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Wound healing acceleration using topical chitosan/ Zinc oxide nanocomposite membrane and local insulin injection in diabetic rats via modulation of genes expression targeting angiogenesis

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ABSTRACT

Diabetic wounds regularly take a longer time to heal than wounds in healthy animals. Chitosan (CS) interacts with many cellular processes and it elevates the necessary expression of growth factors in wound healing. The topical utility of zinc was suggested to limit inflammation, enhance re-epithelialization, and lower bacterial growth in chronic wounds. Also, there is evidence that insulin has effects on healing wounds. Therefore, the wound healing potential of insulin injection with CS/Zno nanocomposite membrane in diabetic rats was assessed. Diabetes was induced by a single IP injection of Streptozotocin (STZ) at a dose of 50 mg/kg b.wt. after diabetes induction. on the back of eacfull, a full-thickness excisional wound had been made. Forty-eight male rats were divided into Six groups. Group I: normal wound non-treated**,** Group II: diabetic wound non-treated, Group II: normal wound treated with CS /ZnO membrane, Group IV: diabetic wound treated with CS/ZnO membrane, Group V: diabetic wound and local insulin treatment (2 U/rat per day), Group VI: diabetic wound treated with Chitosan /ZnO membrane and insulin injection. The findings revealed a notable decline in Basic fibroblast growth factor(bFGF), Vascular endothelial growth factor (VEGF), Transforming growth factor β(TGF-β), and α-Smooth muscle actin(α-SMA) in diabetic non-treated wounds. However, treatment with CS/ZnO nanocomposite membrane or local insulin injection exhibit a significant increase. Conclusively, CS/ZnO nanocomposite membrane and local insulin accelerate diabetic wound healing through activation of growth factors production and stimulating the proliferation of inflammatory cells during the healing process and angiogenesis, followed by wound maturation.

1. INTRODUCTION

Diabetes frequently causes chronic wounds that don't heal and show some distinctions from typical wounds. Chronic wounds that cannot be healed and return the skin's structural and functional integrity are the most common consequences of diabetes (Tao et al., 2018). It's really that: simple improper extracellular matrix buildup damaged re-epithelialization, aberrant inflammation, and hindered angiogenesis are the main pathophysiological abnormalities of diabetic skin in the wound healing process. (Ozdemir and Feinberg, 2019). Because of its structural resemblance to glycosaminoglycans, a crucial macromolecule for wound repair that is present as a component of the extracellular matrix, chitosan is often employed in wound healing (ECM) (Shariatinia and Mazloom, 2018). Because CS is a biodegradable polymer and its byproducts can trigger the creation of ECM components, indicating that it is safe to use in living organisms. Additionally, among the rare polymers that have extraordinary antibacterial property (Kohsari et al., 2016).

In the animal model, ZnO NPs also have the ability to cure a variety of wound types. Numerous researches have been done to support its role in promoting the epithelialization of injured skin. (Khan et al., 2021). Moreover, Numerous researches suggested that the skin safe insulin boost wound recovery in rats with diabetes skin and people due to the fact insulin promotes tissue development, which backs the improvement of several cellular kinds and development, movement, and secretion by using endothelium, fibroblast and keratinocytes cells (Wang and Xu, 2020) Consequently, the insulin capacity to heal injuries with chitosan/Zno nanocomposite membrane in diabetic rats were evaluated via the molecular determination of (bFGF), (VEGF), (α-SMA) and (TGF-β) gene expression in addition to histopathological examination of skin tissues in diabetic rodents.

2. MATERIAL AND METHODS

2.1. Experimental Animals

Forty-eight male Wister albino rats with a two-month history and average body weights of 180–200g. Rats were housed in separate metal cages and kept at constant environmental and nutritional conditions throughout the period of the experiment. The rats were fed on constant ration and fresh, clean drinking water was supplied ad-libitum. All rats were acclimatized for a minimum period of 15 days prior to the beginning the of study. The experiment was conducted according to the guideline for

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the care of laboratory animals approved by the ethical animal committee of Benha university (Approval no. BUFVTM 02-8-21).

2.2. Chemicals and antioxidant agents

2.2.1. Streptozotocin

One intraperitoneal (i.p.) injection of 50 mg/kg body weight of streptozotocin (STZ), which was originally purchased from Sigma Chemical Co. in the United States, causes hyperglycemia. (Ramanathan et al., 1999).

2.2.2. Insulin

Long-acting insulin was bought from (Lantus Solostar, Sanofi-Aventis, Germany). It is subcutaneously injected at a dose of (2 U/rat per day) (Michael et al., 2012).

2.2.3. Chitosan (CS): was obtained from (Sigma-Aldrich, Chemical Company).

2.2.4. Zinc oxide nano-particles (nZnO): were acquired from (Sigma-Aldrich).

2.2.5. Poly vinyl alcohol (PVA): was purchased from (Loba, chemie).

2.3. Preparation of PVA/CS and PVA/CS/ZnO composite membranes

By dissolving 1g of chitosan in 1% (v/v) acetic acid, a chitosan solution with a concentration of 2% was created. The PVA solution was created by dissolving 5 g of PVA powder in 100 ml of distilled water. In order to create a homogenous polymer solution, 20 ml of polymer mixture solution were blended in the ratios of 1:3 and 1:4. ZnO nano-powder 100 mg was mixed with blend solution while being stirred. after 30 minutes of magnetic stirring and sonication of the aggregate, the resultant solution was cast onto glass plates, and the evident membranes were obtained when the solvent slowly evaporated in room temperature air. The following PVA/CS, PVA/CS/ZnO 1:3:1, and PVA/CS/ZnO 1:4:1 codes were added to the membrane in line with its composition (Omer *et al*., 2021).

2.4. Induction of diabetes

Male rats were given one intraperitoneal (i.p.) injection of STZ at a dose of 50 mg/kg body weight, which resulted in experimental diabetes, STZ fresh dispersion in a citrate buffer with pH of 4.5 Following STZ injection, the animals were given a glucose solution (5%) w/v. to prevent hypoglycemia, which STZ may have caused the previous day, 48 hours after STZ injection, hyperglycemia was confirmed by blood glucose monitoring with an ACCU-CHEK sensor glucometer. The rats exhibiting hyperglycemia were classified as diabetic when their blood glucose levels exceeded 250 mg/dl (Ramanathan et al., 1999).

2.5. Excisional skin wound Induction

Every rat's back had a full thickness excisional skin wound a week after the diabetes was started. the rats' back hair was removed after being rendered unconscious with thiopental sodium (40 mg/kg/rat). Using a surgical marker pen, a typical wound with a diameter of 6 mm was produced for each rat. After that, full thickness excisional incisions were made by employing a scalpel blade to cut through the muscle layer. (João DeMasi et al., 2016). Additionally, the wound was high on their back, close to their neck, making it impossible for them to access it. Following the procedure, each rat was housed separately. Moreover, they were checked twice daily to ensure that all the membrane applied to the incisions was still in place (Colobatiu et al., 2019).

2.6. Experimental design

The rats were randomly divided into six groups of eight rats each, kept in separate cages for every group, and categorized as following:

Group I (Normal wound): wounded, non-treated, non-diabetic rats.

Group II (Diabetic wound non-treated): wounded, non-treated, diabetic rats.

Group III (Normal wound treated with chitosan/ZnO nanocomposite membrane): wounded, non-diabetic rats treated with the topical application of chitosan/ZnO nanocomposite membrane. The wounded area was covered with a membrane, and the membrane was replaced with a fresh one at 3,5, 7, 10, 12, and 14 days.

Group IV (Diabetic wound treated with chitosan/ZnO nanocomposite membrane): wounded, diabetic rats with topical application of chitosan/ZnO nanocomposite membrane.

Group V (Diabetic wound treated with local insulin): wounded, diabetic rats treated with local insulin injected subcutaneously (2U/rat per day) for 14 days.

Group VI (Diabetic wound treated with local insulin and chitosan/ZnO nanocomposite membrane): wounded, diabetic rats treated with topical application of chitosan/ZnO nanocomposite membrane and local insulin injection.

2.7. Estimation of wound healing

To record the physical appearance and healing of the wounds, photos of the wound area were taken using a digital camera (Sony, Japan) on days 0, 3, 7, 10, 12, and 14 were previously selected for wound contraction measurement. vertically focusing the camera to the center of the wound allowed for the taking of the pictures. The part of the wound that had not healed was measured using an unusual millimeter-graded ruler. The success of the therapy was evaluated by the amount of wound shrinking. to calculate the wound contraction, which was expressed as a percentage reduction of the wound area, a formula was applied:

Wound healing % = [(Wound area on day 0 –Wound area of day n) /Area on day0] ×100%.

Where; day 0 = immediately after wounding; n = 3, 7,10,12,14 days of measurement (Hasamins *et al*., 2010).

2.8. Sampling

Skin Tissue samples: An overdose of pentobarbital was administered i.p. to euthanize the animals (Zhou et al., 2017) after 14 days of wound treatment. Skin specimens were obtained from the widest area of the wound tissue with the surrounding normal skin margin. Skin tissues specimens were immediately kept in liquid nitrogen and stored at -80°C till RNA extraction for determination of the following gene expression: (bFGF), (VEGF), (TGF β) and (α-SMA).

2.9. Analysis

2.9.1. Molecular analysis

Quantitative RT-PCR was used for the determination of *bFGF, VEGF, TGF β and α-SMA*.in Skin tissue according to the method described by Thermo Scientific, Fermentas, # K0731. β-actin was used as load control.RNA Extraction kit according to manufacturer's

instructions. Each cDNA, sample was reverse transcribed using RevertAid TM First Strand CDNA synthesis kit (#EP0451, Thermo Scientific, Fermentas, USA) (Table 1). Then, real-time quantitative PCR amplification was performed on Faststart Universal SYBR Green Master (Roche, GER). The target gene was normalized with β-actin by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 1 Forward and reverse primers sequence for primers in qPCR

Gene	Forward primer ('5 ------ '3)	Reverse primer ('5 ------ '3)
bFGF	TCCATCAAGGGAGTGTGTGC	TCCGTGACCGGTAAGTGTTG
VEGFA	GATCATGCGGATCAAACCTCACC	CCTCCGGACCCAAAGTGCTC
TGFb	AAGAAGTCACCCGCGTGCTA	TGTGTGATGTCTTTGGTTTTGTCA
a SMA	GAGCGTGGCTATTCCTTCGTG	CAGTGGCCATCTCATTTTCAAAGT
B-actin	AAGTCCCTCACCCTCCCAAAAG	AAGCAATGCTGTCACCTTCCC

2.9.2. Statistical analysis

We used ANOVA which is a one-way analysis of variance to calculate the differences in means of variables between the groups. The obtained results were displayed as the mean ± SE and were examined by a software tool named Statistical Package for Social Science (SPSS) V20, at P<0.05 where this probability is considered as significant.

3. RESULTS

3.1- Evaluation of wound closure

A significantly higher wound closure percent were observed in course of 14 days in all diabetic wound treated with CS/ZnO membrane (G4), local insulin injection (G5) and diabetic wound treated with Chitosan /ZnO membrane and insulin injection (G6) and the highest percent of wound contraction were observed in G6, followed by G4 and G5 as compared to diabetic non treated wound rats (G2). Also, normal wound treated with CS/ZnO membrane (G3) displayed a significant wound contraction percent at day14 as compared normal wound non treated (G1) as shown in (Fig. 1a and Fig.b).

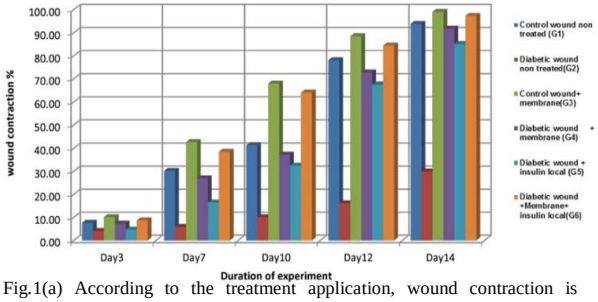

Fig.1(a) According to the treatment application, wound contraction is measured as a percentage of original contraction on days 0, 3, 7, 10, 12, and 14 following surgery.

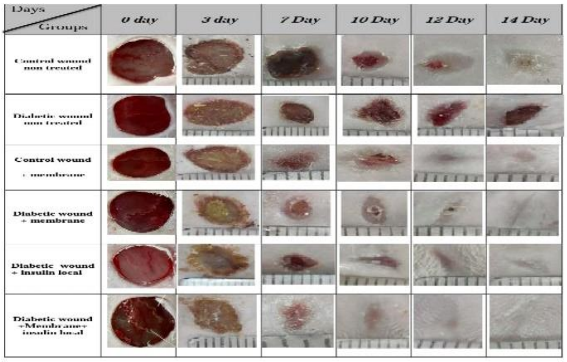

Fig 1(b) Wound closure time at 0, 3,7,10,12 and 14 days after treatment with topical chitosan /ZnO membrane and local insulin injection.

3.2. Molecular analysis results:

Effect of treatment with local insulin injection and topical Chitosan/Zno nanocomposite membrane on *bFG, VEGF, TGF β and α-SMA* genes expression in diabetic wound rats are represented in table (2) and graphically illustrated in figures (2 ,3,4,5).

Markedly down regulation in tissue *bFGF* and *VEGF* genes expression were observed in skin tissue of diabetic wounds compared with normal wound. However, treatment with insulin (GV) and CS/ZnO membrane with insulin injection (GVI) to diabetic wound rats showed a noticeable improvement in tissue *bFGF* and *VEGF* gene expressions as compared with diabetic wound non-treated group (GII) With highest down regulation in (GVI) followed by (GV) and finally (GIV). Also, a significant up regulation in *bFGF* and *VEGF* gene expressions were observed in CS/ZnO membrane treated normal wound rats (GIII) comparing with normal non treated group (GI).

Table 2 Effect of treatment with local insulin injection and topical chitosan/Zno nanocomposite membrane on *bFGF, VEGF, TGF β and α-SMA* gene expression in experimental model of diabetic wound in rats.

Animal Groups	bFGF Fold change ± SE	VEGF Fold change ± SE	TGF β Fold change ± SE	α-SMA Fold change ± SE
Control normal (GI)	1.00 ± 0.06 ^{d}	1.00± 0.06 ^{d}	1.00 ± 0.07 ^{e}	1.00 ± 0.07 ^{d}
Diabetic non treated (GII)	0.08 ± 0.02 ^{e}	0.18± 0.02 ^{e}	0.76 ± 0.05 ^{e}	0.68± 0.05 ^{e}
Control + membrane (GIII)	3.36 ± 0.27 ^{a}	5.39± 0.27 ^{a}	7.78 ± 0.39 ^{a}	4.03± 0.22 ^{a}
Diabetic + membrane (GIV)	1.88± 0.13 ^{c}	1.41± 0.13 ^{c}	2.41± 0.11 ^{d}	0.96 ± 0.08 ^{d}
Diabetic + insulin (GV)	2.38± 0.17 ^{b}	1.64± 0.17 ^{c}	3.32± 0.15 ^{c}	1.67± 0.12 ^{c}
Diabetic + membrane + insulin (GVI)	3.20 ± 0.19 ^{a}	2.13 ± 0.19 ^{b}	5.10 ± 0.23 ^{b}	2.14 ± 0.14 ^{b}

Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

Remarkably down regulation in tissue *TGF β and α-SMA* genes expression were noticed in the skin of diabetic wounds compared with normal wound. However, treatment with insulin (GV) and CS/ZnO membrane with insulin injection (GVI) to diabetic wound rats demonstrated a considerable up regulation in tissue *TGF β and α-SMA* gene expressions as compared with diabetic wound non-treated group (GII) With highest down regulation in (GVI) followed by (GV) and finally (GIV). Also, a significant up regulation in *TGF β and α-SMA* gene expressions were observed in CS/ZnO membrane treated normal wound rats (GIII) comparing with normal non treated group (GI).

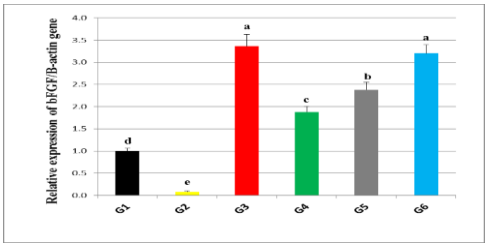

Fig (2) Effect of insulin injection and topical CS/ZnO nanocomposit membrane on bFGF gene expression in experimental model of diabetic wound in rats.

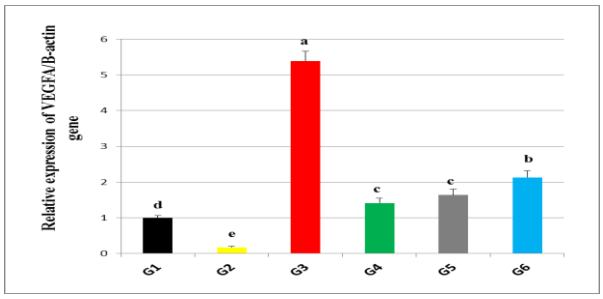

Fig (3) Effect of insulin injection and topical CS/ZnO nanocomposit membrane on VEGF gene expression in experimental model of diabetic wound in rats.

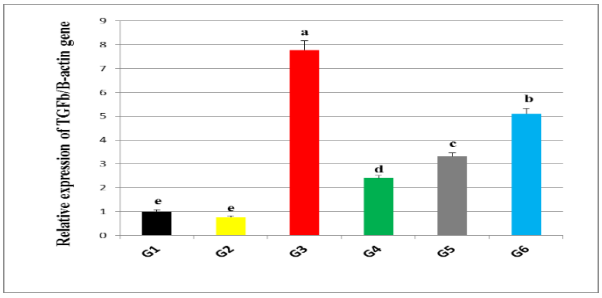

Fig (4) Effect of insulin injection and topical CS/ZnO nanocomposit membrane on TGFb gene expression in experimental model of diabetic wound in rats.

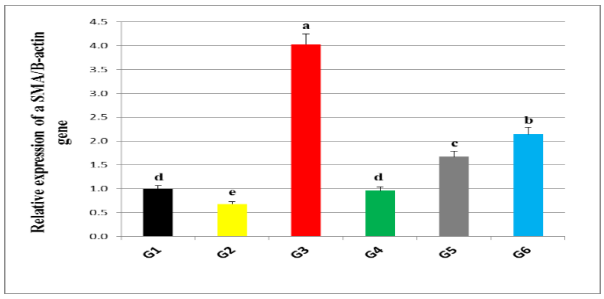

Fig 5 Effect of insulin injection and topical CS/ZnO nanocomposit membrane on α-SMA gene expression in experimental model of diabetic wound in rats.

4. DISCUSSION

Cytokines and various growth factors are released in the wound region to start wound healing (Werner and Grose, 2003). Skin healing is thought to be significantly influenced by two growth factors, VEGF and TGF (Savari et al., 2019). Certain cells release VEGF in response to ischemia and inflammation, which causes endothelial migration, chemotactic agent synthesis, proliferation, angiogenesis, and the development of granulation tissues (Bao et al., 2009). Furthermore, TGF-β activates cells to increase the generation of protein synthesis from the ECM while concurrently reducing collagen proteases (El Gazaerly et al., 2013). Fibroblast cells have the potential to be activated and changed into myofibroblasts at some point in the wound healing process, producing ECM and playing a crucial part in the contraction of granulating tissue. α-SMA expression is considered a particular myofibroblasts indicator. Moreover, TGF- β1 promotes fibroblast differentiation (Wynn, 2008). The results collected showed in table (2) and figure (2,3) revealed that, a noticeably increase in wound tissue bFGF and VEGF expressions in CS/ZnO membrane treated rats comparing with normal group. were markedly, decrease in Diabetic non treated rats comparing with normal group. This result came in accordance with Kang et al., (2013) who attributed, growth factors are polypeptides with biological activity that play a role in cell division, proliferation, migration, and metabolism. According to earlier studies, bFGF is a strong angiogenic factor that helps endothelial cells develop and migrate. It is produced by mast cells and promotes the growth of myocytes and endothelial cells. bFGF is essential for angiogenesis and wound repair (Barrientos et al., 2008), Angiogenesis and wound healing depend on signalling mediated by the fibroblast growth factor receptor (FGFR). Furthermore, VEGF has a significant role in encouraging the growth, migration, and survival of endothelial cells during the wound healing process, which results in neovascularization (Xie et al.,2022). Moreover, Lee et al. (2007) demonstrated that, the function of VEGF-A in the survival of endothelial cells; it is critical for vascular homeostasis. Additionally, Lee et al. (2014) who founded that, bFGF greater the expression of VEGF. by promoting endothelial cell migration and by raising endothelial cell permeability, VEGF plays a significant role in angiogenesis and the healing of wounds (Ferrara, 2001). Additionally, Wiesmann et al. (2019) reported that, ZnO NPs can release zinc ions and bring about the generation of ROS (Heim et al., 2015). It is conceivable that the production of ROS activates the MAPK pathway and causes the release of pro-angiogenic VEGF as a pro-angiogenic mechanism of action in response to radiation (Drigotas et al., 2013). It turned into already proven that, zinc can enhance FGF-2-stimulated VEGF release resulting from upregulating activation of p44/p42 MAP kinase (Hanai et al., 2006), Additionally, Augustine et al. (2014) noted that, ZnO NPs caused an up regulation of VEGF and FGF-2.

First investigations have shown that the addition of a small amount of ZnO NPs to a biomaterial can aid in improving tissue integration of the material by boosting fibroblast adhesion, neo-angiogenesis, and wound healing (Mohandas et al., 2015). ZnO NPs are believed to create reactive oxygen species, which may then promote the upregulation of pro-angiogenic factors such vascular endothelial growth factor (VEGF) (Augustine et al., 2014).

The obtained results showed in table (2) and figure (4,5) revealed that, markedly up regulation of TGF β activity, α-SMA activity was observed in CS/ZnO membrane treated rats comparing with normal control group. The current study agreed with those of Baxter et al. (2013) reported that, chitosan affects how growth factors involved in healing are activated. chitosan has been shown to speed tissue regeneration in mice burn wounds by increasing TGF-b1 expression and collagen production on day 3. chitosan reduces TGFb1 expression in the late post-injury phase (day 7), which would otherwise encourage scar formation. Several cytokines and growth factors are released throughout the healing process. TGF-, a multifunctional cytokine, plays a part in each of the three stages of wound healing among these elements (Wu et al., 2019). Similarly, Shi and Massague (2003) showed that, on day 14 of the experimental paradigm, the animals given the chitosan-zinc complex had increased TGF- β and VEGF mRNA expression. Serine/threonine kinase type I or II receptors that are expressed on the cell surface are the targets of TGF- β. In diabetic cases, wound healing has been linked to a lower concentration of TGF β (Bitar and Labbad, 1996). Moreover, Darby et al. (1997) observed that, decreased alpha-smooth muscle actin (α-SMA) staining in the cells and lack of orientation of fibroblasts in diabetic skin. Importantly, (El Kahi et al., 2009) showed that, TGF-β1 modulated α -SMA expression. The widely recognized marker α-SMA plays crucial role in healing of wounds (Wang et al., 2017).

The current results suggest that, a remarkably increase of TGF β, α-SMA gene expression were discovered in CS/ZnO membrane treated rats comparing with normal group. However, a greater decrease of TGF β and α-SMA were observed in Diabetic non treated rats compared to normal wound group. Treatment with CS/ZnO membrane with insulin injection and insulin to diabetic wound in rats exhibited a significant increase in TGF β and α-SMA gene activity as compared to diabetic wound non-treated group. Similarly, Hinz (2007) reported that, the remodeling phase begins at the give up of the granulation skin improvement. myofibroblasts, which produce (α-SMA) and contract the wound, are differentiate from fibroblasts by being stimulated by Mechanical tension and cytokines like TGF- β. Moreover, treatment with CS/ZnO membrane and insulin injection to diabetic wound in rats showed greater increase in TGF β and α-SMA expression compared with rats treated with CS/ZnO membrane and rats with insulin injection only.

5. CONCLUSION

The present study revealed that chitosan/ZnO membrane and insulin injection have potential accelerate healing of diabetic wounds via activation of growth factors production in wound healing stags, stimulate angiogenesis and inflammatory cells in healing process and augment wound contraction.

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