The data from the *in vivo* wound closure experiment and drug release was analyzed using one-way ANOVA. Independent-sample Student's T-test was used to analyze the difference between groups and a P value <0.05 was considered statistically significant. All results are expressed

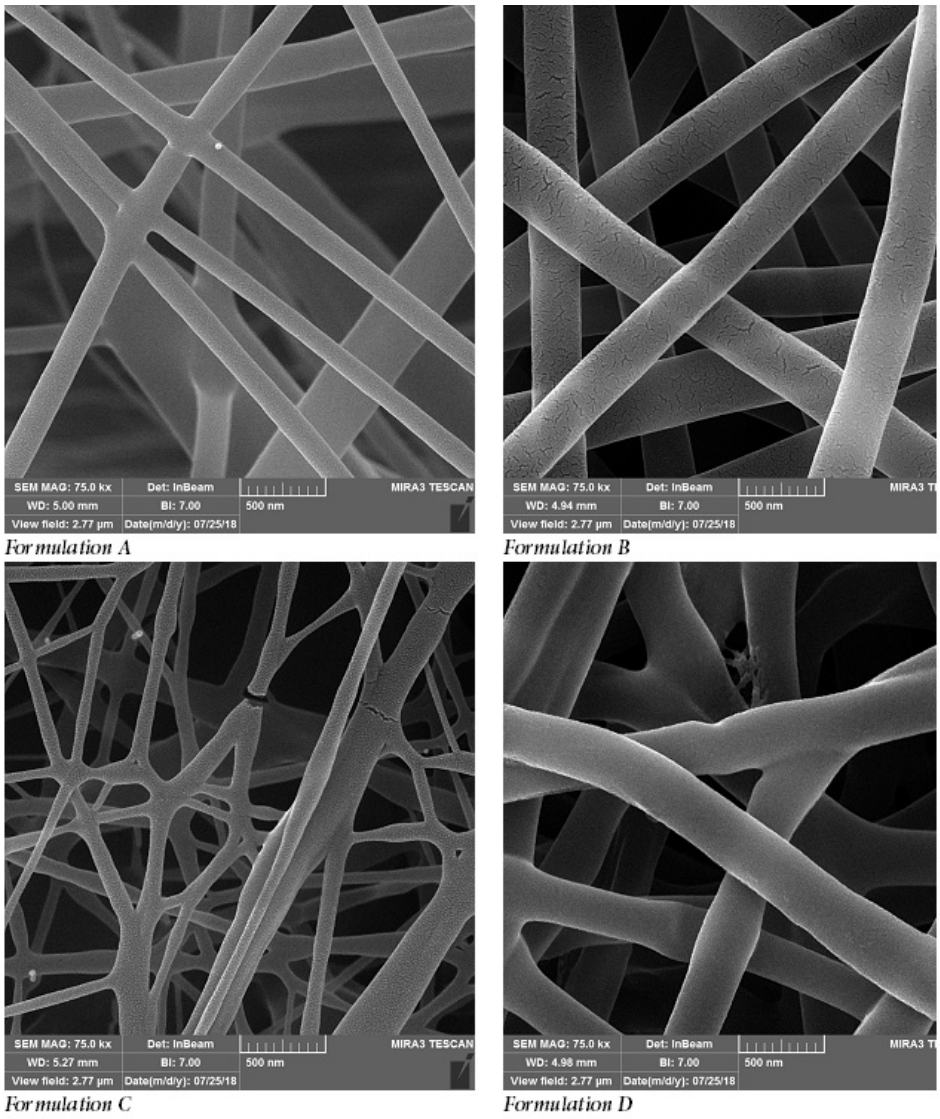

Figure 1. SEM image of wound dressing formulations.

as mean ± standard deviation (SD).

Results

SEM image of the nanofiber dressing

The morphology of the nanofiber wound dressings was determined using SEM as shown in Figure 1. It is seen that all formulations displayed smooth structure and surface without drug crystals ensuring complete encapsulation of the drug within the fibers. The micrographs did not demonstrate the presence of drug crystals and this may suggest that the drug was encapsulated within the nanofibers.[14]

The diameter of the nanofibers containing dexamethasone was larger (307.7±14.2 nm) as compared to the minoxidil-containing formulations (126.53±13.4 nm). These results may be due to the complex structure of dexamethasone and its higher concentration used relative to minoxidil which results in the formation of nanofibers with larger diameters.[15]

Drug release studies

The release profile of nanofiber wound dressings was investigated for over 12 days and the results are depicted in Figure 2. All formulations exhibited a similar drug release pattern. The release was characterized by an initial fast release at the end of day one with around 60% drug release followed by gradual release until day 12. Burst release was observed which may be due to the accumulation of the drug molecule on or near the surface of nanofibers during the electrospinning process. The burst release may be due to the noncovalent loading of the drug molecules.[16] This evidenced the slow degradation of the polymer and the drug release pattern from nanofiber mats. In both formulations higher drug release is seen at 32 °C relative to 25 °C, however, no significant difference is seen in the drug release pattern at the two studied temperatures.

FT-IR analysis

The FT-IR spectra of the nanofibers are depicted in Figure 3. Methylene group of polycaprolactone was detected at

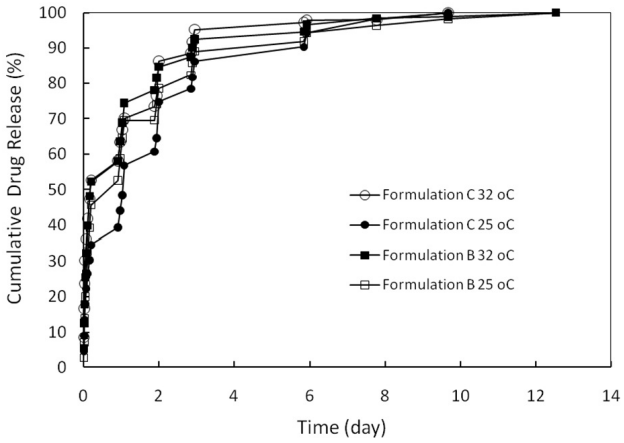

Figure 2. Cumulative drug release from formulation B and C.

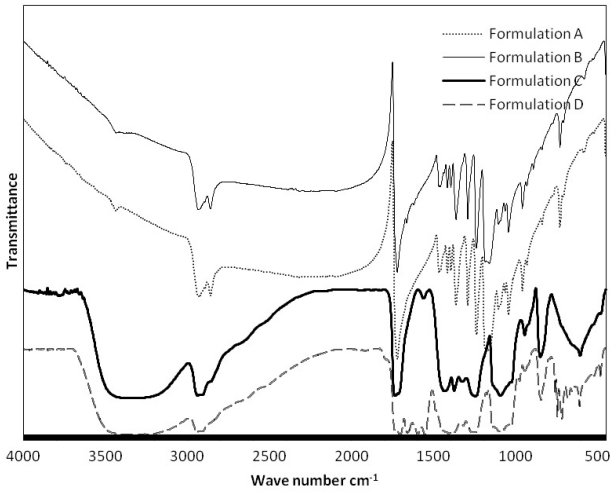

Figure 3. FTIR spectrum of the developed formulations.

2940 cm^{-1} and 2863 cm^{-1}. CH2 bending was seen at 1292 cm^{-1}, 1364 cm^{-1}, and 1469 cm^{-1} while C-O-C stretching was detected at 1187 cm^{-1}. Other peaks including O-C=O stretching and C-C stretching were detected at 1724 cm^{-1} and 1293 cm^{-1} respectively. A small peak at 3435 cm^{-1} represents the O-H stretching vibration.[17,18] These peaks were present in all samples. The peak for minoxidil appears at 3439 cm^{-1} and 2939 cm^{-1} for N-H stretching and aromatic and aliphatic C-H stretching respectively. Aromatic C=N stretching is observed at 1773 cm^{-1} and 1722 cm^{-1} and the pyrimidine N-O aromatic stretching is seen at 1291 cm^{-1}, 1235 cm^{-1} and 1155 cm^{-1}.[19,20]

For dexamethasone samples, a C-H stretching vibration is seen at 2933 cm^{-1}, and the C=O stretching is seen at 1664 cm^{-1}, also the C-F stretching vibration appears at 1248 cm^{-1}. The C=O and the ring C=C stretching vibrations are seen at 1721 cm^{-1} and 1664 cm^{-1} respectively.[21] Results indicate that dexamethasone and minoxidil were successfully integrated within the nanofiber wound dressing via the electrospinning method.

The FT-IR spectra of a sample containing both dexamethasone and minoxidil (formulation D) elucidated that the disappearance of the –OH stretching band at 3525 cm^{-1} confirms that the O-H bonding is the main site for interaction with dexamethasone. Other functional groups remained unchanged, this indicates the stability of the drugs in the developed wound healing mat.

Wound contraction and healing

The wound healing effect was evaluated on full-thickness skin in a rat model. Wound contractions are displayed on days 0, 5, 10, and 15. In the minoxidil group, contraction of the wound was very markedly and on the 5th day, minoxidil-containing dressings show a significant reduction ($p<0.0005$) in wound size relative to the control group while dexamethasone delayed wound contraction (p value not significant). Figure 4 shows the wound- healing effect of various formulations studied.

The macroscopic image of surface healing dressed with formulations at days, 5, 10, and 15 are shown in Figure 5. Statistical analysis indicates significantly slow wound contraction in the group treated with dexamethasone relative to the minoxidil group ($p<0.01$).

Histological analysis

Skin regeneration and inflammation in the wound area were examined 15 days post-surgery. Hematoxylin and eosin stained sections (H&E staining) are shown in Figure 5. Dexamethasone-loaded nanofiber wound dressing group show less inflammatory cells and fibroblasts migrating to the wound site while the control group (nanofiber without drug) exhibited relatively more fibroblast cells which are well attributed to the anti-inflammatory action of dexamethasone. In the group treated with dexamethasone, the epidermis layer was flat and thin. Furthermore, the minoxidil group exhibited high content of blood vessels and hair follicles relative to the control group. Thick epidermis and well-proliferated granulation tissue are seen in the minoxidil-treated group which is an indication of the wound healing process. It is seen that the treatment group receiving both minoxidil and dexamethasone showed complete epithelium structure and better connective tissue arrangement.

Discussion

Minoxidil and dexamethasone-loaded wound dressings were successfully developed and characterized using SEM

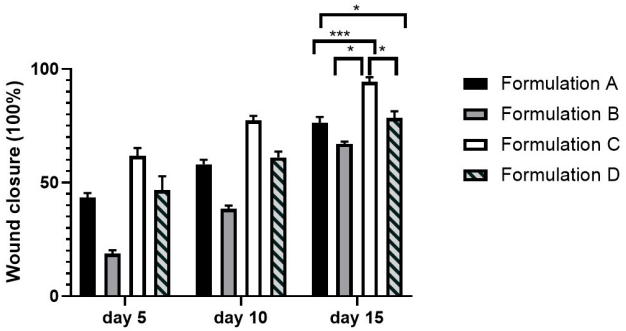

Figure 4. Wound closure rate after 15 day treatment. Statistical analysis was carried out using a students's t test to analyze difference between groups.

Figure 5. Macroscopic image of surface healing dressed with formulations at day, 5, 10, 15.

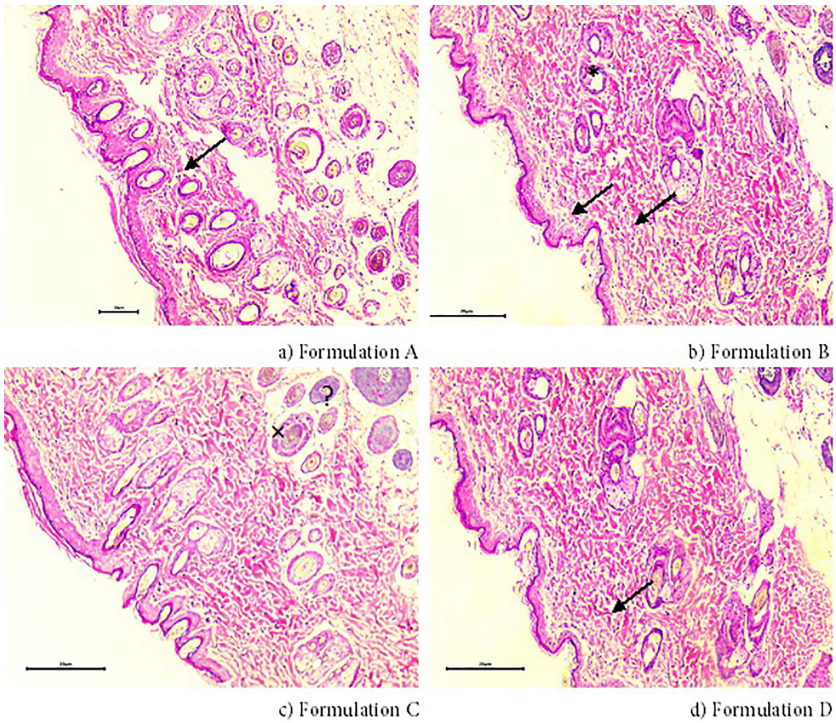

Figure 6. Histomorphological evaluation of wound regeneration on the 15th day with H&E (10X) staining, Granulation tissue thickness, blood capillaries, hair follicles and the boundary of epithelium and dermis are shown. a) Formulation A: Black arrows show granulation tissue mononuclear inflammatory cells, empty white space indicates lack of tissue formation, b) Formulation B: granulation tissue and mononuclear inflammatory cells are shown with black arrows, neovascularisation is shown with * c) Formulation C: the symbol Δ indicates sections of hair follicles close to hair bulb and with most layers visible and × denotes sections of different heights along the hair follicles, d) Formulation D: the application of minoxidil and dexamethasone loaded nanofiber wound dressing in rats show healed skin structure and well-formed, near normal epidermis tissue.

and FT-IR. *In vitro*, drug release studies indicated the gradual release of the drug over 12 days.

Figure 4 indicates that wound closure in the group treated with nanofiber without any drug is relatively good. Electrospun nanofibers are widely employed in various fields including drug delivery, tissue engineering, and wound healing. Electrospinning enables the production of an interconnected network of nano-scale diameter fiber which mimics the ECM and thus promotes the healing of the skin.[10]

Results of the wound closure rate are in good agreement with the H&E and Masson's trichrome staining observations shown in Figures 6 and 7. Wound healing studies on rat models indicate good healing properties of minoxidil-loaded dressing but healing suppression was seen from dexamethasone-loaded nanofibers. Hair re-growth is higher in the minoxidil (formulation C) and minoxidil + dexamethasone group (formulation D) and telogen to anagen conversion is increased in these two groups. This approach is in agreement with the findings that cutaneous wound healing is enhanced during the anagen stage of hair growth.[22] Another finding shows that wound contraction and tensile strength measurements on the excisional wound model treated with minoxidil

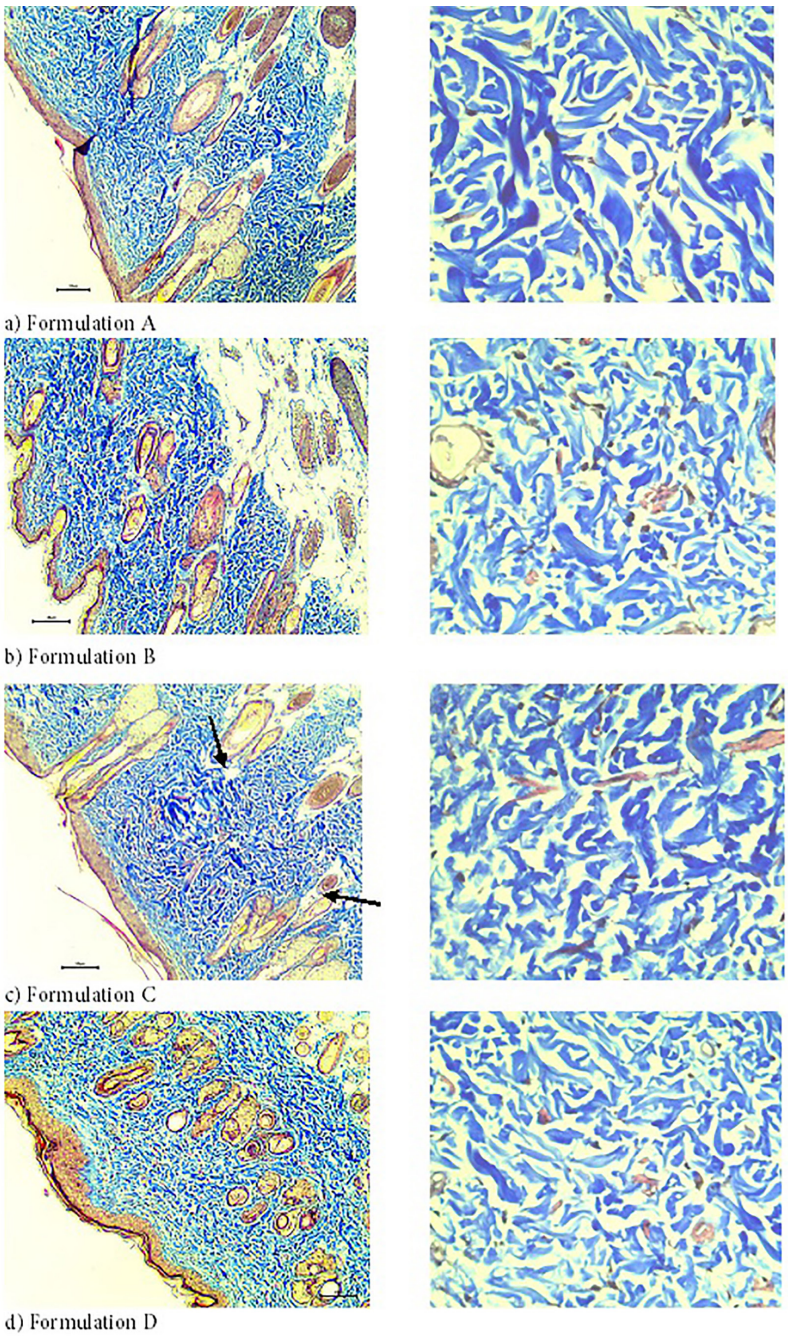

Figure 7. Masson's trichrome staining for collagen deposition; scale bar 20 μm, a) dense and irregular collagen fibers, b) decrease in collagen production, c) mature and well arranged collagen fibers and possibility of scar formation shown with arrows, d) complete arrangement of connective tissue with no sca.

did not show any inhibitory effect on wound healing. A different hypothesis may be that the application of minoxidil parent drug may have an opposite effect relative to its sulfate compound in suppression and inhibition of wound healing.[5] The Photomicrograph of histological analyses for Masson's trichrome staining is shown in Figure 7. A disorganized collagen pattern is seen in the control group. Irregular-shaped collagen fibers with large diameters are seen in the control group in which collagen is represented by blue-colored tissue. Wounds treated with dexamethasone show better organization and distribution of collagen fibers. These findings are in agreement with previous findings.[6] Decrease in collagen type I synthesis has been observed in wounded connective tissue treated with corticosteroids. Histochemical findings indicate that a single dose of dexamethasone IP in rats exhibits significant inflammatory cells and may delay wound healing. Dexamethasone decreases collagen 1 production and may interfere with collagen formation and wound contraction leading to inadequate wound healing.[7]

Scar formation is the consequence of an imbalance in collagen synthesis and degradation which may lead to the accumulation of excess dermal collagen. Type I and III collagen are the main component of the extracellular matrix. Collagen production is necessary for the wound healing process; however, its production in excess can result in scarring. Therefore adequate collagen expression is required for ideal wound healing.

The epidermal thickness index is a marker used for the analysis of hypertrophic scars. This index is a ratio of the epidermal height of scar tissue to the relative epidermal height in normal uninjured skin and values more than 1 indicate scar formation.[23]

Conclusion

This work aimed to demonstrate the applicability of minoxidil and dexamethasone-loaded wound dressing for wound repair. Wound healing activity was assessed using excisional wounds on the dorsal section of male rats. Histological results of Hematoxylin & Eosin and Masson's trichrome staining indicate wound healing suppression in the dexamethasone-treated group and good healing activity in the minoxidil-treated group. Here we identified that the application of topical minoxidil can be effective for wound healing probably driven by the anagen hair growth while dexamethasone suppresses collagen production and prevents scar formation. Results suggest that this minoxidil and dexamethasone wound dressing can potentially be developed as a new treatment modality in the clinic to accelerate wound healing while preventing scar formation.

Ethical Issues

All animal work was approved by the Urmia University of Medical Sciences for ethical and Animal Care and Use Committee (IR.UMSU.REC.1396.302).

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Author Contributions

Saber Khozan: Investigation. Behsa Salehian Investigation, Formal Analysis. Fatemeh Montaghimi: Formal Analysis. Yaeghob Sharifi: Methodology, Visualization. Anahita Fathi-Azarbayjani: Conceptualization, Formal Analysis, Writing - Original Draft.

Conflict of Interest

The authors report no conflicts of interest.

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