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Diabetex: A novel approach for diabetic wound healing

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ABSTRACT

Introduction and aim: Wound healing is an orderly complex process involving inflammation, clotting, re-epithelialization, neovascularization and wound closure. In diabetic patients, such process is impaired and delayed, posing negative economic as well as social consequences. Diabetex, (patency# EP 0877617 A1) composed of L-alanine, D-ribose, nicotinic acid and calcium ascorbate, which was initially introduced to treat cancer is thought to have anti- diabetic effects. The present study was designed to investigate the therapeutic merit of diabetex as well as the cellular mechanisms involved in such effects and its safety profile compared to metformin in wounded diabetic rats.

Main methods: Sixty adult male Sprague-Dawley albino rats were randomly divided into two major groups after induction of full thickness wound; control and treated groups. Liver and kidney function test, as well as cytokines (VEGF, TGF-β, PDGF and MMP2), fasting blood sugar were measured in animal sera. Histopathological studies including hematoxyline and eosin, Masson's trichrome stains were performed on wounded tissue.

Key findings: Diabetex significantly improved wound healing, collagen formation, induced re-epithelialization and neovascularization. Moreover, cytokines involved in wound healing process were increased by the anti-diabetic medication. Noteworthy, the drug exhibited a safe profile on liver and kidney function tests and significantly reduced fasting blood sugar.

Significance: The present study offers a novel approach for treating diabetic resistant wounds with a possible more economic, safe strategy.

1. Introduction

Wound healing is a dynamic interdisciplinary process of several molecular and cellular cascades of events. These events comprise clotting, fibrin-fibrinonectin deposition, inflammation, neovascularization and wound contraction [1]. Notably a plethora of mediators orchestrate this event including; platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF) [2] and matrix metalloproteinase-2(MMP2) [3]. However, this process is impaired in chronic illness such as diabetes mellitus (DM), thus exposing the open wound to infection and trauma, which further delays the healing process [4]. Neuropathy, peripheral vascular disease, minor trauma and socioeconomic status of the patient contribute to the formation and impaired healing of foot ulcers in diabetics [5]. In a vicious cycle, impaired wound healing poses negative socioeconomic effects [6]; prolonged hospitalization and increased time away from work. Accordingly, identifying and avoiding risk factors that are known to cause wounds in diabetic patients may reduce amputation risk. In addition to controlling the glycemic index, current treatment of diabetic foot ulcers includes periodic debridement, infection treatment, pressure-offloading and good wound care. Albeit, this *one size fits all regimen* has failed in some patients owing to impaired microvascular function, diminished activity of growth factors, neuropeptides, and the hypoxic tissue [7].

Diabetex is a patency (EP 0877617 A1) initially introduced to treat cancer and is thought to have anti-diabetic effects. It is composed of L-alanine, D-ribose, nicotinic acid and calcium ascorbate. Each component of the mixture exerts a beneficial effect on accelerating wound healing and further studies are needed to elucidate its wound healing potential. To our knowledge, this is the first study conducted to explore the wound healing effect of diabetex and its dynamics on diabetic rats via collagen metabolism, angiogenesis and the activity of cytokines following identification of its safety profile. Furthermore, the choice of the model is pertinent to human biology in the context that wound healing mimics diabetic foot ulcer. Noteworthy, the choice of Sprague Dawely rats stems from a previous report that utilized the same strain in

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induction of diabetes which lends credence to the susceptibility of the former to diabetic complications [8].

2. Material and methods

2.1. Ethics statement

The present study conforms to the standard ethical procedures and policies approved by the Ethics committee for animal experimentation at the National center for radiation research and technology (NCRRT) # 10A/17 in accordance with the Guide for the care and use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996) [9]. Humane treatment has been conducted to research animals throughout the study.

2.2. Animals care

Sixty adult male Sprague-Dawley albino rats, weighing 200–250 g, 4–5 months old were obtained from Nile Co., for Pharmaceuticals and Chemical Industries, Cairo, Egypt. Rats were at controlled conditions; temperature at 25 ± 1 °C. Light – dark regime: 12 h dark/12 h light, Ventilation: Automated suction/ventilation system Humidity 55% humidity. The rats in the control groups were fed a regular chow diet consisting of a total kcal value of 20 kJ/kg (5% fat, 52% carbohydrate, 20% protein), whereas the rats in the diabetic group were placed on a high-fat diet with a total kcal value of 40 kJ/kg (20% fat, 45% carbohydrate, 22% protein) [10]. Rats were housed in stainless-steel cages (50 cm * 30 cm * 18 cm), five per cage with wood bedding material and left for initial adaptation period of one week before any experiment. Humane end point was carried out throughout the study where; if the blood sugar exceeded 700 mg/dl, in addition to polyuria, hematuria and severe weight loss; animals are discarded from the study and euthanized by thiopental injection.

2.3. Experimental design

Animals were divided into two main groups (GpI Controls and GpII Diabetic), each subdivided as follows (10 rats per group):

Control groups (Gp I)

Negative control (GpIa): received saline for two weeks.

Diabetex (Gp Ib): received diabetex mixture orally twice daily at 500 mg/kg for two weeks.

Metformin (GpIc): received metformin orally once daily at 100 mg/kg for two weeks.

Diabetic and treated groups (Group II)

Diabetic (GpIIa): served as diabetic model and received (30 mg/kg) STZ once orally.

Diabetic Diabetex (GpIIb): received (streptozotocin) STZ and diabetex in the same dose regimen as mentioned above.

Diabetic Metformin (GpIIc): received STZ and metformin in the same dose regimen as mentioned above. All regimens were given between 9:00 am and 12:00 pm.

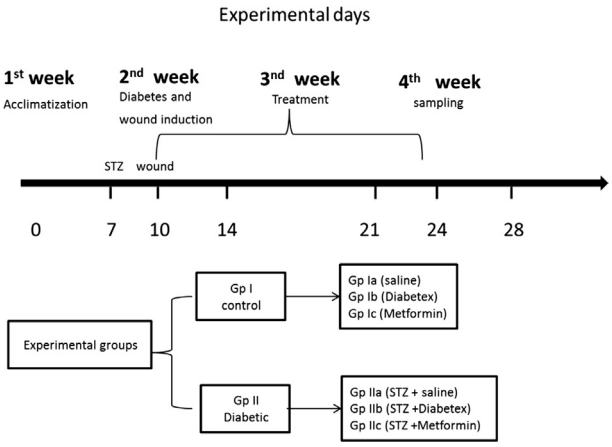

Fig. (a). A timeline representation of the experimental design showing experimental groups and treatments received.

2.4. Diabetes induction

Induction of diabetes mellitus was performed according to Qian et al. [10]; rats were housed in their cages for one week before treatment. Animals were starved for 16 h then injected once intraperitoneally (IP) with STZ (30 mg/kg), (Sigma-Aldrich, Saint Louis, USA) freshly dissolved in sodium citrate buffer. Following STZ injection, rats were given drinking water supplemented with sucrose (15 g/l) for 48 h to limit early mortality. Diabetes in rats was confirmed by measuring the blood glucose 72 h after IP injection of STZ, rats with blood glucose above 280 mg/dl were only used for the experiment. Measurement of blood glucose was carried out by the use of glucose check strips (Bionime glucometer, Validus technology, Berneck, Switzerland).

2.5. Drug preparation

Diabetex: L-alanine, D-ribose, nicotinic acid and calcium ascorbate (Sigma-Aldrich, Saint Louis, USA) were mixed together in a ratio according to the patency number (EP 0877617 A1) and suspended in saline. The mixture was administered orally in a dose of 500 mg/kg twice daily for two weeks.

Metformin preparation: Metformin (CID, Cairo, Egypt) at dose 100 mg/kg was suspended in saline [11].

2.6. Wound induction protocol and treatment

Directly after diabetes induction, animals were anesthetized using thiopental (Sandoz, Switzerland). Hair on the dorsal side was shaved and the skin was cleaned with 70% ethanol. An 8-mm skin biopsy punch (Kai medical- Gifu, Japan) was used to create full thickness cutaneous wounds under aseptic conditions. The exposed skin was covered by Tegaderm (3MTM TegadermTM Non-Adherent Contact Layer 5642, Salt lake, USA) that was maintained for > 24 h in order to preserve tissue fluid. The animals were housed individually to avoid damage to the wounds. The wounds were photographed at day 14 prior to killing with the rats in a standard prone position by using a 4 mega pixels digital camera (Samsung, Suwon, Korea). Notably, thiopental; an ultra-short acting barbiturate [12], provides a good choice for either wound induction or euthanasia to circumvent anesthetic effects on experimental outcome.

Table 1
Effect of diabetex and metformin on liver function tests ALT & AST (IU/L), albumin (g/dl), kidney function tests urea & creatinine (mg/dl) and FBS (mg/dl).

	Control (Gp Ia)	Diabetex (Gp Ib)	Metformin (Gp Ic)	Diabetic (Gp IIa)	Diabetic + diabetex (Gp IIb)	Diabetic + metformin (Gp IIc)
GPT (ALT)	41.00 ± 3.578	35.00 ± 3.937 a	44.00 ± 2.28 **b**	59.50 ± 5.010**a**	38.50 ± 4.626**c**	52.25 ± 3.818**acd**
GOT(AST)	106.8 ± 8.727	101.3 ± 10.31	105.3 ± 2.066	188.8 ± 6.795**a**	144.5 ± 6.18 **ac**	174.7 ± 14.19**ad**
Albumin	3.088 ± 0.1563	3.363 ± 0.1857 **a**	2.900 ± 0.1414 **b**	3.152 ± 0.1887	3.033 ± 0.1966	2.992 ± 0.2245
Urea	44.00 ± 1.703	38.75 ± 3.266**a**	50.77 ± 2.627 **ab**	46.93 ± 1.722	43.85 ± 3.140	58.33 ± 3.225 **acd**
Creatinine	0.5733 ± 0.01633	0.3017 ± 0.03312 **a**	0.653 ± 0.06314 **ab**	2.068 ± 0.1769 **a**	0.7267 ± 0.1786 **c**	0.8383 ± 0.1132 **ac**
FBS	98.33 ± 9.180	88.17 ± 4.708 **a**	94.00 ± 5.020	732.2 ± 49.49 **a**	383.0 ± 33.58 **ac**	380.8 ± 33.62 **ac**

Data are expressed as mean of 6 rats ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey-Kramer Multiple Comparisons test; as compared to negative control (a), diabetex (b), diabetic (c) and diabetic + diabetex (d) groups, $P < 0.05$.

3. In-vivo experiments

3.1. Blood sampling and serum preparation

Directly, after animals were anesthetized using thiopental, blood samples were collected from the heart or tail vein and then centrifuged (Centrurion, Scientific Ltd., city USA) at 373 ×*g* for 20 min. The serum was collected and stored at (−80 °C) until further processing.

3.2. Tissue sampling

After animals were sacrificed, scraps of the wound were dissected in full depth and kept in 10% formalin for histopathological examination.

3.3. Liver and kidney tests

Serum alanine transaminase (ALT), aspartate transaminase (AST), albumin, urea and creatinine were determined using kinetic method test reagent kits (DiaSys Diagnostic Systems GmbH, Germany for liver enzymes, and Randox; city UK for kidney functions), according to the manufacturer's instructions.

3.4. Fasting blood sugar

FBS was measured by GOD-PAP enzymatic colorimetric method using commercially available kit supplied from Spectrum Diagnostics® (GmbH, Schiffgraben, Hannover, Germany) according to the manufacturer's instructions.

3.5. Microscopic evaluation of wound healing

Autopsy samples were taken from the skin and subcutaneous tissue with underlying skeletal muscle of rats in different groups under anesthesia and fixed in 10% buffered formalin. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl alcohol) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin in hot air oven for 24 h. Paraffin bee wax tissue blocks were prepared for sectioning at 4 µm thickness. The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxyline and eosin (H&E) stain for routine examination through the light electric microscope [13] and Masson's trichrome stain for collagen fibers [14].

3.5.1. Cytokines analysis; serum VEGF, TGF-β, PDGF and MMP2 assay

Serum samples were assessed colorimetrically using ELISA kits (CUSABIO, Wuhan, China) according to the manufacturer's instructions.

4. Statistical analysis

Results were expressed as means ± standard deviation. Comparisons between different means were carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test using GraphPad Prism (version 5) software at $p < 0.05$ and $n = 6$. Test for normality was carried out for data.

4.1. Sample size

Sample size was chosen according to the literature so that the power not < 80% [15] where;

$$N = 2(\alpha/\Delta)^2 (z\alpha + z\beta)^2 + 0.25\,(z\alpha)^2$$

One independent test (no replications) was performed for each experiment. Blood samples withdrawn from the heart or tail vein by a well trained technician (Blind experiment).

4.2. Allocating animals/samples to experimental groups

Randamization using random digital generator, allocation in a parallel design order.

5. Results

5.1. Effect of diabetex on toxicity profile tests

Results of the present study revealed that treatment with diabetex (Gp Ib) showed significant reduction in ALT levels as compared to the control group (Gp Ia) and metformin group (Gp Ic) by 14.6% and 25.7% respectively. No significant difference is observed in AST levels among the three control groups (Gp Ia,b,c) (Table 1). Treatment with diabetex (Gp IIb) showed marked reduction in ALT and AST levels compared to diabetic non-treated group (Gp IIa) by 35.29% and by 23.46% respectively. Moreover significant reduction is observed in ALT and AST levels of diabetex group (Gp IIb) compared to metformin group (Gp IIc) by 35.7% and 29.81% respectively (Table 1).

Diabetex (Gp Ib) showed slight significant increase in albumin levels as compared to control group (Gp Ia) and metformin group (Gp Ic) by 8.9% and 13.7% respectively (Table 1) Meanwhile, No significant difference was detected in albumin levels upon comparing all diabetic treated groups (Table 1).

Animals receiving diabetex (Gp Ib) showed significant reduction in urea and creatinine levels as compared to control group (Gp Ia) by 11.93% and 47.35% respectively and metformin group (Gp Ic) by 31% and 116.5% respectively (Table 1). No significant difference is observed in urea levels upon comparing diabetex treated group (Gp IIb) with diabetic group (Gp IIa). Significant reduction is observed in urea levels upon comparing diabetex group with metformin group (Gp IIc) by 33%. Treatment with diabetex (Gp IIb) showed significant reduction in creatinine levels as compared to diabetic group (Gp IIa) by 64.86% and no significant difference compared to metformin group (Gp IIc) (Table 1).

5.2. Changes in FBS mediated by diabetex in control and diabetic rats

Treatment with diabetex (Gp Ib) showed significant reduction in FBS compared to control group (GP Ia) by 10.3% and no significant

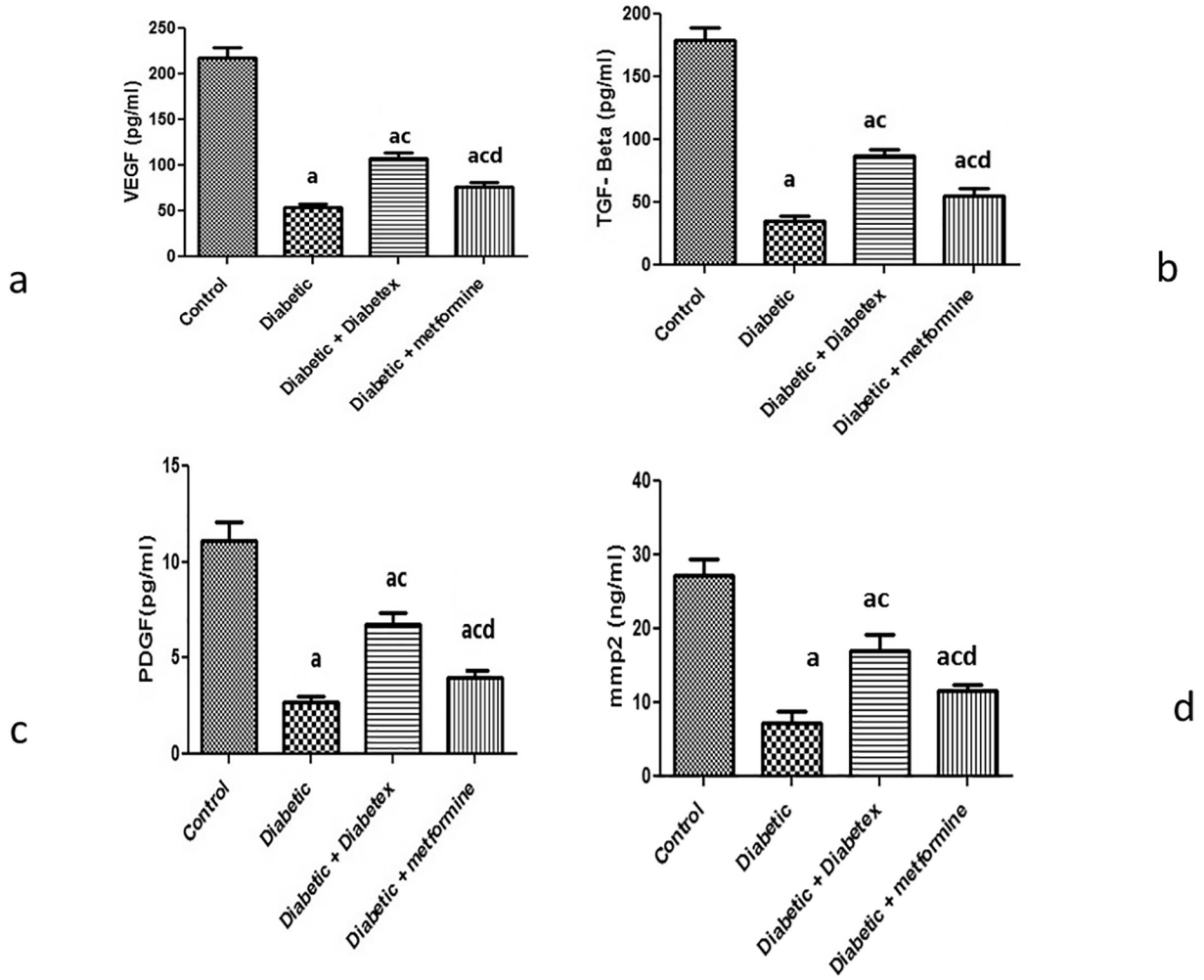

Fig. 1. Effect of diabetex and metformin treatments on VEGF [a], TGF-β [b] and PDGF [c], MMP2 [d] levels in diabetic rats. Data are expressed as mean of 6 rats ± SD. Statistical analysis was performed using one- way ANOVA followed by Tukey-Kramer Multiple Comparisons test; as compared to negative control)(a), diabetex (b), diabetic (c) and diabetic + diabetex (d) groups, $P < 0.05$.

difference compared to metformin group (Gp Ic) (Table 1). Significant elevation is observed in FBS levels of diabetic group (Gp IIa) compared to control group (Gp Ia) by 644.63%. Treatment with diabetex (Gp IIb) and metformin (Gp IIc) showed marked significant reduction in FBS levels as compared to diabetic group (Gp IIa) by 47.7% and 48% respectively. No significant reduction is observed between diabetex (Gp IIb) and metformin (Gp IIc) treatment, suggesting that diabetex has the same therapeutic potential to lower fasting blood sugar as the conventional metformin drug (Table 1).

5.3. Diabetex induces increases in cytokines mediating wound closure

Results of the present study revealed that diabetic rats (Gp IIa) showed marked reduction in VEGF levels as compared to the control group (Gp Ia) by 75.39%. Diabetex administration to diabetic rats (Gp IIb) showed dramatic increase in VEGF as compared to diabetic untreated rats by 100.7%. Treatment of diabetic rats with metformin (Gp IIc) showed significant elevation by 41.7% as compared to diabetic group (Gp IIa). VEGF levels showed marked elevation in the diabetex treated group (Gp IIb) as compared to metformin group (Gp IIc) by 29.4% (Fig. 1a).

In a similar pattern to VEGF, diabetic group (Gp IIa) showed significant reduction in TGF-β levels by 80.36% as compared to control. Diabetex (Gp IIb) and metformin treatments (Gp IIc) showed marked elevation in TGF-β levels by 147.5% and 58% respectively as compared to the diabetic untreated group (Gp IIa). Significant elevation in TGF-β levels upon diabetex treatment (Gp IIb) by 36% as compared to metformin treatment (Gp IIc) (Fig. 1b).

Results of the present study showed that the diabetic group (Gp IIa) displayed significant reductions in PDGF levels as compared to the control group (Gp Ia) by 75.65%. Diabetic rats treated with diabetex (Gp IIb) showed significant elevation in PDGF levels as compared to diabetic untreated rats by 151.2%. Treatment of diabetic rats with metformin (Gp IIc) showed significant elevation by 46.9% as compared to diabetic group (Gp IIa). Diabetex treated group (Gp IIb) showed significant elevation in PDGF levels as compared to metformin group (Gp IIc) by 41.5% (Fig. 1c).

The present investigation revealed a significant decrease in MMP2 levels of the diabetic group (Gp IIa) compared to the control (Gp Ia) by 73%. Metformin treated rats (Gp IIc) showed significant increase in MMP2 levels compared to diabetic one by 57%. Moreover, treatment of diabetic rats with diabetex showed marked elevation in MMP2 levels by 132% and 32% compared to diabetic (Gp IIa) and metformin (Gp IIc) respectively (Fig. 1d).

5.4. Visual inspection (Fig. 2a,b,c,d), histopathological alterations and changes in collagen deposition induced by diabetex

Negative control group displayed an intact epidermis while the underlying dermis had edema, extravasated red blood cells, and focal

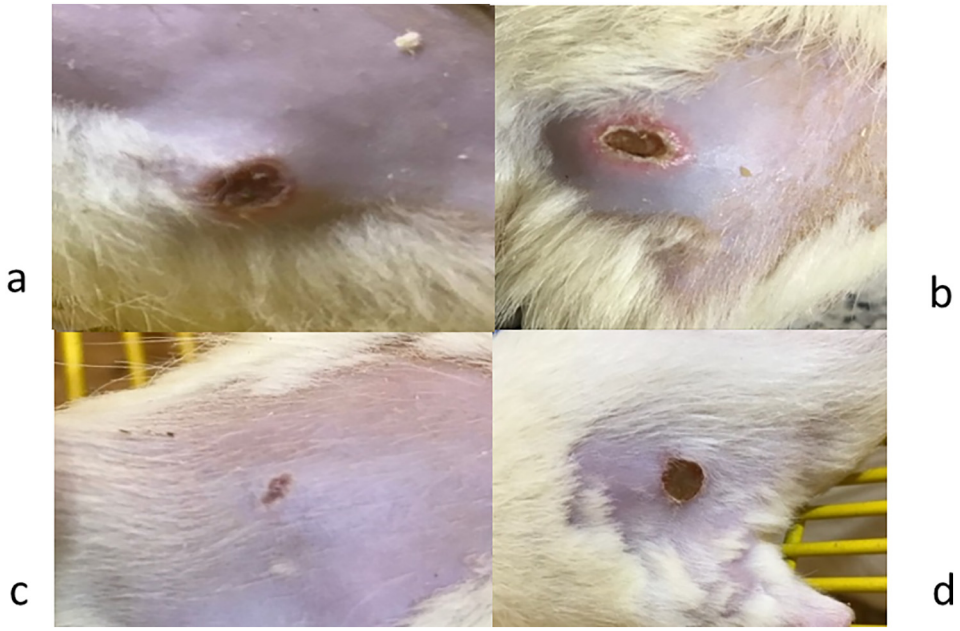

Fig. 2. Visual macroscopic examination of rat wounds; control [a], diabetic [b], diabetic treated with diabetex [c], diabetic treated with metformin [d].

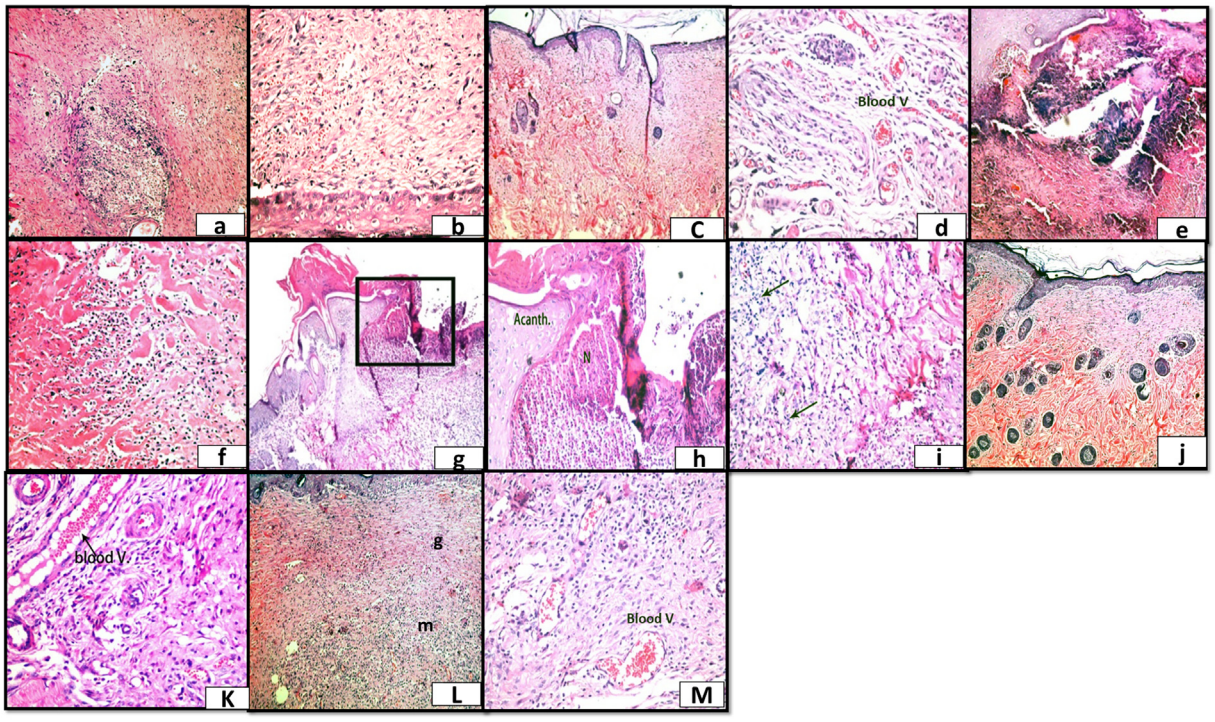

Fig. 3. Histopathological examination using H &E staining of different rat wounds; control [a,b], diabetex [c,d], metformin [e,f], diabetic [g,h,i, diabetic + diabetex [j,k] and diabetic + metformin [el,m] groups. Blood V: blood vessel, Acanth: acanthosis, N: neutrophils, h: Hair follicle, g: granulation tissue, Sb: sebaceous gland.

aggregation of inflammatory cells (Fig. 3a,b). The Diabetex control animals showed a similar pattern to negative control group with inflammatory cells infiltration, fibrosis and newly formed blood capillaries in the subcutaneous tissue (Fig. 3c,d). By the same token, metformin given to naïve animals displayed a similar pattern to the two other negative control groups (Fig. 3e,f). In STZ group, the epidermal and dermal layers of the skin showed focal ulceration and necrosis with adjacent acanthosis in the epidermis. Massive inflammatory cells infiltration and fibrosis were detected in the underlying subcutaneous tissue (Fig. 3g,h,i). However, animals receiving diabetex showed an intact epidermal layer while the underlying focal area of the dermis showed fibrosis, granulation tissue (g) with newly formed blood capillaries, new hair follicles (h), sweat and sebaceous glands (s) (Fig. 3j,k). As for the STZ animals that received metformin, acanthosis was observed in the prickle cell layer of the epidermis associated with massive inflammatory cells (m), fibrosis and dilated blood vessels in the underlying dermis. The dermal tissue showed granulation tissue formation (g) without regeneration of sweat gland and sebaceous gland. (Fig. 3L,m).

Results of the present investigation revealed an increase in the collagen percent upon comparing the diabetex treated group (Gp Ib) to the negative control group (Gp Ia) and the metformin treated group (Gp

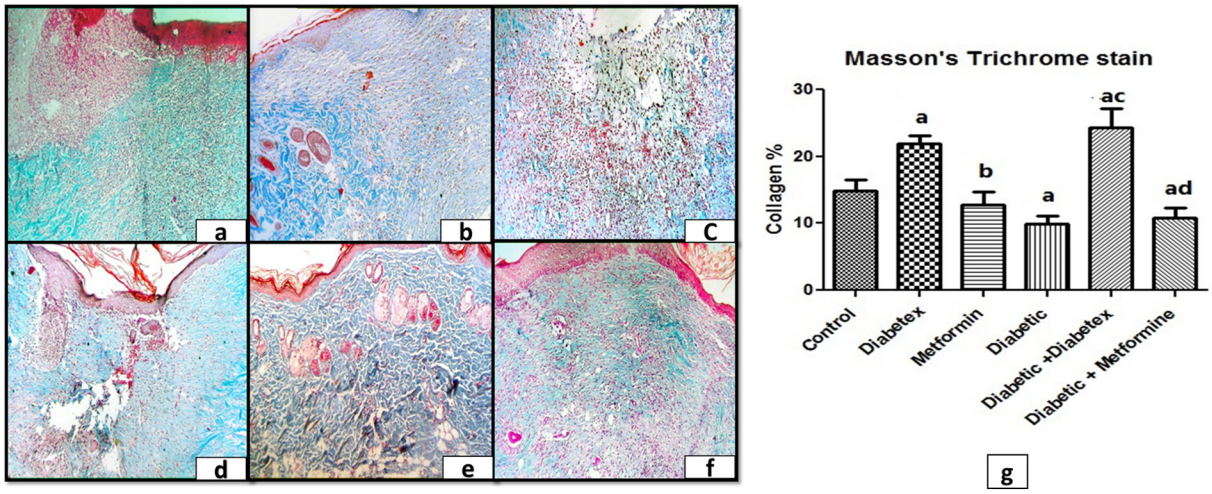

Fig. 4. Masson trichrom's staining of different rat wounds. Control [a], diabetex [b], metformin [c], diabetic [d], diabetic + diabetex [e] and diabetic + metformin [f] groups. g: Graphical representation of rat wounds Masson trichrom's staining showing percent of collagen in each group. Data are expressed as mean of 6 fields ± SD. Statistical analysis was performed using one- way ANOVA followed by Tukey-Kramer Multiple Comparisons test; as compared to negative control (a), diabetex (b), diabetic (c) and diabetic + diabetex (d) groups, $P < 0.05$.

Ic) by 47% and 42% respectively. Diabetic rats treated with diabetex (Gp IIb) showed significant elevation in the collagen percent compared to the diabetic untreated group (Gp IIa) and the metformin diabetic group (Gp IIc) by 145.5% and 55.8% respectively. (Fig. 4a-g).

6. Discussion and conclusion

Wound healing in diabetic patients is impaired and delayed due to defects in cellular infiltration and consequently inadequate granulation tissue formation as well as impaired connective tissue repair [16]. Due to the negative socioeconomic impact of delayed wound healing, new pharmacological agents that accelerate recovery via regulating cytokines/growth factor is an appealing approach for researchers. In this context, the current investigation addressed the safety and toxicity profiles of diabetex in addition to its mechanistic actions on wound healing process.

In the present study, diabetex reducd ALT and AST levels compared to non-treated diabetic group as well as those receiving metfomin as treatment. Although animals receiving metformin showed increments in liver function tests compared to control values, however, naïve animals treated with diabetex showed protection against the changes in liver function. By the same token, naiive animals receiving diabetex showed significant increase in albumin levels as compared to negative control and metformin treated group. The aforementioned results support the hepato- protective effects of the individual components, niacin, ascorbic acid, D-ribose that comprise diabetex [17–19] Likewise, creatinine and urea blood tests used in the current investigation, revealed that diabetex had no negative impact on kidney function. Such results suggest a safety profile of diabetex on the kidney in accordance with Dufva et al. who stated that niacin treatment showed marked reduction in creatinine levels compared to the control group animals [20]. Chanshuai et al.,2011 reported that D-Ribose supplementation did not exhibit deleterious effects on the kidney of mice [19]. Moreover, Mongi et al., 2011 reported the protective role of ascorbic acid treatment on urea and creatinine levels compared to deltamethrin treated group [18]. Notably, metformin is cautiously used in patients with mild to moderate chronic kidney disease [11] which lends further support to the merit of using diabetex. Following the establishment of the safety profile, it was imperative to investigate the effect of diabetex on FBS as a potential milestone in the protection against diabeticinduced complications. Indeed, diabetic animals treated with diabetex showed marked reduction in FBS compared to diabetic non treated groups which was not significant from metformin treatment. In support of this notion, several studies reported that the individual components of diabetex lowered blood sugar levels [21, 22] Clinical and experimental studies demonstrated that ascorbic acid supplementation to type 2 DM patients resulted in significant lowering of fasting and post prandial blood glucose levels [23]. This notion can be attributed to inhibition of oxidative stress by scavenging of ROS, thus protecting beta-cell mass protein and DNA from oxidative damage, hence preserving insulin content [24]. Consequent to the lowered FBS by diabetex, visual macroscopic inspection of the wounds in rats receiving diabetex revealed healthier; contracted lesions in comparison with diabetic non treated group. Moreover, diabetex showed merit over metformin in improving and accelerating wound healing which suggests a mechanism that extends far and beyond FBS control (Fig. 2a,b,c,d).

Several cytokines play a major role in the process of wound healing such as, TGF-β, PDGF, VEGF [25, 26] and MMP2 [3] downstream from macrophage activation. Diabetex resulted in significant elevation in VEGF, TGF-β, PDGF and MMP2 levels as compared to diabetic non treated/metformin groups. In a feed forward cycle, improved angiogenesis via stimulated VGEF recruits macrophages to the wound area which further induces PDGF that in turn activates TGF-β thus enhancing collagen deposition and ultimately wound closure. Noteworthy, the aforementioned cytokines transcriptionally activate MMP2 [3].

In another context, VEGF stimulates endothelial cells to proliferate and migrate. Moreover, VEGF has been shown to stimulate keratinocyte migration and collagen production via fibroblasts. VEGF secretion also induces release of other growth factors which further stimulate healing [27]. Results of the present study are supported by previous findings indicating that the single components of diabetex enhance neutrophil function and increases anigiogenesis [28] as well as its strong antioxidant effect [29]. Aside from enhancing wound healing cytokines, several studies demonstrated that ascorbic acid is necessary for the hydroxylation of proline and lysine residues in procollagen, which is necessary for its release and subsequent conversion to collagen [24, 30]. Hydroxyproline also stabilizes the collagen triple-helix structure. Another proposed mechanism of ascorbic acid stimulation of collagen synthesis is via induction of lipid peroxidation leading to increased transcription of the collagen genes. It is unclear how lipid peroxidation stimulates collagen transcription; however, an alteration of cell membranes may affect the activity of serum growth factors leading to a change in gene expression [31]. In addition, Benfer et al., 1978 reported that xanthinol nicotinate, the most potent form of niacin, stimulated

collagen deposition and angiogenesis in primary closed wounds in rats [32]. As mentioned earlier, VEGF enhances the transcription of MMP2 [3]. Noteworthy, enhanced angiogenesis by niacin might contribute to the increased levels of MMP2 seen in the present study.

Increased inflammatory cytokines, oxidative stress, and nuclear NF-κB in diabetic wounds subsequently leads to the suppression of VEGF and TGF-β signaling impairing wound healing [33]. Sharp and Clark 2011 suggested that inflammatory cytokines remain in the diabetic wound much longer than normal to compensate for the reduced leucocytic activity, thus prolonging the inflammatory phase, resulting in suppression of granulation tissue formation with its main cytokines VEGF, TGF-β [16] and MMP2 [3]. Such findings are in line with the present study, where levels of VEGF, TGF-β and MMP2 are suppressed in the diabetic group. Moreover, Wall et al., 2002 reported that MMP2 levels are suppressed in acute wounds mimicking that of the present study and increased to toxic levels in chronic diabetic foot ulcers [34]. The observed biochemical changes in the present investigation are further consolidated with results from Masson trichrom's staining showing that treatment with diabetex (Gp Ib) and (Gp IIb) displayed marked elevation in the collagen percent compared to negative control group (Gp Ia) and diabetic group (Gp IIa). Interestingly, diabetex treatment demonstrated significant elevation in collagen percent compared to metformin which lends credence to the suggested healing potential of diabetex (Fig. 4a-g).

Histopathologically, control animals that received diabetex showed new blood vessel formation, fibrosis and the intact epidermis. On the other hand, control animals that received metformin showed ulceration, necrosis and massive inflammatory cells in subcutaneous and muscle layer. Interestingly, wounds of diabetic rats treated with diabetex displayed, unbroken epidermal and dermal tissue, with regenerated hair follicle, sweet gland and sebaceous gland as wells as newly formed blood vessels. Surprisingly, wounds of diabetic rats treated with metformin did not show regeneration for hair follicles, sweat and sebaceous glands. Moreover, massive inflammatory cells were detected which reveals that the remodeling stage of wound healing -where inflammatory cells are resolved by apoptosis- was not achieved. Fig. (3a-m).

Taken all together, results of the present study advocate the use of diabetex in wound healing not only indirectly through its glucose lowering effects but also through direct effect on the key cytokines orchestrating the healing process manifest as elevated PDGF, VEGF, MMP2 and TGF-β. Histopathological studies showed acceleration in the healing process and more collagen deposition compared to metformin. The present study offers a novel approach for treating diabetic resistant wounds with a possible more economic, safe strategy.

Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
FBS	Fasting blood sugar
GOD-PAP	Glucose oxidase phenol 4-aminoantipyrine peroxidase
MMP2	matrix metalloproteinase-2
PDGF	Platelet derived growth factor
ROS	Reactive oxygen species
STZ	Streptozotocin
TGF-β	Transforming growth factor beta
VEGF	Vascular endothelial growth factor

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Conflict interest statement

The authors declare that there are no conflict interests in the submitted manuscript.

Contribution statement

The three authors were involved in the study design, conception, drafting and final approval of the version to be published.

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