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Enhancing Diabetic Wound Healing by 3D Organo-hydrogel Through Reducing Apoptosis and Increasing Growth Factors Expression

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Abstract
Chronic wounds place a heavy strain on patients and society.Diabetic woundsare a developing health concern that contribute significantly to the disability and mortality of diabetic patients. The study displayed the possible mechanisms by which 3D organo-hydrogel nanocomposite(PDA-TiO_2@Ag) accelerate wound healing in diabetic rats. Streptozotocin (STZ) was used to induce diabetes through a singleI.P. injection. A fullthickness wound measuring 10mm was formed one week after induction of diabetes. Thirty adult male albino rats divided into: Normal control rats (not diabetic withnon-treated skin wounds),Diabetic control rats(diabetic with non-treated skin wounds), organo-hydrogel treated rats (diabeticwoundswere treated withorgano-hydrogelplacedtopically on the woundonce daily). Seven days after inducing the wound, samples from the wound treated by organo-hydrogelexhibitedmarked increase in the wound closure percentage as compared to normal and diabetic control groups. The finding was supported by histological analysis.Also, the gene expression of Bcl-2 Associated X-protein (BAX) and Insulin like growth factor (IGF) were assessed by qRT -PCR. The organo-hydrogel increased IGF levels and downregulated BAX levels. Hence, the organo-hydrogel can acceleratediabetic wound healing through reducing apoptosis and increasing the growth factors like IGF.

Keywords:
Diabetic; 3D organo-hydrogel; BAX; IGF; wound healing.
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1. Introduction

Chronic wounds place a heavy strain on patients and society. They are typically divided into three primary categories: diabetic ulcers, vascular ulcers and pressure ulcers, depending on the etiologies that caused them. One of the most common chronic wounds, diabetic wounds.Diabetic wounds are a developing health concern that contribute significantly to the disability and mortality of diabetic patients[1].

Wound healing is a complex physiological process which occur in order to restore skin integrity after injury. Numerous cell types with different functions are involved in the stages of wound healing which include, hemostasis, inflammation, proliferation, re-epithelialization and remodeling. These cells must be spatially and temporally synchronized during this process[2].

Due to neuropathy and vascular dysfunction, the wound healing process is slowed down in diabetes patients. Diabetes-related wounds are characterized by a persistent inflammatory response and an angiogenic imbalance, which inhibit tissue remodeling and proliferation[3]. These diseased conditions require immediate clinical care. Although there are a variety of treatments for diabetic wounds, the most effective ones are currently being discovered[4].

powder was disolved in freshly prepared cold citrate buffer with pH 4.5 and then injected immediately [10].

The rats developed weight loss and hyperglycemia, which are typical as diabetic humans[11,12]. The fasting blood glucose levels (FBG) were tested using a glucometer (Accu-Chek Active, Roche Diagnostics, Germany) three days after STZ injection to make sure that the diabetes was developed. The rats with a blood glucose level more than 250 mg/dl were considered diabetic. As a continual monitoring, the FBG was measured again on day 7 with the timing of scarification[13]

2.4. Creationof full thickness wound

The wounds were created after ensuring that diabetes had been established. It was made one week after the diabetes was induced. The rats were given ketamine hydrochloride I.P. injections (90 mg/kg/rat) before shaving their the back hair[14]. Afterwards, full thickness circular wounds measuring about 10mm were made in the rats upper back with a scalpel blade [12,15].The rats recievedanalgesic (Meloxicam 1-2 mg/kg S.C) for three days after forming the wound.

2.5. Synthesis of organo-hydrogel

It was synthesized in Institute of Nanoscience & Nanotechnology, Kafrelsheikh University[16].

2.6. Groups and Treatment

The animals were divided into two groups (n=10 rats/ group).

- Normal control group: non diabetic rats with non-treated skin wounds.
- Diabetic control group: diabetic rats with non-treated skin wounds.
- Organo-hydrogel group:diabetic rats treated with (PDA–TiO2@Ag) organo-hydrogel which was placed topically on the wound once daily for one week [12,17].

2.7. Assessment of wounds closure

The wounds were photographed with digital camera to measure diameters of wounds [18]. The percentage (%) of wounds closure was evaluated by the Wilson's formula mentioned as % of wounds closure = [(Area on 0 day − Area of X days)/Area on 0 day] × 100% [14].

2.8. Histological Studies

Specimens from the wound tissue were taken with about 1 cm normal skin margin. Thespecimens were placed immediately in 10% buffered formalin for 1 day.Paraffin blocks were processed and sections of 5µm thick were subjected to Hematoxylin and Eosin (H&E) stain for recognition of histological changes and Masson's trichrome to detect collagen[19].

2. 9. Quantitative Realtime Polymerase Chain Reaction (qRT -PCR).

Quantitative determination of the levels of Bcl-2 Associated X-protein(BAX) and insulin like growth factor (IGF) were performed using quantitative qRT -PCR technique. Samples of the wounded tissue were taken as soon as the sacrifice was made (on day 7) and were

Growth factors are natural signalling molecules that control cellular reactions during the healing process of wounds. These proteins, which are secreted by platelets, leukocytes, fibroblasts, and epithelial cells, are increased in response to tissue injury. Growth factors bind to membrane or cytoplasmic receptors after they are secreted and then work by autocrine, paracrine, or endocrine pathways[5].

Nanoparticles and nanoscaffolds are modern promising treatment plans for hastening diabetic wound healing. Their diameter ranges from 1–100. Nanoparticles have small size and high surface area to volume ratio. This increases the possibility of biological contact and infiltration at wound site[6].

Hydrogels are materials composed of polymeric network. They areformed by simple reactions of one or more monomers. hydrogels have the ability to absorblarge amounts of water in their structure due to the prescence of hydrophilic groups so they can be effectively used in wound healing[7].

Metal nanoparticles, particularly silver nanoparticles (AgNPs), have been described as a broadspectrum antimicrobial agent improving the antibacterial effect during the wound healing treatment[8]. In addition, Polydopamine (PDA) has been linked to wound healing because of its unique structure and abundance of phenol groups. PDA can be used with a number of substances to create wound dressings that promote wound healing. Furthermore, PDA complex has remarkable mechanical and self-healing characteristics[9].

In this study, we studied the mechanism by which the 3D organo-hydrogel accelerated wound healing in diabetic rats.

2.Methodology

2.1. Drugs

The suppliers of the drugs were as follows. Citrate buffer (pH 4.5) and Streptozotocin (STZ): (Sigma Chemicals Co., St. Louis, MO, USA).Meloxicam:2ml ampule (Amriyapharm.ind.). Pentobarbital: (Al - Gomhoria Company for medicines and medical supplies). It is a short-acting barbiturate, used as a preanesthetic. In high doses it causes death by respiratory arrest, it is used for veterinary euthanasia. Ketamine hydrochloride: 50 mg/ml. Rotexmedica. Trittau. Germany.PDA–TiO_2@Ag organo-hydrogel was obtained from institute of Nanoscience & Nanotechnology.

2.2. Animals

Thirty adult male albino rats with average weight (180-200 grams) were used in this study. They were kept in animal houseof Faculty of Pharmacy; Kafrelsheikh University. All the experimental procedures were done according to guidelines approved by the Animal Use Committee of Kafrelsheikh University.

2.3. Induction of diabetes mellitus

Before receivingSTZ injection,the rats were fasted overnight. STZ was given intraperitoneal(I.P.) injection at a dose of 60 mg/kg body weight. STZ

then used to determine the quantity and quality of the extracted RNA.

2. 9.2Reverse transcription of extracted RNA followed by PCR in one step

Reverse transcription of extracted RNA was performed using the Super Script IV One-Step RT-PCR kit (Cat# 12594100, Thermo Fisher Scientific, Waltham, MA, USA) and followed by PCR in a single step.

kept in Qiazol Lysis Reagent (Cat. #: 79306, Qiagen, Cairo, Egypt) and stored at -80 ◦C until the time of analysis[20].

2. 9.1RNA extraction

Following the manufacturer's instructions, total RNA was extracted from all enrolled samples using Direct-zol RNA Miniprep Plus (Cat. # R2072, ZYMO RESEARCH CORP. USA). The Beckman dual spectrophotometer USA was

Table 1. Primers sequence of all studied genes:

	ard sequence	se sequence
	CAGGCCCATACTGAAT	GACAGGGCAGATAGCC
	TGGTTGATGAATGGT	GGGCAGGGCTAAT
H	CTGTTGCTGTAGCCATATTC	TCAAGAAGGTGGTGAAGCAG

group and (462.5±30.39 mg/dl)in the organo-hydrogel group. There was significant difference in serum glucose levels between normal control group, diabetic control and organo-hydrogel groups.The animals had weight loss and similar physiological symptoms of diabetes that are seen in people with disease; the mortality rate was 12.5%. The animals' weight loss is linked to severe dehydration and inadequate insulin after STZ injection, which inhibits the body's cells' ability to absorb blood glucose. The animals death occurs due to toxicity of STZ on numerous body organs and not from complications of diabetic mellitus (DM)[11,21].

3.2. Wound closure assessment

After seven days,on examination of the wound in the different groups, the diabetic non - treated skin wounds showed a marked delay of wound healing with significant decrease in the wound closure % as compared to other groups. In this regard, the study performed by[22]reported that the diabetic untreated skin wounds showed wide wound gaps with inadequate wound closure. The diabetic skin wounds treated with organo-hydrogel revealeda substantialincrease in the wound closure % as compared to normal and diabetic control groups.This finding was on line with[23] who observed significant increase in the wound closure % that reached 80 % with the use of polydopamine on day 7(Fig. 1). The hydrogel has self-healing and self-adhesive characteristics due to the presence of PDA [9].

2.9.3. Calculation of Relative Quantification (RQ) (relative expression):

After the RT-PCR run, the data were expressed in Cycle threshold (Ct). The PCR data sheet includes Ct values of assessed gene (***BAX and IGF)***versus the corresponding the house keeping gene (*GAPDH*) (Table 1). In order to measure the gene expression of certain gene, a control sample should be used. The RQ of each target gene is quantified and normalized to housekeeping gene according to the calculation of delta-delta Ct (ΔΔCt). We calculated the RQ of each gene by taking $2^{-\Delta\Delta Ct}$.

2.10. Statistical Analysis

Allresults wereexpressed as mean ± standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA). A P-value less than 0.05 was considered to be statistically significant.

3. Results and discussion

3.1.Laboratory results

STZ-injected rats showed hyperglycemia and maintained it during the study. Five days after STZ injection, the mean serum glucose level was (98.63±1.09mg/dl)in the normal control group,(415.8 ±17.13 mg/dl) in the diabetic control group and (413.2 ±18.20 mg/dl) in the organo-hydrogel group. On day seven, the mean serum glucose levelswere(99.02±0.76mg/dl)in the normal control group,(455.3 ±31.41 mg/dl) in the diabetic control

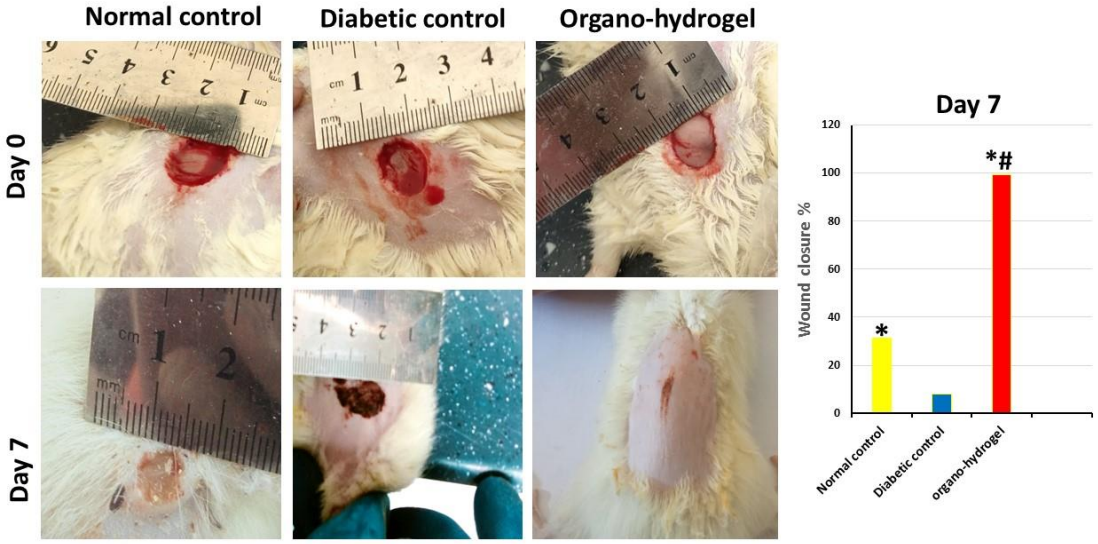

Fig. 1. Showing wound areas in normal control, diabetic control and organo-hydrogel groups from days 0 and 7 post wound induction. Histogram of wound closure % in the different groups from day 7 after wound induction, *: significant compared to diabetic control group, #: significant compared to normal control group (p value ≤ 0.05).

migration and proliferation of keratinocytes, which results in inadequate re-epithelialization and a longer healing period[24]. On examination of the normal control group, it showed numerous inflammatory cells with granulation tissue and collagen fibers. The organo-hydrogel group revealed complete epithelial layers with healthy granulation tissue containing numerous fibroblasts. Similarly, closure of the wound with regeneration of collagen fibers and hair follicles was observed [25]. Also, acceleration of wound healing by polydopamine-assisted Au-hydroxyapatite nanorods and quaternized chitosan / PDA was observed[26,27].

3.3. Hematoxylin and Eosin (H&E) results

H&E stained sections from diabetic non treated wounds showed disorganized granulation tissue with numerous inflammatory cells appeared(Fig. 2). In addition to releasing oxygen free radicals and proteases, the numerous neutrophils that invade the wound also release inflammatory mediators. High concentrations of reactive oxygen species (ROS) result from this. These ROS can cause DNA cleavage, lipid peroxidation and the inactivation of enzymes that scavenge free radicals and also activation of inflammatory mediators that impede tissue remodeling[10].Hyperglycemia hinders the

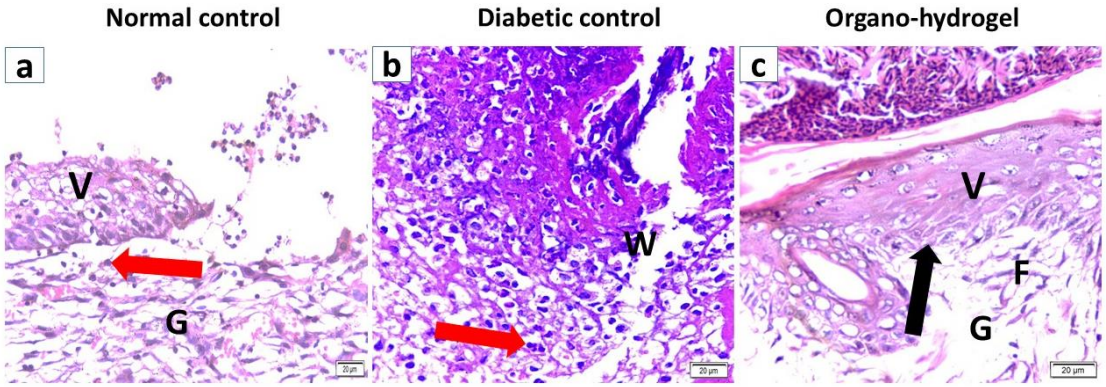

Fig. 2. H&E stained skin sections from normal control, diabetic control and organo-hydrogel treated groups on day 7. (a) Normal control rat showing granulation tissue (G) filling the wound gap with numerous inflammatory cells (red arrow) and cytoplasmic vacuolations in the epithelium in the edge of the wound (V), (X:400, scale bar 20 µm). (b) Diabetic control rat showing diffuse infiltration with inflammatory cells (red arrow) in the wound gap (W), (X:400, scale bar 20 µm). (c) organo-hydrogel treated rat showingcomplete re-epithelialization (black arrow). The underlying dermisshowinggranulation tissue(G) with fibroblasts (F), (X:400, scale bar 20 µm).

Additionally, the Masson's trichrome stained skin sections of organo-hydrogel treated group showed deposition of more organized collagen fibers. This was confirmed by significant increase in mean area % of collagen deposition compared to diabetic control and normal control groups (Fig. 3). Masson staining of PDA complex treated wound displayed regularly arranged and abundant collagen fibers similar to natural skin tissue. It can clearly improve the deposition and arrangement of collagen [28].

3.4. Masson's trichrome results

Masson's trichrome stained skin sections from diabetic non treated rats showed that the wound beds after 7 days was filled with fine, irregularly distributed collagen fibers with significant decrease in the mean area % of collagen deposition as compared with normal control and organo-hydrogel groups (Fig. 3). Consistent with this finding[10,12]noticed fine immature collagen with randomly scattered manner.In this study, the normal controlrevealed significant increase as compared to diabetic control group and significant decrease as compared to organo-hydrogel group.

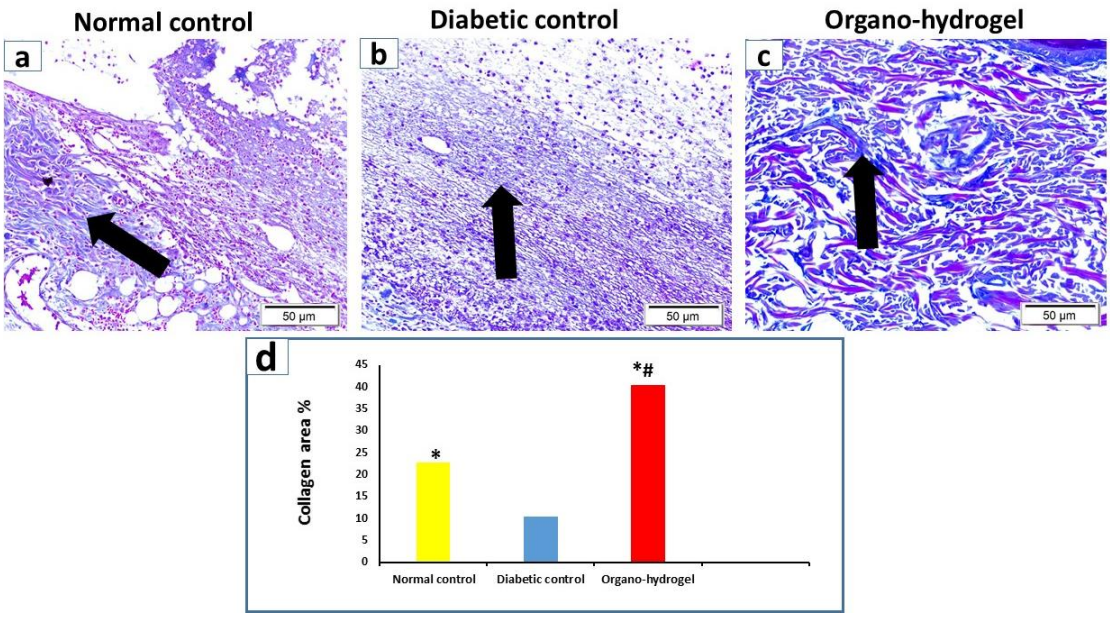

Fig. 3.(I) Masson trichrome stained skin Images (a, b, c) from normal control, diabetic control and organo-hydrogel groups on day 7. (a) Normal control rat showing few irregular collagen fibers (arrow), (b) Diabetic controlrat showingthe wounds bedis filled with thin, faint and irregularly distributed collagen fibers(arrow). (c)Diabetic rats treated with organo-hydrogel showing the wound beds filled with dense collagen bundles (arrow), (X:200, scale bar 50 µm). (d) statistical analysis of collagen area % of the normal control, diabetic control and organo-hydrogel groups, *: Significant compared to diabetic group, #: Significant compared to normal control group(p value ≤ 0.05).

compound made up of the peptides IGF-1 and IGF-2. IGF-1 promotes endothelial cell chemotaxis, keratinocyte and fibroblast proliferation and cell granulation and re-epithelization, all of which are involved in wound healing. IGFincreases the strength of the wound[29]. On the other hand, IGF-1 expression is downregulated in diabetics, which could account for defective cell granulation. The acidity of the wound environment affects its affinities[30]. PDA complex increases the levels of growth factors [9].

3.5. RT-PCR results

The gene expression of IGFwas assessed in the different groups on day 7 using RT-PCR as shown inTable 2, Fig. 4. The normal control group revealed significant increase in gene expression of IGF as compared to diabetic control group. In contrast, the diabetic control groupprevealed marked decrease in gene expression of IGF when compared to organo-hydrogel and normal control groups. Moreover, the organo-hydrogel group showed marked increase inIGF levels as compared to other groups.Insulin-like growth factor (IGF) is a

Table 2. The mean values of BAX and IGF mRNAexpression levels ±SD, on day 7 post wound induction

Groups		Normal con group	Diabetic control group	Organo-hydrogel group
Day 7	BAX	1.98± 0.08	3.94 ± 0.24	0.96 ± 0.07
	IGF	1.01 ± 0.04	0.16 ± 0.05	2 ± 0.12

permeabilization of mitochondrial outer membrane in response to different cellular stresses[31].Diabetic wounds are characterized by excessive apoptosis due to oxidative stress leading to increased susceptibility to infection. This leads to prolonged inflammatory process and delayed wound healing[32].PDA complex can effectively promote cell proliferation and reduce the apoptosis rate[33]. Silver nanoparticles (AgNPs) encourage the proliferation and inhibit apoptosis and relocation of keratinocytes[34].

In the current study. The expression of BAX in the different groups is also evaluated using RT-PCRon day 7(Table 2, Fig. 4). The normal control group revealed significant decrease in gene expression of BAXas compared to diabetic control group. In contrast, the diabetic control grouprevealed markedincrease in gene expression of BAX when compared to organo-hydrogel and normal control groups. Moreover, the organo-hydrogel group showed marked decrease inBAXlevels as compared to other groups.Bax is a member of Bcl-2 family. Baxencourages cell death through

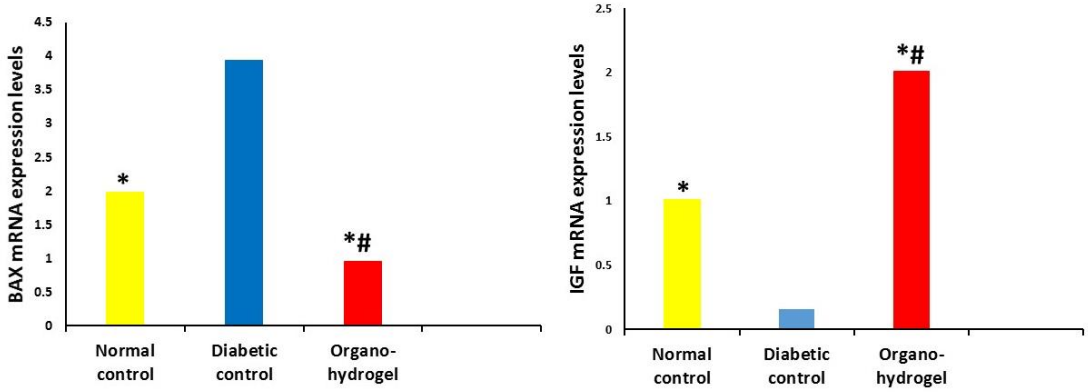

Fig. 4. Showing RT-PCR analysis of BAX and IGF sequentially in the normal control, diabetic control and organo-hydrogel groups at 7 day after wound induction, *: significant to diabetic control group, #: significant to normal control group (*P* value < 0.05).

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4. Conclusion

The present study provided experimental evidence that 3D organo-hydrogel nanocomposite effectivelytreated the diabetic wound through reducing apoptosis and increasing gene expression of insulin like growth factor. Further studies needed to use 3D organo-hydrogel nanocomposite as a new approach targeting other diseases in humans.

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