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Topical metformin accelerates wound healing by promoting collagen synthesis and inhibiting apoptosis in a diabetic wound model

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Abstract

The wound healing process, which is a pathophysiological process that includes various phases, is interrupted in diabetes due to hyperglycemia, and since deterioration occurs in these phases, a normal healing process is not observed. The aim of the current study is to investigate the proliferative and antiapoptotic effects of metformin on wound healing after topical application on diabetic and non-diabetic wounds. For this purpose, we applied metformin topically on the full-thickness excisional wound model we created in diabetic and nondiabetic groups. We investigated the effects of metformin on the apoptotic index by the Terminal deoxynucleotidyl transferase mediated dUTP Nick-End Labeling method and on collagen-I, collagen-III, p53, and c-jun expression levels by quantitative reverse transcription polymerase chain reaction technique in wound biopsy tissues. Our results showed that c-jun and p53 mRNA levels and apoptotic index increased with the effect of diabetes, while collagen synthesis was disrupted. As a result of the study, we showed that metformin increases cellular proliferation and has anti-apoptotic effects by increasing collagen-I/III expression and decreasing p53/c-jun level, especially in diabetic wounds and also in normal wounds. In conclusion, the topical effect of metformin on diabetic wounds reversed the adverse effects caused by diabetes, increasing the wound healing rate and improving the wound repair process.

KEYWORDS

apoptosis, collagen, diabetes mellitus, metformin, wound healing

Key messages

- Since chronic hyperglycemia, which is the clinical outcome of diabetes, causes prolongation of the inflammatory phase in the proliferative phase and apoptosis of important cells, a healthy wound healing is not observed.
- In order to prevent the prolonged inflammation phase in diabetic wounds and to accelerate the wound healing process by increasing collagen

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production, we applied metformin, which is known to have anti-inflammatory properties, topically on the diabetic wound model we created in rats.

- The wound repair process is improved due to the normal occurrence of apoptosis through the inhibition of p53 and the increase in COLI/III expression due to the downregulation of c-jun.

1 | INTRODUCTION

Wound healing, which is a pathophysiological process that includes various stages such as haemostasis, inflammation, proliferation and maturation, is impaired due to hyperglycemia, which is the most prominent clinical outcome of diabetes mellitus (DM).[1,2] When the inflammation subsides, the wound enters the proliferative phase.[3] While type III collagen, the main component of granulation tissue, is mainly produced in the proliferation phase, it is gradually replaced by type I collagen in the remodelling phase, resulting in increased tensile strength and maturation of the dermal tissue or scar.[4,5] On the other hand, the activator protein-1 (AP-1) transcription factor, composed of homo- and heterodimers of Fos and Jun, plays a role in the regulation of genes important for differentiation, cell migration, wound re-epithelialization, apoptosis and proliferation processes, and is involved in wound healing.[6–8] Especially in the proliferating cell, the expression of c-jun and c-fos is highly increased and acts as an important player in cell cycle progression.[9,10] In contrast, AP-1 also modulates the transcription of several inflammatory genes, particularly matrix metalloproteases (MMPs).[11] MMP-9 and MMP-2 degrade denatured collagens during the granulation events of cell migration and wound healing. It has been shown that the tensile strength of nonhealing wounds in DM is lower than normal and this is associated with a decrease in collagen synthesis.[12,13] In addition, AP-1 components also have some opposing functions inside the cell. The most well-known of these functions is that it triggers apoptosis with its ability to regulate the expression and function of one of the cell cycle regulators, such as p53, when exposed to both cellular and environmental stress factors.[14,15]

Apoptosis, which is defined as three main extrinsic, intrinsic and p53-dependent pathways, is a cell death process that is necessary for normal physiological function and to maintain cellular homeostasis and occurs in response to various causes.[16] Cells with specialized functions that increase at different stages of the wound healing process should be removed from the wound after each stage after their task is completed. Apoptosis, which is the healthiest method of this elimination, performs without tissue damage or inflammation.[17,18] However, oxidative stress induced by a hyperglycemic condition in DM causes DNA damage and induces apoptosis as a result of the transcription of apoptosis-related genes through mechanisms associated with the activation of p53.[19–21] Eventually, excessive apoptosis due to oxidative stress in diabetic patients causes susceptibility to infection during wound healing and paves the way for delayed wound healing due to the prolonged inflammation process in wound healing.[22] For this reason, it is seen that there is a need for locally applied agents with anti-inflammatory properties for a faster healing result, especially in inadequate wound healing caused by diabetes.

Metformin, which has recently received increasing attention for its anti-inflammatory properties, is one of the most commonly used oral medications for the treatment of type 2 DM (T2DM). Recently, it has been reported in studies that topically administered drugs are known to be effective in faster wound contraction, wound closure, and overall healing due to the desired local effect directly at the wound site. Although it is known to have the potential to improve inflammation, there are few studies on the molecular mechanism of wound healing of metformin, one of the oldest antidiabetic agents.[23–30] In light of this information, this study evaluated the proliferative and antiapoptotic effects of topical application of metformin, a widely used therapeutic drug for T2DM, on the wound healing process in healthy and Streptozotocin (STZ)-induced diabetic Wistar albino rats.

2 | METHODS

2.1 | Animal study design

The experimental protocols of this study were approved by the Laboratory Animals Local Ethics Committee of Bezmialem University (2020/15). In the study, 24 healthy adult male Wistar Hannover rats weighing between 300 and 350 g were used. Six rats were randomly distributed to each group, divided into four groups as non-diabetic control group (NC), non-diabetic treatment

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group (NT), diabetic control group (DC) and diabetic treatment group (DT). Rats randomly allocated to the diabetic groups were injected intraperitoneally (IP) with a single dose of 60 mg/kg STZ dissolved in 0.1 M sodium citrate buffer (pH 4.5).[31] Non-diabetic groups were given only saline (IP). After ~72 h, glucose levels of blood samples taken from the tail vein of rats were measured, and those higher than ~250 mg/dL were accepted as diabetes and included in the experiment, rats below this limit were excluded from the group.[31,32] 50 mg/kg sodium pentobarbital was administered IP to the rats for anaesthesia before each operation. Three circular 12 mm^2 full-thickness excisional wound models were created using a sterile punch on the back of all rats in the experimental groups, and the wound healing process was followed for 14 days.[33] While only saline was applied topically to the wounds of the rats in the control groups and 3 mM metformin was applied to the rats in the treatment groups every day on the wounds by absorbing sterile surgical sponges. On the 3rd, 7th, and 14th days, the rats were reoperated for wound healing follow-up and wound biopsy samples. After the last wound biopsy was taken on the 14th day, the experiment was terminated without any treatment, and the rats were sacrificed with high-dose anaesthesia. Wound diameter measurements were made using the AutoCAD® 2021 software. The wound closure percentage was calculated according to the following formula[34]:

$$\text{Percentage wound contraction on } N\text{th day} = 100 - \frac{\text{Wound area on } N\text{th day}}{\text{Wound area on first day}} \times 100$$

2.2 | RNA isolation from tissue

In order to isolate total RNA from the tissue, the wound biopsy tissues were homogenized using zirconium beads in a homogenizer. RNA was purified from the lysates, using the total RNA isolation kit from tissue (High Pure RNA Tissue Kit-Roche) as described in the steps in the manufacturer's protocol. Purified RNAs were stored at −80°C until the day of analysis.

2.3 | Quantitative reverse transcription polymerase chain reaction

The purified RNAs were taken from −80°C where they were stored, and the samples were thawed, and quantitative changes in the amounts of P53, COLI, COLIII and AP-1 mRNA were determined by reverse transcription real-time polymerase chain reaction (RT-qPCR). This analysis was performed on a Roche LightCycler 480 II device using the LightCycler® EvoScript RNA SYBR® Green I Master kit and primers (Merck) designed with the Primer3 program (Table 1). In order to check for contamination in each reaction, the reaction mixture without RNA was added as a negative control. In order to control the specificity of the primers, a melting curve was added after the amplification, and the binding of the primers was checked by Tm analysis. $2^{-\Delta Ct}$ results of TP53, COLI, COLIII and cJUN relative mRNA expression levels were calculated by the system compared to G6PD.

2.4 | Terminal deoxynucleotidyl transferase mediated dUTP Nick-End Labeling

Terminal deoxynucleotidyl transferase (TdT) mediated dUTP Nick-End Labeling (TUNEL) was used for the determination of apoptosis. Tissue sections taken into Coplin jars were deparaffinized and rehydrated according to the standard protocol. Antigenic masking was removed

TABLE 1 Primers of genes JUN, COL1A1, COL3A1, TP53 and G6PD for performing quantitative reverse transcription polymerase chain reaction.

Gene name	Gene symbol	Primer sequence (5′-3′)
Transcription factor Jun (AP1 subunit)	JUN	F: TGACCAGAAGATGGTGCAGT R: CACAGCGCATGCTACTTGAT
Collagen type I alpha 1	COL1A1	F: ACATCTCTGTGAAGGGGTGG R: GCACTCTCTCCTCCCTTGTT
Collagen type III alpha 1	COL3A1	F: GACTCGGGATCTGTCCTCTG R: CAGGTGTAGAAGGCTGTGGA
Tumour protein 53 (p53)	TP53	F: TATCTGGACGACAGGCAGAC R: CGTGATGATGGTAAGGATGG
Glucose 6-phosphate dehydrogenase	G6PD	F: ACATCCGCAAACAGAGTGAG R: GCTGTTGAGGTGCTTGTAGG

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after boiling for 5 min in the microwave with citrate buffer (pH 6.0). Then the tissues were washed in PBS for 2×5 min and the TUNEL method was applied according to the recommended procedure in the kit (Chromogenic Apoptosis Detection Kit, A049, ABP Bioscience).

One negative and one positive control were used for the specificity of the immunostaining. PBS was added to the section used as a negative control instead of the primary antibody, and all the steps were applied exactly. Mayer's haematoxylin dye was used for counterstaining. Apoptotic index detection was performed by counting brown-stained nuclei in 10 randomly selected areas under a light microscope (LEICA DM500) at ×40 magnification. The apoptotic index was calculated by taking the arithmetic mean of the 10 counted areas.

2.5 | Statistical evaluation

According to the data of our previous similar study, using G Power 3.1 software, the sample size was calculated as a minimum of $n = 6$ in each group, assuming the significance level $\alpha = 0.05$ and $\beta = 0.20$.[35] Statistical analysis of the data obtained at the end of the study was performed using GraphPad Prism 8.0.2 software. The differences between the groups were evaluated with the analysis of variance test, paired comparisons were made with the Tukey test for significant results. Pairwise comparison of normal and diabetic groups at day 0 was analysed by *t*-test. The mean ± SD value was given as descriptive statistics. $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Wound healing

Figure 1A shows the percent values of wound healing rates as a line graph, Figure 1C shows these percentages as mean + SD bar graph on the graph, and Figure 1B shows the comparative photographs of wound healing by groups and days (Table 2). In both untreated control groups, only a significant increase was observed on days 7 and 14 compared to day 0 ($p < 0.0001$ for N0; $p < 0.0001$ for D0). In the NT group, on the 3rd, 7th, and 14th days, it was shown that metformin accelerated wound healing at a statistically significant level ($p < 0.0001$; $p < 0.0001$; $p < 0.0001$, respectively) compared to day 0. Similarly, the percentage of wound closure in the DT group showed a significant decrease every 3 days after treatment compared to day 0 ($p = 0.0112$; $p < 0.0001$; $p < 0.0001$, respectively).

3.2 | Demonstration of TP53, COL1A1, COL3A1, JUN expression by RT-qPCR method

According to the results of JUN mRNA expression levels (Figure 2A, Table 3); a significant increase was observed in the N0 group compared to day 3 ($p = 0.0409$), while a dramatic decrease was observed on treatment days 7 and 14 compared to day 0 (respectively; $p = 0.0008$; $p = 0.0008$). While a statistically significant increase was detected only on day 3 compared to day 0 in the D0 group ($p = 0.0004$), although there was a decrease on days 7 and 14, no statistical significance was observed. In the NT group, a significant decrease was observed on the 3rd, 7th and 14th days of wound healing compared to day 0 (respectively; $p = 0.0011$; $p < 0.0001$; $p < 0.0001$). When evaluated according to the statistical analysis in the DT group, a significant dramatic decrease was observed on the 3rd, 7th and 14th days compared to day 0 (respectively; $p < 0.0001$; $p < 0.0001$; $p < 0.0001$).

According to the results of COL1A1 mRNA expression levels (Figure 2B, Table 3); a significant increase was observed on the 14th treatment day compared to the 0th day in the N0 group ($p = 0.0384$). In the NT group, a linear significant increase was observed on the 3rd, 7th and 14th days of wound healing compared to day 0 (respectively; $p = 0.0051$; $p < 0.0001$; $p < 0.0001$). No significant change was observed on all days compared to day 0 in the D0 group. On the other hand, when the DT group was evaluated compared to days, a significant increase was observed on days 7 and 14 compared to day 0 (respectively; $p = 0.0039$; $p < 0.0001$).

According to the results of COL3A1 mRNA expression levels (Figure 2C, Table 3); when in-group comparisons were made according to days in the N0 and D0 groups, no significant change was observed on any day compared to day 0. In the NT group, which is one of the treatment groups, a significant increase was observed on the 3rd and 7th days of wound healing compared to the 0th day (respectively; $p = 0.0011$; $p = 0.0115$). In the DT group, which is another treatment group, a significant increase was observed on the 3rd day compared to the 0th day when evaluated compared to the days ($p = 0.0263$).

According to the results of TP53 mRNA expression levels (Figure 2D, Table 3); in the N0 group, a significant increase was observed on the 3rd day compared to the 0th day ($p = 0.0103$). A significant reduction was observed in the NT group on days 3 and 7 of wound healing compared to day 0 (respectively; $p = 0.0329$; $p = 0.0057$). A statistically significant increase was found in the D0 group on days 3 and 7 compared to day 0

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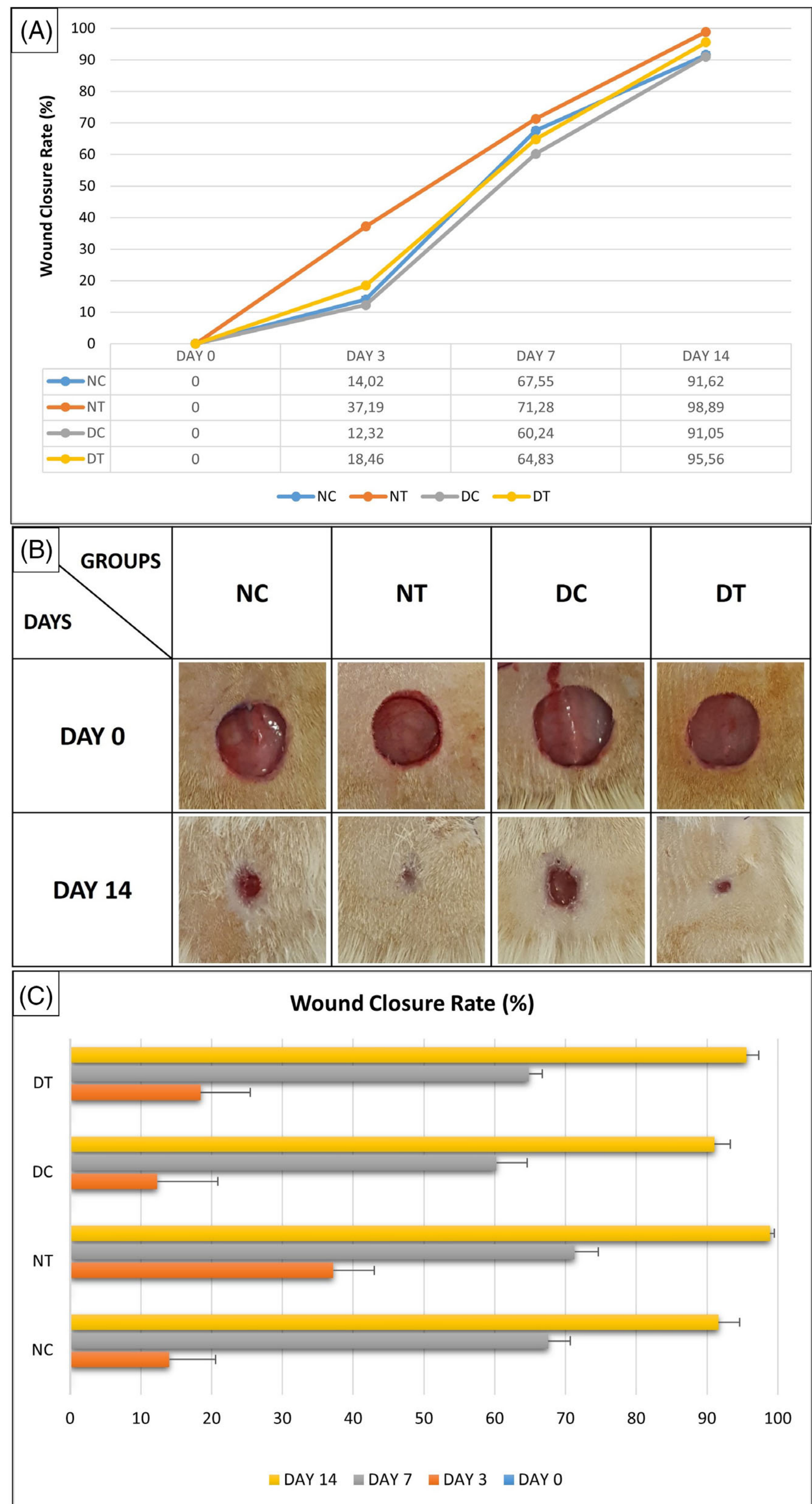

FIGURE 1 Graphical representation of the change in wound rate in all control and treatment groups (A). Photographs of wounds treated with topical metformin and saline on days 0, 3, 7, and 14 after creating a full-thickness excisional wound model (B). Intragroup graphical representation of the wound healing rates of the experimental groups (C). DC, diabetic control group; DT, diabetic treatment group; NC, non-diabetic control group; NT, non-diabetic treatment group.

(respectively; $p = 0.0004$; $p = 0.0007$). When the DT group was evaluated compared to days, a significant dramatic decrease was observed on days 3, 7 and 14 compared to day 0 ($p < 0.0001$; $p < 0.0001$; $p < 0.0001$, respectively).

3.3 | Demonstration of apoptosis change by TUNEL method

In Figure 2D, in-group evaluations of the apoptotic index compared to days are graphed, and in Figure 3,

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TABLE 2 Change of wound rate in all control and treatment groups.

	Day 3	Day 7	Day 14
Non-diabetic control group	14.02 ± 6.523	67.55 ± 3.14****	91.62 ± 3.004****
Non-diabetic treatment group	37.19 ± 5.818****	71.28 ± 3.375****	98.89 ± 0.6156****
Diabetic control group	12.32 ± 8.543	60.24 ± 4.325****	91.05 ± 2.272****
Diabetic treatment group	18.46 ± 7.004*	64.83 ± 1.864****	95.56 ± 1.778****

Note: Values are stated as the mean ± SE. All values were compared with respect to day 0.
*$p < 0.05$; ****$p < 0.0001$.

microscopic images of apoptotic cells after TUNEL staining in wound biopsy tissue sections taken on days 0, 3, 7, and 14 from all groups are presented (Table 3). The results of the analysis of tissue samples on the day of the first wound (day 0) without any treatment showed a significant increase in the diabetic group compared to the normal group ($p = 0.0005$). When compared within the group according to the days; In the N0 group, a significant increase was seen at day 3 compared to day 0 ($p = 0.0214$). In the NT group, however, no significant difference was found on treatment days compared to day 0. While a significant increase was observed on the 3rd and 7th treatment days in the D0 group compared to the 0th day (respectively; $p = 0.0021$; $p = 0.0355$); A significant decrease was observed on days 3, 7, and 14 in the DT group compared to day 0 (respectively; $p = 0.0002$; $p < 0.0001$; $p < 0.0001$).

4 | DISCUSSION

Under normal conditions, wound healing occurs with the regular combination of many cellular activities such as phagocytosis, chemotaxis, mitogenesis, collagen synthesis and synthesis of some matrix elements. In contrast, wound healing in diabetic patients is disrupted at different stages and is described as 'delayed wound healing'.[12] In this study, we examined the proliferative and antiapoptotic effects of metformin, which is used as a first-line oral antidiabetic in the treatment of type 2 diabetes, by applying it topically on healthy and diabetic wounds. While the percentage of wound closure showed a significant increase in all three treatment days in the treatment groups in which we applied metformin topically, it showed a significant difference in the control groups, with a percentage below the treatment groups only on the 7th and 14th days. Thus, it is clearly seen as a result of the study that topical metformin treatment accelerates the wound healing process in healthy tissue, as well as accelerates the diabetic wound healing process even more than the spontaneous wound healing process.

Oxidative stress induced by high glucose causes DNA damage, and occurred cell damage by genotoxic events activates p53, a transcription regulator gene, and can induce apoptosis by p53-related mechanisms.[19] Apoptosis prevents excessive inflammation by killing inflammatory cells such as neutrophils and macrophages. If the inflammation proceeds continuously, normal wound healing will not occur. Although limited apoptosis is beneficial in wound healing, too much or abnormal apoptosis negatively affects wound healing, as in many pathological conditions, including diabetes with poor glycemic control.[22] Also, the increased AGE level caused by chronic hyperglycemia causes prolongation of the inflammatory phase in the proliferative phase, decreased migration and wound contraction, and apoptosis of important cells.[36] Early apoptosis of fibroblasts prevents the formation of granulation tissue. In a study evaluating the effect of apoptosis on wound healing in a diabetic condition, Desta et al. showed that impaired gingival wound healing is common in diabetic mice due to increased fibroblast apoptosis.[37] In another wound model in which melatonin was applied topically, it was reported that the amounts of caspase-3 and p53 decreased significantly in the treatment groups, but these effects did not accelerate wound healing.[38] A cutaneous wound model study in mice showed that transient inhibition of p53 with pifithrin-α (PFT-α) promotes early cell proliferation, which is essential for rapid tissue repair.[39] In our study, we observed that hyperglycemia-induced apoptosis in parallel with the increase in the amount of p53 compared to control. On the other hand, the amount of p53 mRNA and the ratio of apoptotic cells were significantly reduced in the diabetic and non-diabetic groups treated with metformin compared to the controls. In light of the data of our study; metformin accelerated wound healing by suppressing apoptosis via the p53 pathway in the pathophysiological processes of wound healing, especially due to the stress induced by diabetes.

AP-1, which plays an active role in cell proliferation; is activated by phosphorylating the c-Jun subunit. However, against this feature, it is thought that c-jun activity plays a role in the development of stress-induced

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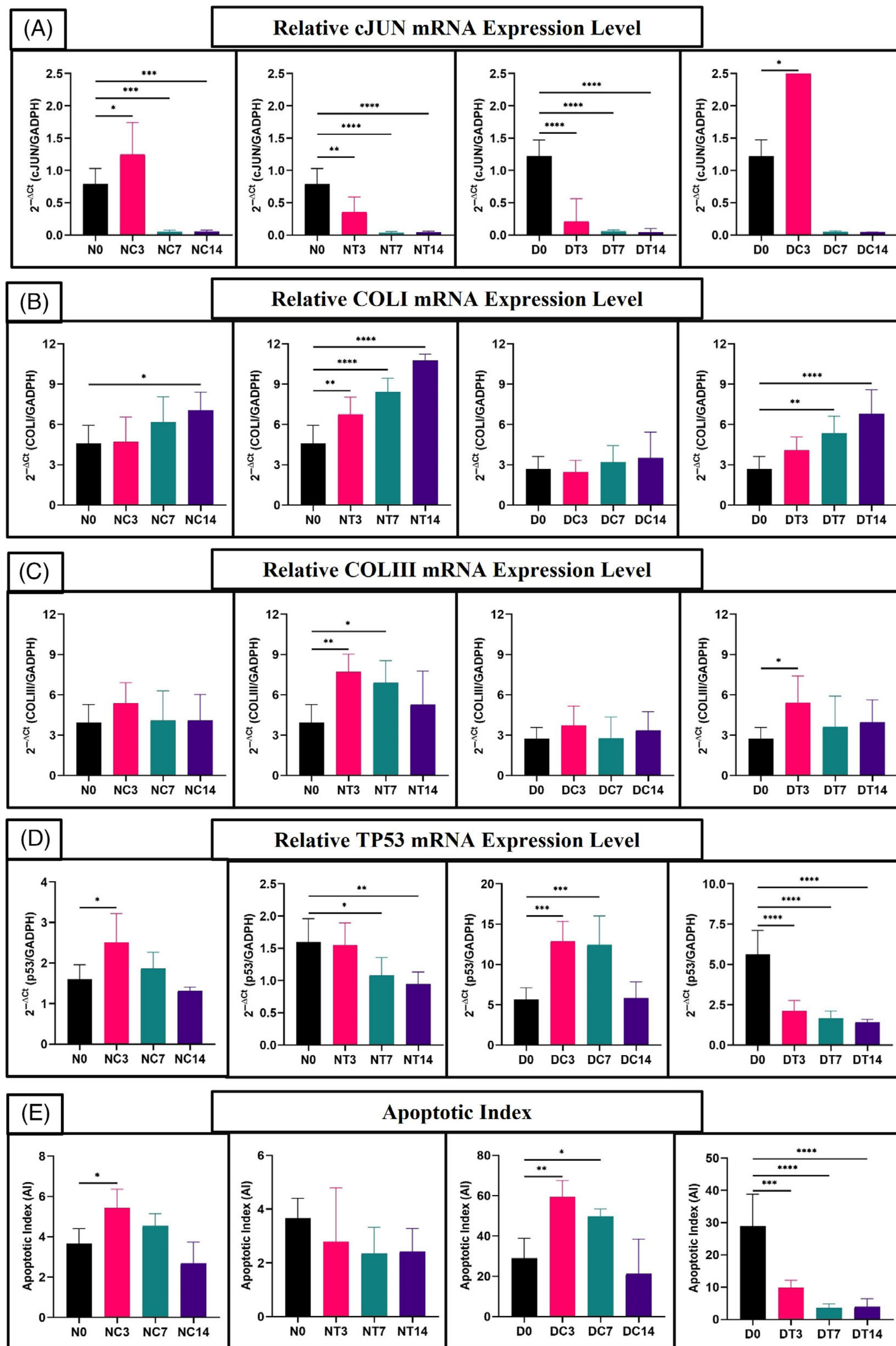

FIGURE 2 Graphical representation of mRNA levels and apoptotic index change in all control and treatment groups. Relative mRNA levels following full-thickness excisional wound formation and treatment with topical metformin, as determined by quantitative reverse transcription polymerase chain reaction. Apoptotic index following full-thickness excisional wound formation and treatment with topical metformin, as determined by TUNEL staining. Values are stated as the mean ± SD. All values were compared with respect to day 0. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$. D0, diabetic group on the first day of wound opening; DC, diabetic control group; DT, diabetic treatment group; N0, non-diabetic group on the first day of wound opening; NC, non-diabetic control group; NT, non-diabetic treatment group.

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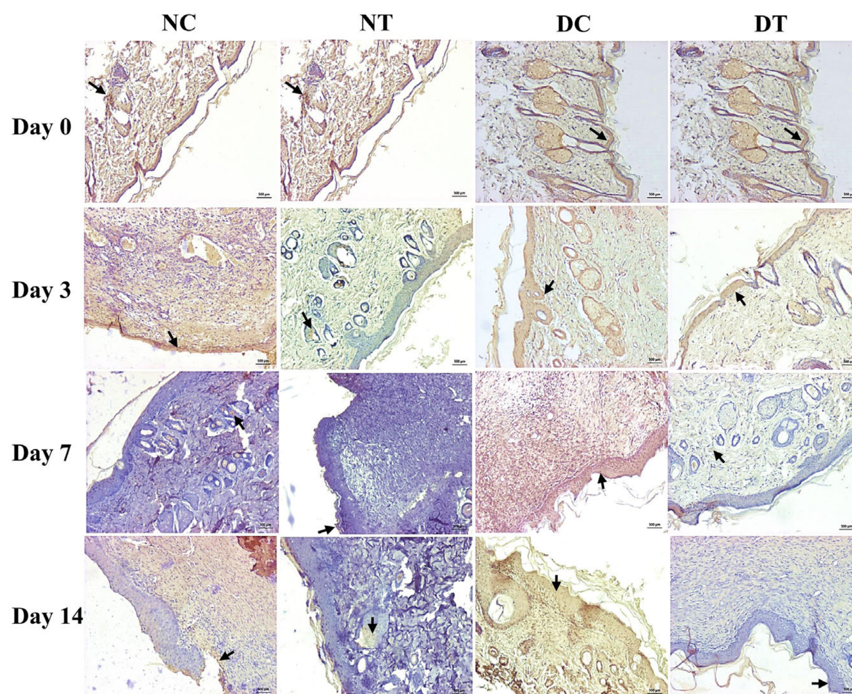

FIGURE 3 Microscopic images of apoptotic cells after TUNEL staining in all control and treatment groups. As a result of counterstaining with Mayer's haematoxylin, we considered blue-stained nuclei as healthy cells and brown-stained nuclei as apoptotic cells. Apoptotic cells are shown in detail with the arrow in the figure. Apoptotic nuclei are indicated with an arrow (⟶) as an example in each group. DC, diabetic control group; DT, diabetic treatment group; NC, non-diabetic control group; NT, non-diabetic treatment group.

TABLE 3 Evaluations of relative mRNA levels and apoptotic index following full-thickness excisional wound formation and treatment with topical metformin on days 0, 3, 7, and 14.

		Groups			
Parameters	Days	Non-diabetic control group	Non-diabetic treatment group	Diabetic control group	Diabetic treatment group
COLI	Day 0	4.59 ± 1.346		2.688 ± 0.9387	
	Day 3	4.708 ± 1.84	6.761 ± 1.274**	2.453 ± 0.8849	4.083 ± 0.9835
	Day 7	6.185 ± 1.869	8.437 ± 1.004****	3.199 ± 1.222	5.345 ± 1.28**
	Day 14	7.08 ± 1.329*	10.75 ± 0.4903****	3.501 ± 1.932	6.802 ± 1.771****
COLIII	Day 0	3.951 ± 1.321		2.724 ± 0.8524	
	Day 3	5.388 ± 1.515	7.711 ± 1.312**	3.723 ± 1.444	5.416 ± 1.994*
	Day 7	4.088 ± 2.213	6.907 ± 1.64*	2.774 ± 1.577	3.628 ± 2.29
	Day 14	4.096 ± 1.944	5.265 ± 2.5	3.35 ± 1.389	3.959 ± 1.676
c-JUN	Day 0	0.791 ± 0.239		1.223 ± 0.25	
	Day 3	1.25 ± 0.494*	0.361 ± 0.227**	2.779 ± 1.542*	0.212 ± 0.352****
	Day 7	0.051 ± 0.021***	0.037 ± 0.016****	0.515 ± 0.015	0.059 ± 0.021****
	Day 14	0.054 ± 0.021***	0.045 ± 0.016****	0.047 ± 0.001	0.044 ± 0.055****
p53	Day 0	1.6 ± 0.36		5.624 ± 1.481	
	Day 3	2.507 ± 0.713*	1.55 ± 0.35	12.87 ± 2.479***	2.127 ± 0.637****
	Day 7	1.867 ± 0.40	1.082 ± 0.273*	12.45 ± 3.597***	1.672 ± 0.447****
	Day 14	1.315 ± 0.096	0.943 ± 0.188**	5.853 ± 1.983	1.423 ± 0.171****
Apoptotic index	Day 0	3.66 ± 0.748		28.91 ± 9.952	
	Day 3	5.43 ± 0.931*	2.787 ± 2.005	59.38 ± 8.144**	9.783 ± 2.315***
	Day 7	4.54 ± 0.61	2.35 ± 0.981	49.79 ± 3.698*	3.588 ± 1.199****
	Day 14	2.697 ± 1.045	2.427 ± 0.859	21.31 ± 17.12	3.887 ± 2.5****

Note: Values are stated as the mean ± SD. All values were compared with respect to day 0.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

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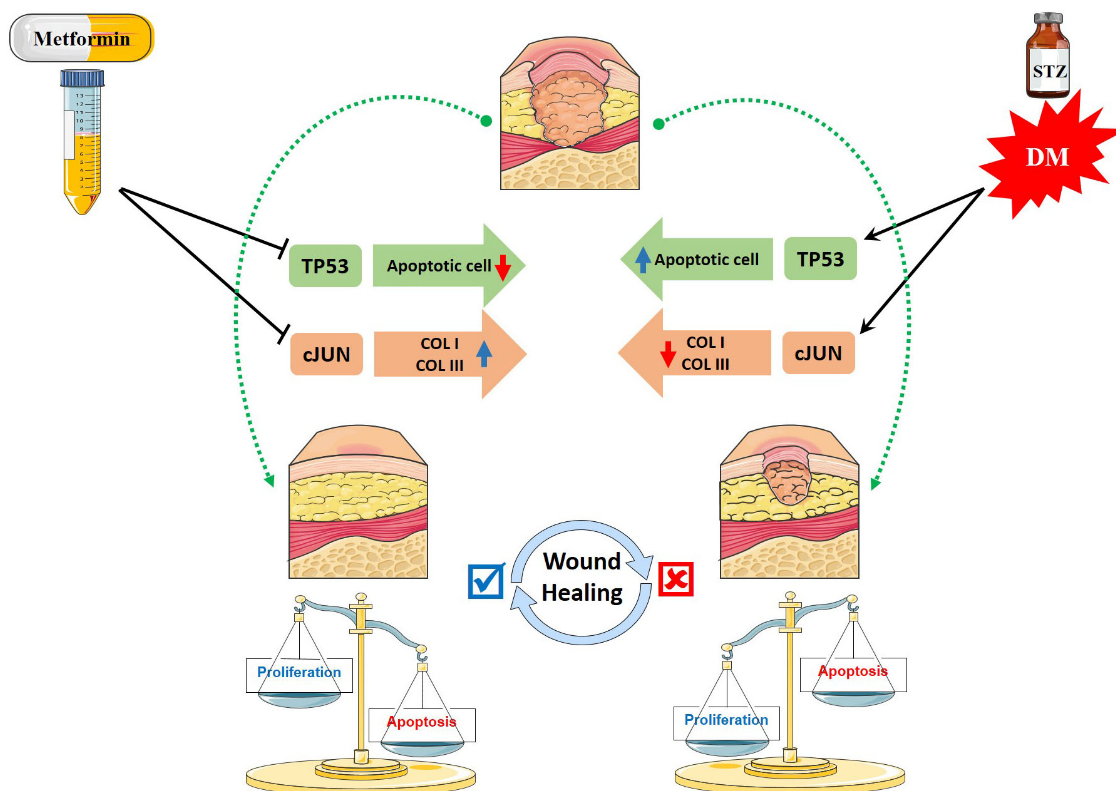

FIGURE 4 Graphical abstract of the results of our study.

apoptosis, DNA repair steps in response to genotoxic damage, and cell cycle arrest.[14,15] In addition, phosphorylated AP-1 is also known to regulate genes involved in wound healing, such as growth factors and MMPs.[9] Evidence from recent in vitro wound model studies have shown that c-Jun expression increases as a result of treatment with different agents, which promotes the migration and proliferation of keratinocytes, thereby accelerating wound healing.[40–42] However, in another study, they showed that high glucose increased the mRNA and protein level of AP1 subunits c-Fos/c-Jun, resulting in the activation of AP1 in diabetic skin tissue.[43] In an in vivo wound model, photobiomodulation treatment combined with a topical hydrogel prepared from the fruit of Lycium barbarum was shown to decrease MMP-1/-2/-9 expression as well as c-Fos/c-Jun, and increase collagen I/III level, inhibit skin thickening caused by ultraviolet radiation.[44] In the results of our study, we showed that the mRNA expression of c-jun was significantly higher in the tissue of hyperglycemic rats, and it decreased significantly with metformin treatment on the 3rd day compared to the 0th day. In addition, there was a significant decrease in c-jun expression on all three treatment days compared to day 0, while a significant increase was observed in the control groups on the 3rd day. In our previous study, we showed that while c-jun, MMP2, MMP9 expression decreased, COLI and COLIII expression increased with treatment in the wound model in which we applied larval secretion, which, in turn, accelerated wound healing.[35] In addition, in the wound model study in which we applied topical metformin, we showed that it reduced MMP2/9 levels and therefore suppressed inflammation.[45]

Collagen is the essential and major component of the extracellular matrix and contributes significantly to wound integrity, wound remodelling process, and healing process, thus largely dependent on its regulated biosynthesis, accumulation, and subsequent maturation.[46] In the early phase of wound healing, Type III collagen is synthesized and replaced by Type I collagen in the later phase of wound healing. Thus, the highest percentage of type I collagen compared to Type III collagen indicates the collagen maturation status.[47,48] Increasing the amount and organization of collagen with many agents used has been directly associated with accelerating and improving the wound repair process.[49–55] In an in vivo model, in which the effect of calcium alginate on wounds was examined and parallel to our study, it was reported that COLI expression increased from the 3rd day to the 14th day in the treated group, and there was no significant difference in COLIII expressions between the two groups.[56] Ganeshkumar et al. showed that

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topically applied Acalypha indica extract improved cellular proliferation by significantly increasing the expression of COLI (150%) and COLIII (180%) on the 7th day of scar tissue.[57] We also found that metformin, which we applied topically on the wounds, showed a linearly significant increase in COLI mRNA expression every three treatment days in non-diabetic wounds and on the 7th and 14th days in diabetic wounds compared to the first day. In the untreated groups, we found that there was a significant increase in the healthy wound only on the 14th day, while no significant difference was observed in the diabetic control wound. While COLIII mRNA level increased significantly on the 3rd and 7th days in the treated healthy wound and on the 3rd day in the diabetic wound, no significant changes were observed in the control groups.

New treatment options and their effects are still being investigated to improve the wound repair process, especially in chronic wounds caused by hyperglycemia, which is one of the physiological consequences of diabetes. There is still a molecular gap in the literature to be filled in order to increase treatment options and to propose new potential therapeutic agents. As a result of this study, in which we aimed to show the effect of metformin on the created full-thickness excisional wound model, metformin showed a therapeutic effect that accelerates wound healing by increasing collagen production, which is an important biomarker in the proliferation phase, due to inhibition of apoptosis in both spontaneous wound healing and diabetic wound healing (Figure 4). In addition, the reason for the increase in wound healing rate with metformin can probably be related to the inhibition of the activation of MMPs due to the decreased expression of AP-1 as a result of treatment with metformin, and thus the increase of collagen synthesis. Thus, we showed that metformin is not only an orally used antidiabetic agent but also a potential topical agent for wound healing. In conclusion, metformin increases cellular proliferation and accelerates wound healing by increasing collagen 1/3 expression and decreasing the p53/c-jun level.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restrictions.

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REFERENCES

1. Krzyszczyk P, Schloss R, Palmer A, Berthiaume F. The role of macrophages in acute and chronic wound healing and interventions to promote pro-wound healing phenotypes. *Front Physiol*. 2018;9:419.
2. Frykberg RG, Banks J. Challenges in the treatment of chronic wounds. *Adv Wound Care*. 2015;4(9):560-582.
3. Koh TJ, DiPietro LA. Inflammation and wound healing: the role of the macrophage. *Expert Rev Mol Med*. 2011;13:e23.
4. Enoch S, Leaper DJ. Basic science of wound healing. *Surgery (Oxford)*. 2008;26(2):31-37.
5. Li J, Chen J, Kirsner R. Pathophysiology of acute wound healing. *Clin Dermatol*. 2007;25(1):9-18.
6. Hess J, Angel P, Schorpp-Kistner M. AP-1 subunits: quarrel and harmony among siblings. *J Cell Sci*. 2004;117:5965-5973.
7. Jaakkola P, Kontusaari S, Kauppi T, Määttä A, Jalkanen M. Wound re-epithelialisation activates a growth factor-responsive enhancer in migrating keratinocytes. *FASEB J*. 1998;12:959-969.
8. Tran PO, Hinman LE, Unger GM, Sammak PJ. A wound-induced $[Ca^{2+}]_i$ increase and its transcriptional activation of immediate early genes is important in the regulation of motility. *Exp Cell Res*. 1999;246:319-326.
9. Yates S, Rayner TE. Transcription factor activation in response to cutaneous injury: role of AP-1 in reepithelialization. *Wound Repair Regen*. 2002;10(1):515.
10. Yamashita J, McCauley LK. The activating protein-1 transcriptional complex. *Clin Rev Bone Miner*. 2006;4:107-122.
11. Qin Z, Balimunkwe RM, Quan T. Age-related reduction of dermal fibroblast size upregulates multiple matrix metalloproteinases as observed in aged human skin in vivo. *Br J Dermatol*. 2017;177(5):1337-1348.
12. Brown DL, Kane C, Charnamsek SD, Greenhalgh D. Differantial expression and localization of insulin like growth factors 1 and 2 in cutaneous wounds of diabetic and non diaabetic mice. *Am J Pathol*. 1997;151:715-724.
13. Wieman TJ. Clinical efficacy of Becaplarmin (rhPDGF-BB) gel. *Am J Surg*. 1998;176(suppl 2A):74-79.
14. Reno EM, Haughian JM, Jackson TA, Thorne AM, Bradford AP. c-Jun N-terminal kinase regulates apoptosis in endometrial cancer cells. *Apoptosis*. 2009;4(6):809-820.
15. May GH, Allen KE, Clark W, Funk M, Gillespie DA. Analysis of the interaction between c-Jun and c-Jun N-terminal kinase in vivo. *J Biol Chem*. 1998;273:33429-33435.
16. Redza-Dutordoir M, Averill-Bates DA. Activation of apoptosis signalling pathways by reactive oxygen species. *Biochim Biophys Acta Mol Cell Res*. 2016;1863(12):2977-2992.
17. Greenhalgh DG. The role of apoptosis in wound healing. *Int J Biochem Cell Biol*. 1998;30(9):1019-1030.
18. Wu YS, Chen SN. Apoptotic cell: linkage of inflammation and wound healing. *Front Pharmacol*. 2014;21(5):1.
19. Allen D, Yaqoob MM, Harwood SM. Mechanisms of high glucose-induced apoptosis and its relationship to diabetic complications. *J Nutr Biochem*. 2005;16:705-713.
20. Volpe E, Sambucci M, Battistini L, Borsellino G. Fas–fas ligand: checkpoint of t cell functions in multiple sclerosis. *Front Immunol*. 2016;7:382.
21. Benchimol S. p53-dependent pathways of apoptosis. *Cell Death Differ*. 2001;8:1049-1051.

1742481x, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/iwj.14345 by Istanbul Aydin University Kutuphane Ve Dok Dai Bsk, Wiley Online Library on [02/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

22. Arya AK, Pokharia D, Kumar H, Mishra M, Tripathi K. Impact of lymphocyte apoptosis in diabetes mellitus. *Asian J Med Sci*. 2011;2(1):1-6.
23. Han X, Tao Y, Deng Y, Yu J, Sun Y, Jiang G. Metformin accelerates wound healing in type 2 diabetic db/db mice. *Mol Med Rep*. 2017;16(6):8691-8698.
24. Schuiveling M, Vazirpanah N, Radstake TR, Zimmermann M, Broen JC. Metformin, a new era for an old drug in the treatment of immune mediated disease? *Curr Drug Targets*. 2018;19(8):945-959.
25. Ursini F, Russo E, Pellino G, et al. Metformin and autoimmunity: a "new deal" of an old drug. *Front Immunol*. 2018;9:1236.
26. Yang Q, Yuan H, Chen M, et al. Metformin ameliorates the progression of atherosclerosis via suppressing macrophage infiltration and inflammatory responses in rabbits. *Life Sci*. 2018;198:56-64.
27. Lee CH, Hsieh MJ, Chang SH, et al. Enhancement of diabetic wound repair using biodegradable nanofibrous metformin-eluting membranes: in vitro and in vivo. *ACS Appl Mater Interfaces*. 2014;6(6):3979-3986.
28. Isoda K, Young JL, Zirlik A, et al. Metformin inhibits proinflammatory responses and nuclear factor-kappaB in human vascular wall cells. *Arterioscler Thromb Vasc Biol*. 2006;26(3):611-617.
29. Zhao P, Sui BD, Liu N, et al. Anti-aging pharmacology in cutaneous wound healing: effects of metformin, resveratrol, and rapamycin by local application. *Aging Cell*. 2017;16(5):1083-1093. doi:10.1111/acel.12635
30. El-Bahy AAZ, Aboulmagd YM, Zaki M. Diabetex: a novel approach for diabetic wound healing. *Life Sci*. 2018;207:332-339.
31. Metwally MM, Ebraheim LL, Galal AA. Potential therapeutic role of melatonin on STZ-induced diabetic central neuropathy: a biochemical, histopathological, immunohistochemical and ultrastructural study. *Acta Histochem*. 2018;120(8):828-836.
32. Elsharawy MA, Naim M, Greish S. Human CD34+ stem cells promote healing of diabetic foot ulcers in rats. *Interact Cardiovasc Thorac Surg*. 2012;14(3):288-293.
33. Sumitra M, Manikandan P, Suguna L. Efficacy of Butea monosperma on dermal wound healing in rats. *Int J Biochem Cell Biol*. 2005;37:566-573.
34. Datta HS, Mitra SK, Patwardhan B. Wound healing activity of topical application forms based on ayurveda. *Evid Based Complement Alternat Med*. 2011;2011:134378.
35. Tombulturk FK, Soydas T, Sarac EY, et al. Regulation of MMP 2 and MMP 9 expressions modulated by AP-1 (c-jun) in wound healing: improving role of *Lucilia sericata* in diabetic rats. *Acta Diabetol*. 2019;56(2):177-186.
36. Peppa M, Stavroulakis P, Raptis SA. Advanced glycoxidation products and impaired diabetic wound healing. *Wound Repair Regen*. 2009;17(4):461-472.
37. Desta T, Li J, Chino T, Graves DT. Altered fibroblast proliferation and apoptosis in diabetic gingival wounds. *J Dent Res*. 2010;89(6):609-614.
38. Şener A, Çevik Ö, Doğan Ö, Altindiş NG, Aksoy H, Okuyan B. The effects of topical melatonin on oxidative stress, apoptosis signals, and p53 protein expression during cutaneous wound healing. *Turk J Biol*. 2015;39(6):888-895.
39. Vollmar B, El-Gibaly AM, Scheuer C, Strik MW, Bruch H-P, Menger MD. Acceleration of cutaneous wound healing by transient p53 inhibition. *Lab Invest*. 2002;82(8):1063-1071.
40. Stelling-Férez J, Gabaldón JA, Nicolás FJ. Oleanolic acid stimulation of cell migration involves a biphasic signaling mechanism. *Sci Rep*. 2022;12(1):1-16.
41. Meng Z, Li Z, Guo S, et al. MED1 ablation promotes oral mucosal wound healing via JNK signaling pathway. *Int J Mol Sci*. 2022;23(21):13414.
42. Kim M, Kim JG, Kim KY. *Trichosanthes kirilowii* extract promotes wound healing through the phosphorylation of ERK1/2 in keratinocytes. *Biomimetics*. 2022;7(4):154.
43. Lang J, Yang C, Liu L, et al. High glucose activates ERK1/2 to stabilize AP1 and increase MMP9 expression in diabetic foot ulcers. *Exp Cell Res*. 2021;403(1):112550.
44. Neves LMG, Parizotto NA, Tim CR, et al. Polysaccharide-rich hydrogel formulation combined with photobiomodulation repairs UV-induced photodamage in mice skin. *Wound Repair Regen*. 2020;28(5):645-655.
45. Tombulturk FK, Todurga-Seven ZG, Huseyinbas O, Ozyazgan S, Ulutin T, Kanigur-Sultuybek G. Topical application of metformin accelerates cutaneous wound healing in streptozotocin-induced diabetic rats. *Mol Biol Rep*. 2022;49(1):73-83.
46. Maehata Y, Takamizawa S, Ozawa S, et al. Type III collagen is essential for growth acceleration of human osteoblastic cells by ascorbic acid 2-phosphate, a long-acting vitamin C derivative. *Matrix Biol*. 2007;26:371-381.
47. Robson MC, Steed DL, Franz MG. Wound healing. *Curr Probl Surg*. 2001;38:73-94.
48. Busnardo VL, Biondo-Simões MLP. Effects of low-level helium-neon laser on induced wound healing in rats. *Rev Bras Fisioter*. 2010;14:45-51.
49. Cam ME, Ertas B, Alenezi H, et al. Accelerated diabetic wound healing by topical application of combination oral antidiabetic agents-loaded nanofibrous scaffolds: an in vitro and in vivo evaluation study. *Mater Sci Eng C Mater Biol Appl*. 2021;119:111586.
50. Qiu S, Jia Y, Sun Y, et al. Von Hippel-Lindau (VHL) protein antagonist VH298 improves wound healing in streptozotocin-induced hyperglycaemic rats by activating hypoxia-inducible factor-(HIF-) 1 signalling. *J Diabetes Res*. 2019;2019:1897174.
51. Alhakamy NA, Caruso G, Eid BG, et al. Ceftriaxone and melittin synergistically promote wound healing in diabetic rats. *Pharmaceutics*. 2021;13(10):1622.
52. Sun X, Yang Y, Liu T, Huang H, Kuang Y, Chen L. Evaluation of the wound healing potential of *Sophora alopecuroides* in SD rat's skin. *J Ethnopharmacol*. 2021;273:113998.
53. Afshar M, Hassanzadeh-Taheri M, Zardast M, Naderi Z. Effect of earthworm oil on formation of collagen type III during wound healing process in BALB/c mice. *Folia Med (Plovdiv)*. 2022;64(2):267-274. doi:10.3897/folmed.64.e62272
54. Jeong W, Yang CE, Roh TS, Kim JH, Lee JH, Lee WJ. Scar prevention and enhanced wound healing induced by polydeoxyribonucleotide in a rat incisional wound-healing model. *Int J Mol Sci*. 2017;18(8):1698.
55. Paraguassú GM, Xavier FCA, Cangussu MCT, et al. Effect of laser phototherapy (λ660 nm) on type I and III collagen expression during wound healing in hypothyroid rats: an

1742481x, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/iwj.14345 by Istanbul Aydin University Kutuphane Ve Dok Dai Bsk, Wiley Online Library on [02/05/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

immunohistochemical study in a rodent model. *Photomed Laser Surg*. 2014;32(5):281-288.

56. Wang T, Gu Q, Zhao J, et al. Calcium alginate enhances wound healing by up-regulating the ratio of collagen types I/III in diabetic rats. *Int J Clin Exp Pathol*. 2015;8(6):6636.
57. Ganeshkumar M, Ponrasu T, Krithika R, Iyappan K, Gayathri VS, Suguna L. Topical application of *Acalypha indica* accelerates rat cutaneous wound healing by up-regulating the expression of Type I and III collagen. *J Ethnopharmacol*. 2012;142(1):14-22.

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