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ORIGINAL ARTICLE

Topical application of metformin accelerates cutaneous wound healing in streptozotocin-induced diabetic rats

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Abstract

Background Diabetic chronic wound, which is one of the diabetic complications caused by hyperglycemia, characterized by prolonged inflammation has become one of the most serious challenges in the clinic. Hyperglycemia during diabetes not only causes prolonged inflammation and delayed wound healing but also modulates the activation of nuclear factor-kappa B (NF-κB) and the expression of matrix metalloproteinases (MMPs). Although metformin is the oldest oral antihyperglycemic drug commonly used for treating type 2 diabetes, few studies have explored the molecular mechanism of its topical effect on wound healing. Therefore, we aimed to investigate the molecular effects of topical metformin application on delayed wound healing, which's common in diabetes.

Methods and results In this context, we created a full-thickness excisional wound model in Wistar albino rats and, investigated NF-κB p65 DNA-binding activity and expression levels of RELA (p65), MMP2 and MMP9 in wound samples taken on days 0, 3, 7, and 14 from diabetic/non-diabetic rats treated with metformin and saline. As a result of our study, we showed that topically applied metformin accelerates wound healing by suppressing NF-κB p65 activity and diminishing the expression of MMP2 and MMP9.

Conclusions Diabetic wounds treated with metformin healed even faster than those in the control group that mimicked standard wound healing.

Keywords Diabetes mellitus · Metformin · MMP · NF-kB · Wound healing

Introduction

Diabetes mellitus is considered to be associated with a series of changes in the metabolism of connective tissue, as a result of which diabetics face the problem of delayed wound healing, which is stalled in the persistent inflammatory phase with elevated levels of pro-inflammatory cytokines and proteases. The wound healing process does not proceed toward the next phases to heal. A frequent and severe problem in patients with diabetes is delayed or poor wound healing, affecting 15–20 per cent of all people with diabetes [1–3].

The wound healing process is dominated by the coordination of numerous cell-signalling events, growth factors, pro-inflammatory cytokines, NF-κB signalling pathway, is one of a key regulator of the inflammation [4, 5]. Increased inflammatory cytokines, and activated NF-κB pathway in diabetic wounds subsequently leads to the suppression of some growth factors signalling impairing wound healing and cause a prolonged inflammation process, creating a result that negatively affects wound healing [6, 7]. Besides, hyperglycaemia in diabetes increases MMP activity directly or indirectly through oxidative stress or advanced glycation end products (AGEs). The wounds need a certain number of these enzymes for an efficient healing process, contrarily, may be damaging at high levels, causing disrupted wound healing [8–10]. It is known

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that the expression of MMPs is regulated by NF-κB until now most of the researchers have focussed on the expression patterns of MMP-9 and -2. There are reports showing that MMP2 and MMP9 levels are higher in diabetic wounds, thus causing delayed wound healing and risked repair. Furthermore, MMP9 and MMP2 are the best known pro-inflammatory members of proteases that degrade ECM components. Disruption of the optimization of inflammation due to high MMP levels in the scar tissue delays wound healing process [1, 9–12].

Metformin is one of the most constantly prescribed first-line oral anti-hyperglycemic drug for the management of type 2 DM for many years [13, 14]. Although metformin is one of the oldest and most effective agents in the treatment of patients with diabetes, there are few studies on the potential effect of its on wound healing. 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. Few studies have reported that metformin showed accelerated wound-healing effects in healthy animals diminished activation of NF-κB, inhibited the expression of pro-inflammatory mediators such as MMP [15–19].

As a result, considering drug interactions and systemic side effects of pharmacological agents, due to the known anti-inflammatory effect of metformin, it's likely that applying it topically will show its effect faster than systemic circulation. Also, agents that decrease inflammatory molecules expression such as inhibiting the NF-κB signalling pathway and decreasing the MMP levels may be a useful effective therapeutic method to cure the impaired wound healing in diabetic patients. Thus, wound healing can pass from the prolonged inflammatory phase to the proliferative phase in an optimal time. The preventive efficacy of metformin against wound healing has been supported by several investigations, but there are many unanswered questions to illuminate the underlying impaired wound healing in diabetes. Therefore, the target of this study is to evaluate the effect of metformin in the STZ-induced diabetic rat wound model and to figure out the role of NF-κB, MMP2 and MMP9.

Materials and methods

Animals

The experimental protocols of this study were reviewed and approved by the Laboratory Animals Local Ethics Committee of Bezmialem University (2019/216). Adult, male, with an average weight of 250–300 g Wistar albino rats were obtained. The rats included in the study were housed in a temperature-controlled (22–24 °C) room within individually polycarbonate cages on a 12-h light/dark cycle. Standard pellet and water were provided ad libitum.

Six rats were randomly distributed to each group, and these rats were divided into two main groups as diabetic and non-diabetic rats. Each group was again divided into two groups as metformin (treatment groups) and saline applied groups (control groups) once daily for 14 days. The four groups were as follows:

Non-diabetic control group-NC: Only sterile saline was applied to the wounds.

Non-diabetic Metformin treatment group-NT: Rats were treated topically with 3 mM metformin.

Diabetic control group-DC: Only sterile saline was applied to the wounds.

Diabetic Metformin treatment group-DT: Rats were treated topically with 3 mM metformin.

Induction of diabetes

At the beginning of the study, blood glucose levels and body weights of all animals were measured. Diabetes was induced in rats constituting the experimental group with a single dose of intraperitoneal (IP) 60 mg/kg STZ (Cayman) in citrate buffer solution (0.1 M, pH 4.5), which is immediately once prepared. After 72 h, blood samples drawn from the tail vein of rats were measured for fasting glucose levels using a glucometer (Accu-Check, Roche). Those higher than ~ 250 mg/dL were considered diabetes and selected for further experiment. Rats with blood glucose levels below this level were excluded from the study [20].

Creation of excisional wound model

Rats were administered intraperitoneally 50 mg/kg pentothal sodium for anaesthesia before each operation. After the dorsal region of each rat undergoing anaesthesia was shaved off with an electric razor, the wound site was made ready for operation by disinfection with povidone-iodine and 70% alcohol. Three circular full-thickness excisional wounds were created in the back region of the animals by 12 mm^2 diameter sterile skin biopsy punch on each rat in each group. This first operation was accepted as 0 days and the experimental process was started. The biopsy days were planned for the 3rd, 7th, and 14th days. The biopsy procedure was performed according to the clock position, sequentially in the wounds. The wound tissue upper left on the 3rd day, the wound tissue upper right on the 7th day, and wound tissue lower midline on the 14th day were taken under sterile conditions. The wound area was traced on days 0, 3, 7, and 14 post-wounding by taking photos from a stable height. Wound size measurements were made with the Autocad® program.

Application of the treatment

Sterile surgical sponges were absorbed with saline and applied on the wound to control groups once daily throughout 14 days. Metformin-HCl (Aarti Drugs) prepared at a concentration of 3 mM was absorbed into sterile surgical sponges and topically applied on the wound to treatment groups once daily throughout 14 days. The blood glucose levels of the rats were checked before the surgical procedure on each operation day, and their blood glucose levels of the diabetic rats were continuously monitored to be above ~ 250 mg/dL. After the wound biopsy was taken on the 14th day, the experiment was terminated. Rats were sacrificed with a high dose anaesthetic on day 14 post-wounding.

Total RNA isolation and RT-qPCR

Total RNA was isolated from all snap-frozen wound tissues using High Pure RNA Tissue Kit (Roche) according to the manufacturer's protocol and stored at − 80 °C until the experiment day. The RNA quality was determined via NanoDrop-One (Thermo-Scientific) by measuring the OD260/OD280 ratio (> 2.0). mRNA expression levels of RELA, MMP2 and MMP9 in wound tissues were determined by quantitative reverse transcriptase PCR. RT-qPCR analysis was accomplished using LightCycler 480 System 1.5 (Roche) by LightCycler® EvoScript RNA SYBR® Green I Master Mix. For each sample, RT-qPCR assay was done at least in duplicate. The final reaction volume was 20 µl. All quantifications were normalized to the β-actin as housekeeping gene. $2^{-\Delta Ct}$ results of relative mRNA expression levels were calculated using Lightcycler 480 Software Release 1.5.0 SP4 system compared to ACTB.

Nuclear extraction and ELISA

Separation of nuclear fractions from tissue was performed to the manufacturer's instructions (Abcam). NF-kB (p65) activity in the nuclear extracts was determined using NF-κB p65 Transcription Factor Assay Kit according to the manufacturer's instructions (Abcam). The optical density of the peroxidase product was read using an ELISA reader at 450 nm. The results were expressed as OD.

Statistical analyses

The number of animals in this study was determined by power analysis (power of 0.8 with alpha value 0.05) (G Power 3.1). The sample size was estimated from data of a similar previous study [21] to detect a 50% difference in the average wound healing, with an α of 0.05 and β of 0.20. Statistical analyses were performed using Graphpad Prism 8.0.2. Results were expressed as mean ± SEM, where "n" is the number of individual animals per group. A p-value of ≤ 0.05 was considered statistically significant. The differences between the groups were examined using ANOVA test followed by Tukey test to compare groups with each other for meaningful results.

Results

Effect of topically applied metformin on wound healing

The improvement in DC is much behind on the 14th day compared to the other groups. In the NT, almost all animals had closed wounds on day 14, while a faster wound healing was observed in the DT than the NC, which represents the spontaneous treatment process (Fig. 1A, B).

Each group was evaluated in comparison to the days within itself (Fig. 1A). On the 7th day and the 14th day, there was a significant decrease compared to the 0th day ($p < 0.0001$) in the NC group. In the NT group, it was shown that metformin at a statistically significant level ($p < 0.0001$) accelerated wound healing on the 3rd, 7th, and 14th days compared to the 0th day. In the DC group, the speed of wound healing was prolonged, and no significant difference was found on the 3rd day. But, on the 7th and 14th days, wound healing showed a significant decrease ($p < 0.0001$). Because of metformin administration in the DT group, a significant decrease was observed on the 3rd, 7th, and 14th days compared to the 0th day ($p = 0.0049$, $p < 0.0001$, $p < 0.0001$; respectively).

When comparing the groups on the 3rd, 7th, and 14th days of healing; when the wound measurement results on the 7th day are evaluated; there is a significance between the DC group and NC group ($p = 0.0119$), this finding shows the negative effect of diabetes on wound healing. The DT group also showed a significant wound healing result ($p = 0.0001$) compared to DC. Also, there is a significant difference between treatment groups ($p = 0.0038$). On the 14th day; The NT group showed a dramatic decrease ($p = 0.0005$) compared to the NC, and a significant improvement was also found in the DT group compared to the DC ($p < 0.0001$). Also, a significant difference was found when the two controls were compared ($p = 0.0083$).

Effect of metformin on NF-κB p65 activity

According to these results, when each group is compared within itself at the level of days (Fig. 2A); in the NC group, p65 activity on day 3 increased significantly compared to day 0 ($p < 0.0001$). On the 14th day, it showed a significant decrease compared to the 0th day ($p = 0.0026$, respectively). In addition, a significant decrease was seen

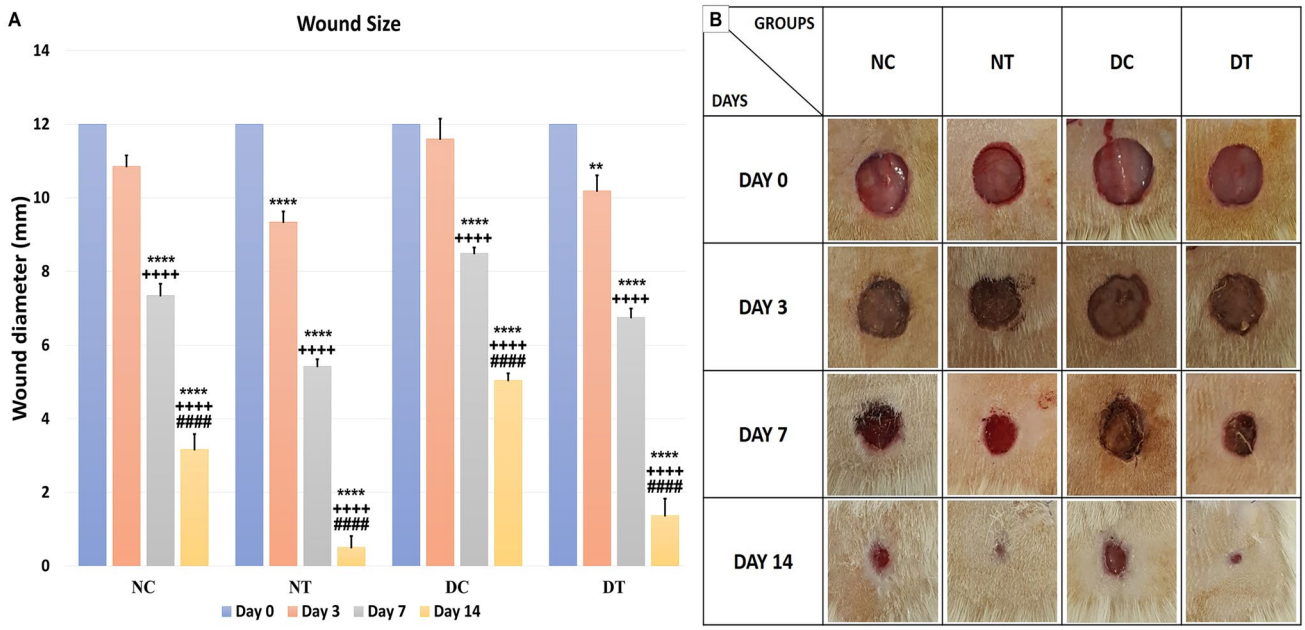

Fig. 1 A Graphical evaluation of the healing effect of topically applied metformin on cutaneous wound healing in the experimental groups. Data shown are the mean ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; compared to day 0.+p < 0.05;++p < 0.01;+++p < 0.001;++++p < 0.0001; compared to day 3. #p < 0.05; ##p < 0.01; ###p < 0.001; ####p < 0.0001; compared to day 7- within-group significance level is indicated). **B** Photo images of wound contraction of metformin-treated (3 mM) rats on days 0, 3, 7, and 14

on days 7 and 14 compared to day 3 ($p < 0.0001$). It was observed that there was a significant increase on the 3rd day and 7th day in the DC group compared to the 0th day ($p < 0.0001$, $p = 0.0005$, respectively). In the NT and DT groups, expression values on the 3rd, 7th, and 14th days were significantly decreased compared to the 0th day ($p < 0.0001$).

When the comparison between groups was made on the 3rd, 7th, and 14th days of wound healing (Table 1A); On the 3rd day of wound healing, the NT and DT groups decreased dramatically compared to their control groups and statistically significant ($p < 0.0001$). According to the evaluation results on the 7th day; No significance was found between the NT group and its control (NC). In the DT group, compared to the control (DC), the significant decrease continued ($p < 0.0001$). According to the evaluation on the 14th day of wound healing; a significant decrease was seen in the DT group compared to DC ($p < 0.0001$). On 3., 7., and 14. days of the healing, whereas no significant difference was observed between the treatment groups, among the control groups, the DC group was significantly higher than the NC group ($p < 0.0001$).

Effect of metformin on RELA (p65) mRNA expression level

When comparing within the group according to days (Fig. 2B) in the NC group, it showed a significant increase on the 3rd day compared to the 0th day ($p < 0.0001$). A significant decrease was observed on the 7th and 14th days compared to the 0th day ($p = 0.001$, $p = 0.0259$, respectively). A dramatic decrease was observed on the 7th and 14th days compared to the 3rd day ($p < 0.0001$). A statistically significant increase was found in the DC group on day 3 and day 7 compared to day 0 ($p = 0.0005$, $p = 0.0155$, respectively). When the NT and DT groups were evaluated compared to days, there was no difference on day 3 compared to day 0. However, the dramatically lower levels on the 7th and 14th days compared to the 0th day showed statistical significance ($p < 0.0001$). A significant decrease was also observed on days 7 and 14 compared to day 3 of wound healing ($p = 0.0003$, $p = 0.0004$, $p < 0.0001$, $p < 0.0001$, respectively).

According to the intergroup evaluation results according to the days (Table 1B); Results on day 3 showed a significant decrease in the NT and DT groups compared to their control groups ($p = 0.036$, $p = 0.0007$, respectively). Among the control groups, it was observed that the DC group had a significantly higher expression level than the NC group ($p = 0.0016$). According to the results on the 7th day; It was

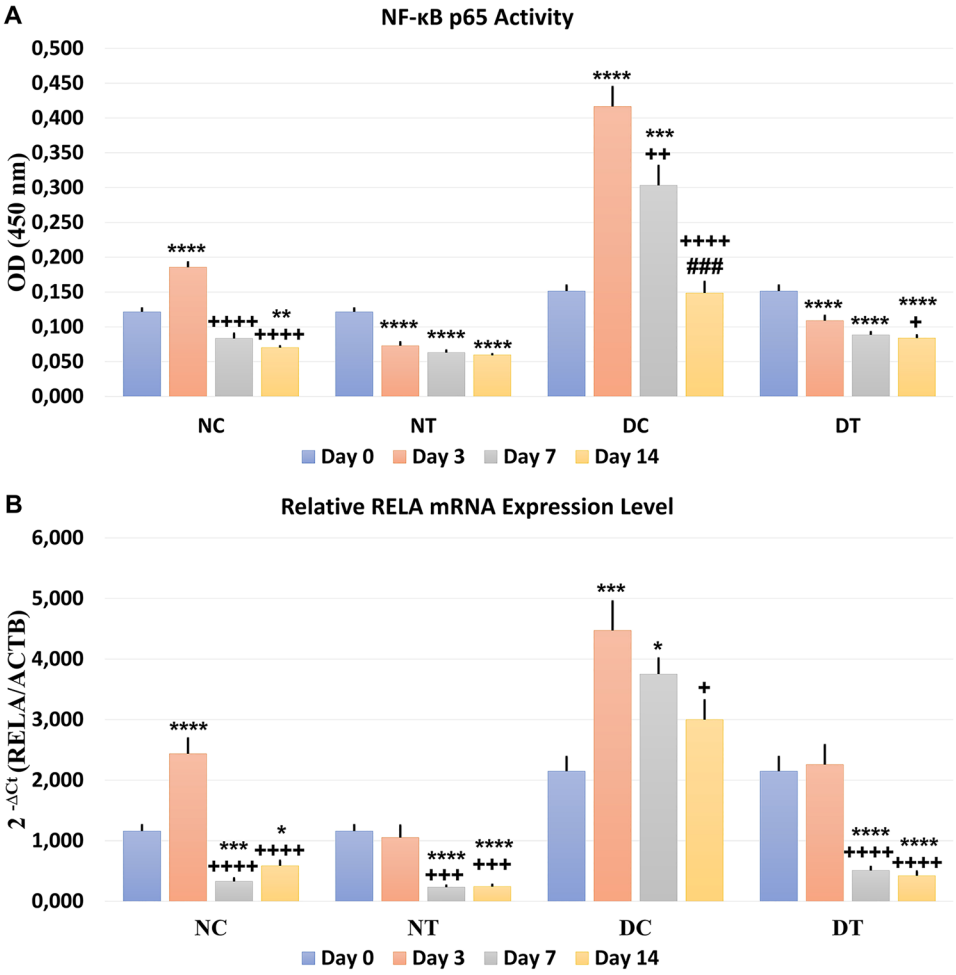

Fig. 2 **A** Graphical evaluation of the NF-κB (p65) activity results detected by ELISA method on days 0, 3, 7, and 14 of wound healing. **B** Graphical evaluation of the RELA mRNA expression level determined by RT-qPCR method on days 0, 3, 7, and 14 of wound healing. Data shown are the mean ± SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; compared to day 0. + $p < 0.05$; + + $p < 0.01$; + + + $p < 0.001$; + + + + $p < 0.0001$; compared to day 3. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$; ####$p < 0.0001$; compared to day 7-within-group significance level is indicated)

found that the expression level in the DT group was significantly reduced ($p < 0.0001$) compared to the control (DC). No significance was found between the NT group and NC. There was also a significant difference between the NC and DC groups due to the increase in the expression level of diabetes ($p < 0.0001$). The RELA expression level results on day 14 of wound healing showed a significant decrease in the DT group compared to the DC ($p < 0.0001$). However, no significant change was observed in the NT group compared with NC. The DC group was found to be statistically significantly higher than the NC group ($p < 0.0001$).

Effect of metformin on MMP2 mRNA expression level

When the comparison results of the experimental groups according to the days are evaluated (Fig. 3A); Results on days 7 and 14 in the NC group showed a dramatic significant decrease compared to day 0 ($p = 0.0012$, $p = 0.0003$, respectively) and day 3 ($p < 0.0001$). Although there was an increase on the 3rd day and a decrease on the 7th day in the DC group compared to the 0th day, no significance was found. However, a significant decrease was observed on day 14 compared to day 0 ($p = 0.0003$). When the results of the NT and DT groups were evaluated, a dramatic decrease was observed on the 3rd, 7th, and 14th days compared to the 0th day and was statistically significant ($p < 0.0001$).

When comparing the groups on the 3rd, 7th, and 14th days of the healing (Table 1C); On day 3, the NT group showed a significant decrease compared to the NC ($p = 0.0051$). In the DT group, it was found that MMP2 gene expression level was considerably decreased compared to DC and was considered significant ($p < 0.0001$). The expression levels of NC and DC groups, which are the control groups, were found to be significantly increased compared to the NC group in the DC group ($p < 0.0001$). According to the results on the 7th day; there was a significant difference in the expression level of the DT group compared to the DC ($p < 0.0001$). There was no significant effect of metformin on MMP2 expression in the NT group compared to its control. Also, a significant difference was found between the control groups ($p < 0.0001$). Looking at the results of the 14th

Table 1 **A** NF-κB (p65) activation results on the 0, 3, 7, and 14 days of wound healing. RELA **(B)**, MMP2 **(C)**, MMP9 **D** mRNA expression levels on the 0, 3, 7, and 14 days of wound healing

	NC	NT	DC	DT	p value
A)	NF-κB (p65) activity				
Day 0	0,106 ± 0,007		0,159 ± 0,006†		0,0001
Day 3	0,186 ± 0,007	0,071 ± 0,004†	0,4164 ± 0,028†, §	0,1089 ± 0,007†, §, ‡	< 0,0001
Day 7	0,083 ± 0,007	0,063 ± 0,003	0,3031 ± 0,028†, §	0,08,843 ± 0,004‡	< 0,0001
Day 14	0,07 ± 0,002	0,0596 ± 0,001	0,149 ± 0,016†, §	0,084 ± 0,004‡	< 0,0001
B)	RELA mRNA expression levels				
Day 0	1,159 ± 0,098		2,148 ± 0,236†		0,0007
Day 3	2,436 ± 0,253	1,052 ± 0,199†	4,471 ± 0,481†, §	2,257 ± 0,322‡	< 0,0001
Day 7	0,327 ± 0,051	0,232 ± 0,026	3,751 ± 0,259†, §	0,508 ± 0,059‡	< 0,0001
Day 14	0,585 ± 0,085	0,243 ± 0,030	2,998 ± 0,321†, §	0,421 ± 0,071‡	< 0,0001
C)	MMP2 mRNA expression levels				
Day 0	0,240 ± 0,043		0,582 ± 0,104†		0,0124
Day 3	0,333 ± 0,039	0,050 ± 0,013†	0,773 ± 0,093†, §	0,065 ± 0,018†, ‡	< 0,0001
Day 7	0,049 ± 0,014	0,049 ± 0,007	0,393 ± 0,040†, §	0,070 ± 0,021‡	< 0,0001
Day 14	0,024 ± 0,002	0,020 ± 0,004	0,061 ± 0,011†, §	0,020 ± 0,002‡	0,0002
D)	MMP9 mRNA expression levels				
Day 0	1,507 ± 0,121		2,494 ± 0,242†		0,0045
Day 3	2,284 ± 0,485	1,404 ± 0,079	4,064 ± 0,197†, §	1,950 ± 0,401‡	0,0001
Day 7	0,839 ± 0,164	0,477 ± 0,040	2,373 ± 0,373†, §	0,510 ± 0,146‡	< 0,0001
Day 14	1,026 ± 0,151	0,362 ± 0,054†	1,343 ± 0,071§	0,683 ± 0,273‡	0,0023

Results are expressed as mean ± standard error values (Mean ± SEM)

†Compared to NC group

‡Compared to DC group

§Compared to NT group- The level of significance between groups is indicated ($p < 0,05$)

day; the decrease in the DT group was significant compared to the DC ($p = 0.0005$). While no significant change was observed in the NT group compared to the control, a significant difference was again observed between the control groups ($p = 0.0014$).

Effect of metformin on MMP9 mRNA expression level

Within-group evaluation results of MMP9 expression levels compared to days (Fig. 3B); it showed that the increase on day 3 and the decrease on days 7 and 14 did not have a significant effect compared to day 0 in the NC group. However, it was found that the decrease observed on the 7th and 14th days compared to the 3rd day was a significant difference ($p = 0.0066$, $p = 0.0192$, respectively). The decrease in the 7th and 14th days in the NT group was considered to be statistically significant ($p < 0.0001$). Similarly, expression levels on the 7th and 14th days were found to be significantly decreased compared to the 3rd day ($p < 0.0001$). Results of the DC group; It showed that the expression levels on day 3 increased significantly to day 0 ($p = 0.0011$). On the 14th day, it was determined that the decrease observed compared to the 0th day was also significant ($p = 0.0169$). In the DT group; The results of the 7th and 14th days decreased significantly compared to the 0th day ($p = 0.0004$, $p = 0.001$, respectively). The decrease observed on the 7th and 14th days compared to the 3rd day was found to be significant ($p = 0.0083$, $p = 0.0218$; respectively).

According to the evaluation results between groups compared to days (Table 1D); On the 3rd day, the decreased expression level of the DT group compared to the DC was found to be statistically significant ($p = 0.0012$). In the NT group, although there was a significant decrease compared to the control (NC), there was no significant difference. The results of the control groups were found to be significantly increased in the DC group compared to the NC group ($p = 0.0058$). While the MMP9 expression level did not show a significant change in the NT group compared to the NC on the 7th day similar to the 3rd day, it was found that the DT group showed a dramatic decrease compared to the DC ($p < 0.0001$). The difference in the control groups was found that the DC group showed a significant increase compared to the NC ($p = 0.0004$). Expression results on day 14 showed a significant change, showing a decrease (NC) compared to the control of the NT group. It was also found that the DT group showed a statistically significant decrease compared to its control ($p = 0.0427$).

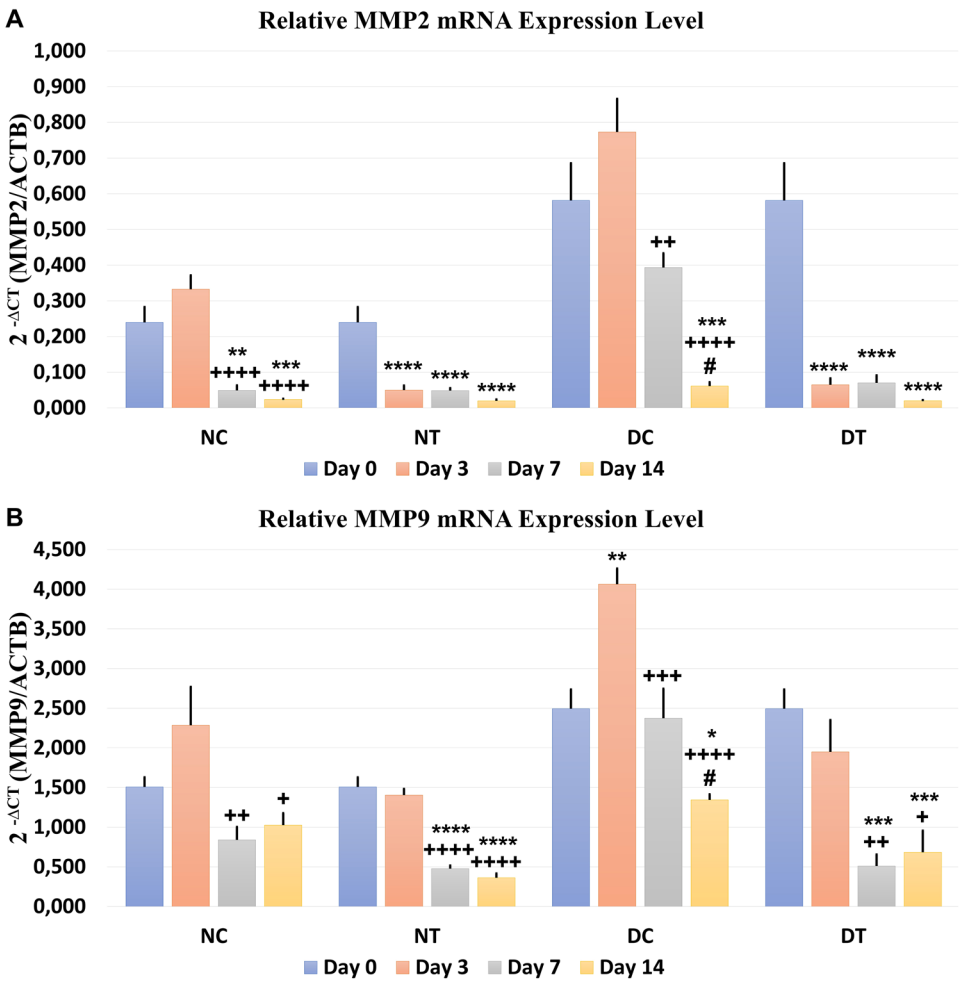

Fig. 3 **A** and **B** Graphical evaluation of the MMP2 and MMP9 mRNA expression levels determined by RT-qPCR method on days 0, 3, 7, and 14 of wound healing. Data shown are the mean ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; compared to day 0. + p < 0.05; + + p < 0.01; + + + p < 0.001; + + + + p < 0.0001; compared to day 3. #p < 0.05; ##p < 0.01; ###p < 0.001; ####p < 0.0001; compared to day 7- within-group significance level is indicated)

Discussion

Hyperglycemia associated with diabetes induces proinflammatory cytokines and an increase in inflammatory cells that disrupt components of the ECM and growth factors necessary for wound healing. As a result, the inflammatory phase of healing is prolonged, granulation tissues cannot develop, and chronic wounds eventually occur. These findings suggest that hyperglycemia is responsible for delayed wound healing process in diabetic patients [22–25]. In present study, we showed that wound healing in STZ diabetic rats was delayed due to hyperglycemia compared to the non-diabetic group.

In vitro and in vivo studies have shown that metformin has a protective effect against oxidative damage induced by hyperglycemia and inhibits the expression of pro-inflammatory cytokines [17–19]. Recently, interest in metformin has been increasing due to its anti-inflammatory properties. It is suggested that metformin achieves its effect on wound healing by changing cytokine and chemokine expression patterns and eliciting some pleiotropic effects [14, 17, 26]. In light of this information, we created a full-thickness excisional wound model in STZ diabetic and non-diabetic rats, and applied 3 mM metformin topically on the wounds for 14 days. It was observed that the improvement in metformin treatment groups was statistically significantly increased. Our striking finding was that diabetic wounds improved in a shorter time with metformin administration, even than the control group. We demonstrated an accelerated wound healing significantly at day 7 and day 14, while there was an acceleration in wound healing from day 3 compared to controls in the diabetic group treated with metformin (Fig. 1A, B). We found that metformin treatment significantly decreased wound diameters in both diabetic and non-diabetic groups. In Lee et al.'s studies, metformin was shown to cause an 85% versus 67% wound closure at day 7 and an 85% versus 97% wound closure at day 14 compared to control wounds [15]. This study supports our findings.

In the study of Qing et al., topically applied Metformin (2 mM) treatment in a pluronic gel formulation in wounds created in diabetic rats was shown to accelerate the healing of excisional wounds in rat skin compared to the control group [27]. In another study, the effects of locally applied metformin on wound healing in elderly skin were investigated. Regarding cutaneous wound healing rates, metformin

significantly accelerated wound healing in aged rats [18]. Although there are few studies showing the potential effect of metformin on wound healing, the data are consistent with the data we obtained as a result of our study. Consistent with our study with STZ diabetic rats, studies show that metformin leads to faster wound healing [15, 18, 28–32]. In addition to these studies, it is shown that administered orally metformin to show its effect accelerates wound healing in different diabetes models compared to diabetic control groups, but when metformin is applied topically on the wound, it is more effective than oral use [28, 33].

Hyperglycemia activates various transcription factors. NF-κB, is a biomarker of inflammation, and can lead to chronic inflammation and hence tissue damage. In this case, it is clear that NF-κB signals have directly critical importance in cutaneous wound healing. The low level of NF-κB in the diabetic wound can help prevent progression to systemic inflammation that initiates the development of diabetic complications [34–37]. In our study, we investigated the NF-κB p65 DNA binding activity and RELA gene expression to show whether it is related to the delays in wound healing seen in STZ-induced diabetes. NF-κB p65 activity was found to be significantly higher on the 0.3, 7 and 14 days in wounds with diabetes compared to the wounds in the non-diabetic group. As a result of metformin treatment, it was observed that NF-κB p65 activity decreased statistically significantly in both groups compared to controls (on day 3: $p < 0.0001$ (NC/NT), $p < 0.0001$ (DC/DT); on day 7: $p < 0.0001$ (DC/DT); on day 14: $p < 0.0001$ (DC/DT)). The NF-κB p65 activity that significantly increased on the 3rd day in the NC group and the 3rd and 7th day in the DC group was quite low in the treatment groups. The significant decrease not only by days but also within the group compared to the starting day every 3 days is noteworthy. Moreover, the activity values in the DT group were even lower than the NC group on the 3rd day, almost the same as the spontaneous wound healing on days 7 and 14. After metformin treatment in RELA gene expression, a significant decrease was observed in the NT group on the 3rd day compared to the first day, while a statistically significant decrease was observed in the DT group on the 7th and 14th days. Such that the expression level of the DT group on the 3rd day was lower than the RELA expression level in the NC group. These results suggest that treatment with metformin resulted in a faster improvement in the diabetic treatment group not only compared to the control but even than the normal control group, which mimics spontaneous healing. In the study conducted by Cam et al., the NF-κB level was significantly lower in the treatment groups where metformin was administered in groups, compared to the control and, showed better anti-inflammatory effects than the other groups [31]. In addition, in the study conducted and in recent studies using different active substances in line with our study, it has been shown that these substances reduce inflammation and accelerate wound healing via an NF-κB inhibitor, as in the use of metformin [31, 36–38].

NF-κB also regulates the expression of MMPs, it has been reported that NF-κB is one of the main components of the intracellular signalling pathways specifically responsible for MMP-2 and MMP-9 [39, 40]. Studies have confirmed that uncontrolled MMP activity causes tissue damage and functional changes, and that increased MMP levels (especially MMP-2 and MMP-9) lead to negative effects on wound healing [9, 41, 42]. Topical application of MMP inhibitors accelerate diabetic wound healing, reduces inflammation, increases fibroblast tissue formation [10, 43]. In our study, as a result of topical metformin administration in diabetic and non-diabetic groups, a significant decrease was observed in MMP2 mRNA expression levels on all days and, in MMP9 mRNA expression levels on the 7th and 14th days compared to the first day. In light of this information, MMPs are important mediators regardless of acute or chronic wounds. Thus, MMP levels are critical biomarkers when analyzing the therapeutic efficacy of wound healing processes. There are many studies specifically aimed at inhibiting MMPs [44, 45]. These results are in line with our results.

The healing effect of topically applied metformin in a full-thickness excisional wound model created on both diabetic rats and non-diabetic rats is the first study in the world that we present to what extent MMP2, MMP9 and RELA expression changes relative to NF-κB activity. Therefore, since there is no study showing that the topical application of metformin affects NF-κB and MMPs together, studies, where different substances were applied, were compared. According to Singh et al. study, the icariin molecule was tested on a wound model created in rats. The results show that NF-κB protein expression is decreased in tissue samples taken from wounds, especially in the icariin-treated groups compared to the control group, and also, it decreases both MMP-2 and MMP-9 activities. Thus, icariin is thought to cause the acceleration of wound healing compared to control [46]. In the study investigating the effects of topically applied mangiferin molecule on excisional wound model in rats with type 2 diabetes model, it was shown that NF-κB p65 protein level was lower and MMP expression was decreased after mangiferin treatment. This suggests that it was sped up the wound healing process [47]. In the cutaneous wound model created by Durmuş et al., as a result of topical application of arginine silicate inositol, the amount of both MMP-2 and MMP-9 gradually decreased on the 5th, 10th, and 15th days, and in parallel, the amount of NF-κB was significantly decreased, hence, they demonstrated that wound healing was accelerated accordingly [48]. A study parallel with our study shows that topical application of syringic acid effectively enhances the wound healing process in diabetic rats. According to the data of the current study;

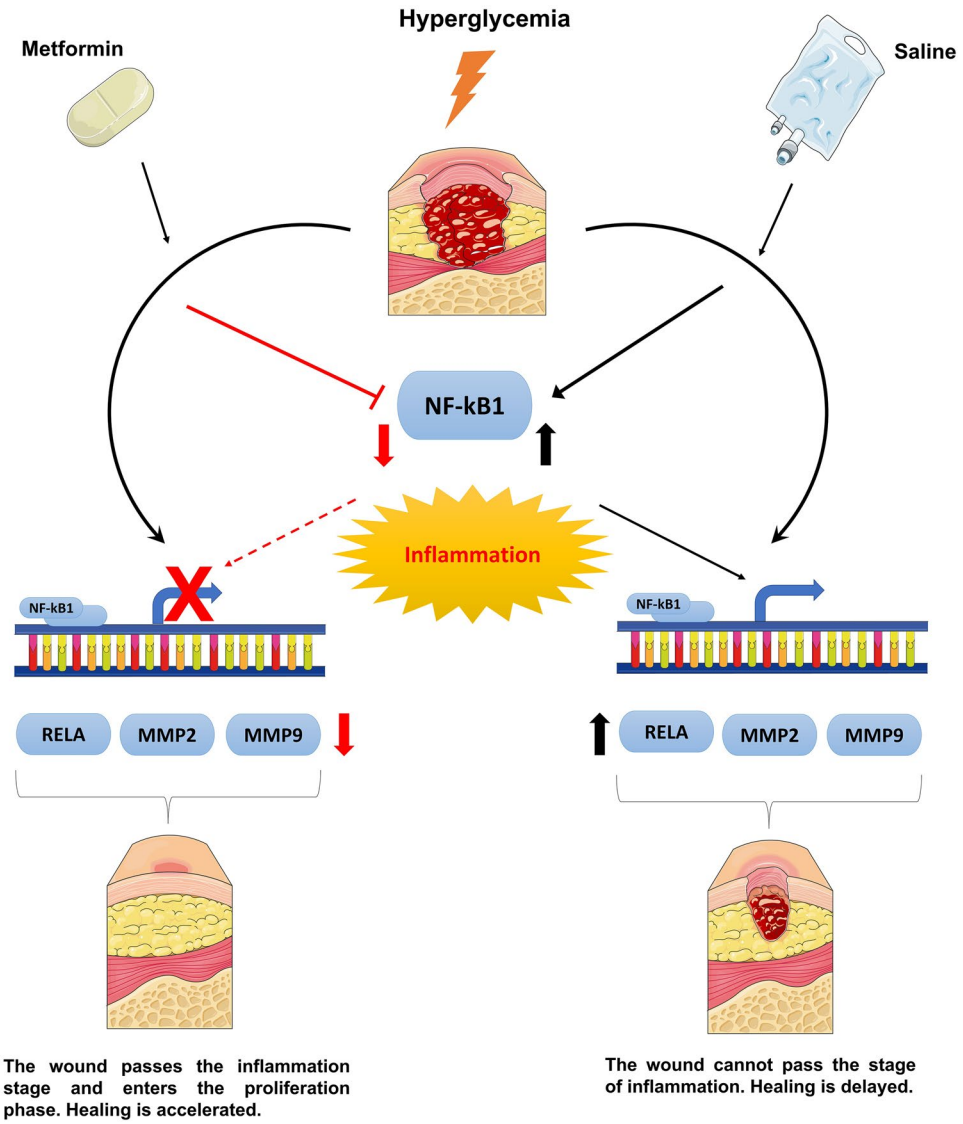

Fig. 4 Topically applied metformin in the treatment of non-healing or delayed-healing wounds because of hyperglycemia affects transcriptional regulation by decreasing NF-kB p65 activity. This leads to decreased expression levels of RELA, MMP2 and MMP9. Thus, it has positive effects on wound healing by passing the inflammation phase, which is a critical stage for wound healing, and accelerating the transition to the proliferation phase

showed that following administration of syringic acid to diabetic wounds for 14 days, decreased NF-κB p65 activity and levels of MMP-2, MMP-9 mRNA expression compared to control [49].

In line with the results of our study; metformin accelerates wound healing by suppressing NF-κB activation and inflammatory response to improve the wound healing process in hyperglycemic rats (Fig. 4). This may be related to anti-inflammatory properties (reduction of NF-κB p65 activity) and inhibition of MMPs (down-regulation in MMP-2 and MMP-9 mRNA). As a result; although the topical application of metformin on wounds is much more effective in diabetic wounds, we have also shown that non-diabetic wounds increase wound healing rate by inhibiting NF-κB p65 activity and decreasing RELA mRNA expression level, and in addition, reducing MMP2 and MMP9 expression levels (Table 2). In the future, used topically metformin may be an effective, inexpensive and reliable potential therapeutic agent applied not only to diabetic wounds but also to all wounds.

Table 2 Showing the levels of the parameters studied according to the physiological phases of wound healing (According to the baseline levels)

Days	Groups	Parameters			
		p65 Actıvıty	RELA	MMP2	MMP9
Day 3	NC	↑	↑	↑	↑
	NT	↓	↔	↓	↔
	DC	↑	↑	↑	↑
	DT	↓	↔	↓	↓
Day 7	NC	↓	↓	↓	↓
	NT	↓	↓	↓	↓
	DC	↑	↑	↓	↔
	DT	↓	↓	↓	↓
Day 14	NC	↓	↓	↓	↓
	NT	↓	↓	↓	↓
	DC	↔	↑	↓	↓
	DT	↓	↓	↓	↓

Authors' contributions FKT and GKS designed the research study concept. FKT, ZTS, OH carried out the animal experiments. Laboratory analyzes were studied by FKT. TU and SO participated in the design. FKT, GKS interpreted the data and drafted the final version of the manuscript. All authors have read and approved the final manuscript.

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Data availability The datasets analysed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interests.

Ethical approval This study was approved by the Laboratory Animals Local Ethics Committee of Bezmialem University (No: 2019/216). The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

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