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Wound healing treatment of local insulin injection with topical chitosan/zinc oxide nanocomposite membrane in diabetic rat model

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ABSTRACT

Diabetes put off healing method as it impairs every section of wound restoration i.e., hemostasis, irritation, proliferation, and remodeling section. Insulin and a natural polymer of chitosan (CS) can potentially repair the integrity of broken pores and skin in the field of wound repair. Zinc oxide (ZnO) possesses both antibacterial and anti-inflammatory properties and accelerates the wounds restoration. The wound recovery capability of insulin injection with chitosan/ZnO nanocomposite membrane in diabetic rats were evaluated. Diabetes was induced by Streptozotocin (STZ) administered intraperitoneally (i.p.) as one dose (50 mg/kg b. wt.). Forty-eight male rats were divided into six groups. Group I: control wounded, non-treated, non-diabetic rats, Group II: wounded diabetic non treated, Group III: Normal wound treated with chitosan/zinc oxide nanocomposite membrane, Group IV: Diabetic wounded treated with chitosan/zinc oxide nanocomposite membrane, Group V: wounded diabetic rats treated with local insulin injection, Group VI: wounded diabetic treated with chitosan/zinc oxide nanocomposite membrane and local insulin injection. A significant decrease in (PI3K), (MAPK) genes expression in addition to miRNA 125 and miRNA 132 in skin tissue of diabetic wounds compared with normal wound. However, increase in PI3K, MAPK, miRNA 125 and miRNA 132 genes were observed in diabetic wounds treated with chitosan/ZnO nanocomposite membrane or local insulin injection as compared to diabetic wounds non treated. The present data indicated that local insulin injection in wound area with chitosan/ZnO nanocomposite membrane might activate the PI3K/MAPK signaling pathway and some miRNA that promote and accelerates diabetic wound healing.

1. INTRODUCTION

Metabolic illness with numerous etiologies and an insufficiency in insulin secretion is known as diabetes mellitus (DM). It is characterized by a persistent rise in blood sugar levels involves interference in the metabolism of carbohydrates, fats, and proteins, which causes insulin to operate abnormally in the body. Diabetes over time causes problems like sluggish wound healing and the emergence of persistent, nonhealing foot ulcers that cause grave depression and death (Vijayakumar *et al*., 2019).

A diabetic wound has an expanded illness and amputation. The delay factors of wound recovery in diabetic sufferers encompass contamination, impaired glucose metabolism and chance of neurovascular complications (Tsourdi *et al.*, 2013).

Hemostasis phase, inflammatory phase, proliferation phase, and remodeling phase are the first four phases of wound healing. (Sun *et al*., 2014).

Chitosan has been decided as a promising biomaterial for carrying proteins and other energetic molecules via physical or chemical manner (Agnihotri *et al*., 2004).

Like every different property, ZnO NPs additionally have the recovery properties of numerous varieties of wounds inside the animal version. Much research has been performed to help its epithelialization-stimulating effect on damaged skin (Khorasani et al., 2019; Khan *et al*., 2021). Insulin is a peptide hormone with extra than one physiological role. It regulates blood glucose stages and is identified to have a truly beneficial feature in wound restoration. When you think about that insulin can probable assist fix the integrity of damaged skin, it is miles of interest interior the self-discipline of wound restores, specifically owing to its low fee relative to distinct increase factors, and as a consequence, is increased probably to be viewed for incorporation into wound dressings, bio adhesive membranes and hydrogels (Hrynyk and Neufeld 2014). On this observe, we intended to discover the wound healing capability of nearby insulin injection with chitosan/ZnO nanocomposite membrane on (PI3k), (MAPK) similarly to epigenetic miR-125 and miR-132 gene expression in diabetic rats.

2. MATERIAL AND METHODS

2.1. Animals:

Forty-eight male Wister albino rats of two months old and common physique weight between 180-200 g rats purchased from Animals Research Center in Cairo University. Animals were kept under good environmental and nutritional conditions, and clean drinking water supplied ad-libitum. Prior to the study's start, rats were given a 2-weeks

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acclimation period. Experiment was conducted according to the guide for 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:

Streptozotocin (STZ) was purchased from (Sigma Chemical Co. P.O. Box. 14508, St. Lowis, U.S.A.) was used to induce hyperglycemia by means of one intraperitoneal (i.p) at dose 50 mg /kg body wt. (Ramanathan *et al.,* 1999).

2.2.2. Insulin:

Long performing insulin bought from Lantus Solostar, Sanofi-Aventis (Germany) was 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 (St. Louis, U.S.A)

2.2.4. Zinc oxide nano-particles (nZnO): was acquired from Sigma-Aldrich Chemical Company.

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

2.2.6. Membrane preparation:

2.2.6.1. Preparation of Poly vinyl alcohol /chitosan and Poly vinyl alcohol / chitosan /zinc oxid composite membranes

Chitosan solution (2%) was prepared via dissolving 1g of chitosan in solution in 1% (v/v) acetic acid. Polyvinyl alcohol (PVA) solution became organized by means of adding 100 ml of distilled water to 5 gm of PVA powder to dissolve. 20 ml mixture solution of polymer changed into combined to put together the ratio of 1:3 and 1:4 of PVA:CS mixture homogenous polymer solution. Under stirring, 100 mg of ZnO nano-powder was dispersed in blend solution. The aggregate changed into sonicated for 30 min after magnetic stirring and the resulting solution was cast onto glass plates and the obvious membranes were received after solvent evaporating slowly in air at room temperature. Picture changed into marked and coded in accordance with composition as the subsequent PVA/CS, PVA/CS/ZnO 1:3:1 and PVA/CS/ZnO 1:4:1 (Omer *et al*., 2021).

2.2.6.2 Characterization of PVA/CS and PVA/CS/ZnO composite membranes:

2.2.6.2.1. Surface roughness:

Using a surface roughness teste (SJ-201P, Japan) the average roughness of membranes was assessed. Double-sided tape was used to attach the membranes to the glass slide. Minimum sample dimensions were 20 mm × 20 mm, The average of five measurements was used in all results. (Hassan *et al*., 2021).

2.2.6.2.2. Contact angle measurements:

Water contact angle determinations were done using advanced Gonimeter model 500-F1 at room temperature in the form of sessile drops (using Milli-Q water), connected to a camera and image processing software. For each film, at least five droplet photos were collected and examined (Eldin *et al*., 2008).

2.3 Induction of diabetes:

One intraperitoneal (i.p.) injection of the STZ caused experimental diabetes in male rats at dose of 50 mg /kg body wt., fresh dispersion in a buffer of citrate, pH 4.5. After STZ injection the glucose solution (5% w/v) was provided to the animals in a single day to keep away from hypoglycemia which is probably induced by means of STZ. Hyperglycemia turned into confirmed 48 h after STZ injection by monitoring the blood glucose by using ACCU-CHEK sensor glucometer. The rats displaying hyperglycemia were glucose level greater than 250 mg/dl were considerate diabetic (Ramanathan et al., 1999).

2.4. Experimental design:

The rats were randomly divided into six groups of eight rats each, placed in separated cages for each rats, and categorized as following:

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

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

Group III (Normal wound treated with membrane): wounded, non-diabetic rats treated with the topical application of chitosan/ ZnO nanocomposite membrane, The wounded area was covered with membrane and membrane was replaced with 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): included 8 diabetic wound rats treated with topical application of chitosan/ ZnO nanocomposite membrane and local insulin injection.

Excisional skin wound Induction:

Following a week of initiating diabetes, every rats' back had a full thickness excisional skin wound. Thiopental sodium (40 mg/kg/rat) intraperitoneally (Zhou *et al*., 2017) was used to make the rats unconscious and hair on the back was removed. a typical wound with a diameter of 6 mm for each rat, was created using a surgical marker pen. Next, using a scalpel blade to reach the muscle layer, full thickness excisional incisions were created. (João DeMasi *et al*., 2016). In addition, they couldn't reach the wound because it was high on their back, close to their neck. After the surgery each rat housed individually. They were also observed twice daily to sure that all the membrane utilized on the wounds (Colobatiu *et al*., 2019). It was continually monitored to make sure the rats weren't removing the membrane.

2.5 Skin tissue sampling: Pentobarbital were injected intraperitoneally and used to put the animals to sleep after 14 days of wound treatment. Skin specimens were obtained from the widest area of the wound tissue with surrounding normal skin margin. The samples of skin tissues were immediately placed in liquid nitrogen and maintained at -80 °C until the RNA extraction for determining the expression of the following genes: PI3K, MAPK, miRNA-125 and miRNA -132 (Table 1).

2.6. Molecular analysis:

Quantitative RT-PCR was used for determination of PI3K, MAPK, miRNA-125 and miRNA -132 (Table 1) 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. With each cDNA, sample was reverse

transcribed using Revert Aid TM First Strand CDNA synthesis kit (#EP0451, Thermo Scientific, Fermentas, USA). Then, real-time quantitative PCR amplification was performed on Fast start 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)
PI3K	AACACAGAAGACCAATACTC	TTCGCCATCTACCACTAC
MAPK	AGGGCGATGTGACGTTT	CTGGCAGGGTGAAGTTGG
B-actin	AAGTCCCTCACCCTCCCAAAAG	AAGCAATGCTGTCACCTTCCC
miRNA132	TAACAGTCTACAGCCATGGTCG	CCAGTCTCAGGGTCCGAGGTATTC
miRNA125b	TCCCTGAGACCCTAACTTGTGA	CCAGTGCAGGGTCCGAGGTATT
U6	TGACACGCAAATTCGTGAAGCGTTC	ACGTAGCTAGCCAGCTAGCTCA

2.6. Statistical analysis:

To determine the differences in variable means between the groups, we performed a one-way analysis of variance (ANOVA). Statistical Package for Social Science (SPSS, V20) was used to analyze the results, which were presented as the mean and standard error of the mean (SE). at $P< 0.05$ where this probability is considered as significant.

3. RESULTS

3.1. A description of the CS/PVA/ZnO membrane:

The surface roughness of wound dressing membranes has a great role in fixed attached cells on the surface. The roughness recorded 0.68µm for PVA/CS, 0.87 µm for PVA/CS/ZnO 1:3:1 and 1.34 µm for PVA/CS/ZnO 1:4:1. The obtained results demonstrate an increase in surface roughness of membranes that leads to the rise in membranes surface area.

Water contact angle with PVA/CS/ZnO composite membranes was measured with advanced Gonimeter model 500 F1 (Table 2). There are increases of the contact angle by introducing the ZnO nanoparticles in the membrane blends, the hydrophobicity of membranes was increased in the CS content in the composite membranes.

The color of the membrane is an important index regarding general appearance. Table (2) showed the square coordinates (L, a, and b) and the color difference (ΔE) of chitosan/PVA/ZnO composite membranes. introducing of PVA/chitosan membranes with ZnO demonstrated a decrease in membrane brightness (ΔL) with an increase of red color direction (Δa), yellow color direction (Δb), and decrease in total color difference (ΔE).

3.2. Molecular analysis results:

Effect of treatment with local insulin injection and Chitosan/ZnO nanocomposite membrane on PI3K, MAPK, miRNA-125 and miRNA -132 genes expression in diabetic wound rats are represented in tables (3) and figures (1-4).

Marked down-regulation in tissue PI3K and MAPK 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 in rats exhibited a significant up-regulation in PI3K and MAPK 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 PI3K and MAPK gene expressions were observed in CS/ZnO membrane treated normal wound rats (GIII) comparing with normal non treated group (GI).

A significant down-regulation in tissue miRNA 125 activity, miRNA 132 genes expression were exhibited 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 in rats exhibited a significant up-regulation in miRNA 125 activity, miRNA 132 gene expression as compared with diabetic wound non-treated group (GII) With highest down-regulation in (GVI) followed by (GV) and finally (GIV). Also, exhibited a significant up-regulation in tissue miRNA 125 activity, miRNA 132 gene expression were observed in CS/ZnO membrane treated normal wound rats (GIII) comparing with normal non treated group (GI).

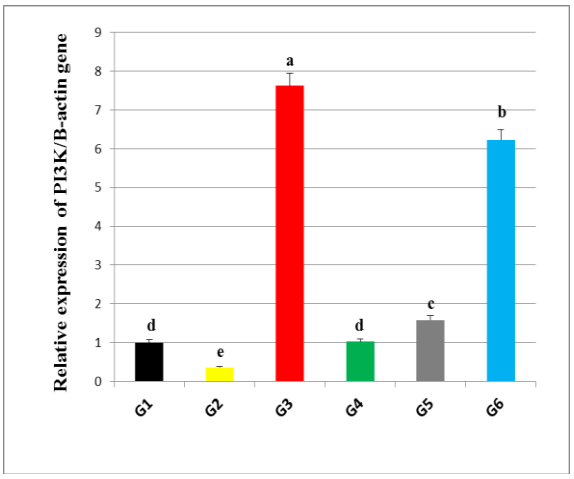

Fig. (1): Effect of insulin injection and CS/ZnO nanocomposite membrane on PI3K gene expression in experimental model of diabetic wound in rats

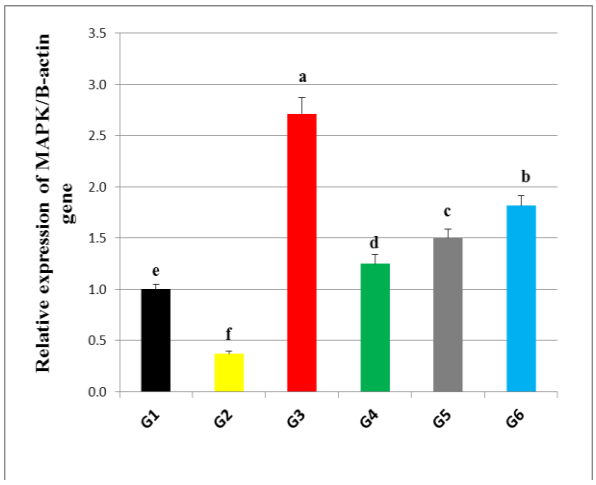

Fig.: (2): Effect of insulin injection and CS/ZnO nanocomposite membrane on MAPK gene expression in experimental model of diabetic wound in rats.

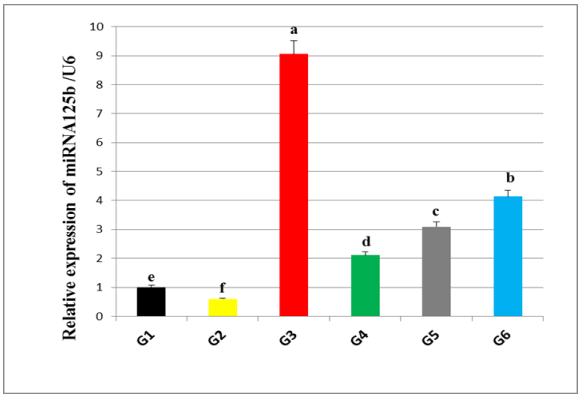

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Fig.(3): Effect of insulin injection and CS/ZnO nanocomposite membrane on miRNA 125b gene expression in experimental model of diabetic wound in rats.

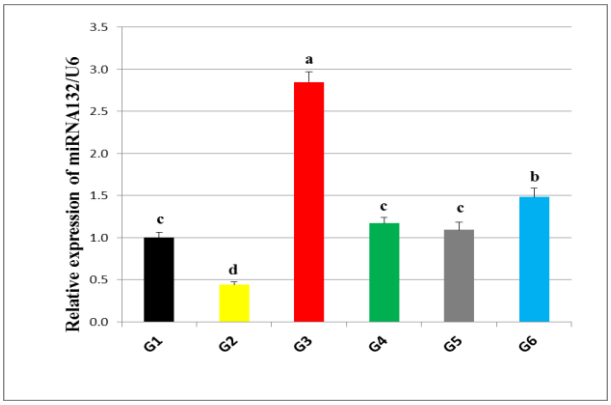

Fig. (4): Effect of insulin injection and CS/ZnO nanocomposite membrane on miRNA 132 gene expression in experimental model of diabetic wound in rats.

Table2: Physical parameters of PVA/CS/ZnO composite membranes.

	Contact angle			Roughness (µm)	Color parameters			
	θ_L	θ_R	θ_{mean}		L	Δa	Δb	ΔE
PVA/CS	72.27	69.12	70.695	0.68	86.86	-0.69	-8.07	38.45
PVA/CS/ZnO 1:3:1	72.07	71.72	71.895	0.87	85.83	-0.61	-8.29	37.5
PVA/CS/ZnO 1:4:1	79.38	78.06	78.72	1.34	81.33	2.82	11.48	34.18

Table3 Effect of treatment with local insulin injection and chitosan/Zno nanocomposite membrane on PI3K, MAPK, miRNA125 and miRNA 132 gene expression in diabetic wound rats.

Animal groups	PI3K	MAPK	miRNA 125	miRNA 132
	Fold change ± SE	Fold change ± SE	Fold change ± SE	Fold change ± SE
Control wounded, non-treated, non-diabetic rats (GI)	1.00 ± 0.08 ^{d}	1.00 ± 0.05 ^{e}	1.00 ±0.08 ^{e}	1.00 ± 0.06 ^{c}
Diabetic wounded, non-treated rats (GII)	0.36 ± 0.03 ^{e}	0.37 ± 0.03 ^{f}	0.60 ± 0.03 ^{f}	0.44 ± 0.03 ^{d}
Control wounded, non-diabetic rats treated with membrane (GIII)	7.62 ± 0.33 ^{a}	2.71 ± 0.16 ^{a}	9.06 ± 0.45 ^{a}	2.85 ± 0.12 ^{a}
Wounded, diabetic rats with topical application of membrane (GIV)	1.02 ± 0.08 ^{d}	1.25 ± 0.09 ^{d}	2.11 ± 0.11 ^{d}	1.17 ± 0.07 ^{c}
Wounded, diabetic rats treated with local insulin injected (GV)	1.58 ± 0.11 ^{c}	1.51 ± 0.08 ^{c}	3.10 ±0.16 ^{c}	1.09 ± 0.09 ^{c}
Wounded, diabetic rats treated membrane and local insulin injection (GVI)	6.23 ± 0.26 ^{b}	1.82 ± 0.1 ^{b}	4.14 ± 0.21 ^{b}	1.48 ± 0.1 ^{b}

4. DISCUSSION

Diabetes mellitus inhibits the healing of wounds and is triggered by hyperglycemia, chronic inflammation, micro- and macro-circulatory dysfunction, hypoxia, autonomic and sensory neuropathy, and faulty neuropeptide signaling (Baltzis et al., 2014).

The development of biopolymer and modified biopolymeric materials with critical biocompatibility and enhanced characteristics has sped up the wound healing process (Casimiro and Gil, 2010).

The results showed in table (3) showed a considerable rise in wound tissue PI3K and MAPK gene expressions in CS/ZnO membrane treated rats comparing with normal control group. These results are nearly similar to those reported by Roy et al. (2014a), who recorded that, primary macrophages were exposed to ZnO nanoparticles, there was a substantial improvement in the expression of Ras, PI3K, intensified phosphorylation, followed by activation of its downstream signaling cascades via ERK1/2, p38, and JNK MAPKs as well as overexpression of c-Jun, c-Fos, and NF-B. Today, it has been established that PI3K/Akt and MAPK play a role in ZnO NP toxicity through use of a number of cellular pressure mechanisms or pro-inflammatory cytokines in autophagy, apoptosis, and cellular survival. (Roy et al., 2014b). In those investigations, treatment with ZnO NP stimulated macrophages' PI3K/Akt and MAPK dual signaling pathways. Similar to this, we discovered that ZnO NPs caused a notable rise of PI3K/MAPK. Also, Zhang et al. (2019) found that, The PI3K/AKT and MAPK pathways are also promoted by chitosan/O-HTCC nanoparticles, which likewise noticeably boost IL-2 transcription and cause lymphocyte proliferation.

Insulin uptake and the improvement of DM are two cellular responses that are diminished by decreased PI3K/AKT signaling (Li and Wang 2014). A number of pathways, including EGFR/PI3K/AKT and ERKs, downstream Bcl2 associated death promoter (BAD) signaling, and epidermal growth factor receptor/PI3K/AKT and extracellular signal regulated kinases (EGFR/PI3K/AKT and ERKs) are activated in response to DM. This causes cellular death to last longer and cellular proliferation to decline, which ultimately delays wound recuperation (Xu and Yu, 2011).

Treatment with insulin injection to diabetic wound in rats exhibited a greatly increase in PI3K and MAPK gene expressions as compared with diabetic wound non treated group. This result came in accordance with Lima et al. (2012), who attributed, in order to promote wound healing, insulin activates the IR/SHC/ERK and IR/IRS/PI3K/AKT pathways for insulin signaling. Furthermore, additional mechanistic studies have demonstrated that nanomaterials should support the complement system in wounds by modulating the TLR/NFB, MAPK/mTOR, and KGF2/p38 signaling pathways, decreasing the expression of pro-inflammatory cytokines like TNF-, IL-1, and IL-6, and increasing the expression of anti-inflammatory cytokines like IL-4 and IL-10. (Sun et al., 2019).

Treatment with insulin and CS/ZnO membrane with insulin injection to diabetic wound in rats exhibited a significant increase in PI3K and MAPK gene expressions as compared with diabetic wound non treated group.

The obtained outcomes demonstrated in table (3) revealed that, remarkably increase in miRNA 125 and miRNA 132

activities were observed in CS/ZnO membrane treated rats comparing with normal control group. The current study agreed with those of Jia *et al.* (2018), reported that, the function of miR-125b in many cell types of various human illnesses has been identified. More specifically, it was discovered that miR-125b controls the expression of MMP-2 and targets the PI3K/Akt/mTOR pathway to suppress keratinocyte proliferation and promote their apoptosis (Wang *et al*., 2016).

Additionally, according to Li et al. (2015), who found that, MiR-132, which is expressed in epidermal keratinocytes, regulates the inflammatory segment in white blood cells (WH) by working on the Nuclear Factor-kappaB (NF-kB) pathway and the associated activation of neutrophils. The activation of chemokines and cytokines, which would stimulate endothelial cells and entice leukocytes and peripheral cells to the injury site, is associated with the functional expression of miR-132. MiRNA 132 was found to have markedly decreased in (Table 3). Similarly, Li *et al*. (2017), who reported that, the role of miR-132 in DM patients and db/db mice compared to healthy controls. A gene ontology analysis of miR-132 revealed that it is associated with inflammatory pathways such as NF-B, NOD-like receptor, TLR, and TNF signaling pathways. Previously, miR-132 expression was lowered in diabetes.

In the present study, the down regulation of miRNA 125 and miRNA 132 in diabetic rats was significantly upregulated on treatment with insulin injection only and CS/ZnO membrane with insulin injection to diabetic wound.

5. CONCLUSION

The present study revealed that the combination of topical chitosan/ZnO nanocomposite membrane and local insulin injection have a greater effect in speeding up tissue repair and wound healing, accelerates angiogenesis by activating the PI3K/MAPK signaling pathway and during the initial stages of the wound healing process, certain miRNA are produced to aid in the healing of diabetic wounds caused by (STZ) with a rat model. These results lay the groundwork for future study into the treatment of diabetic wounds and support the scientific use of local insulin with chitosan/ZnO nanocomposite membrane.

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