Frontiers in Life Sciences and Related Technologies 5(1) (2024) 65-73

Contents lists available at Dergipark

Frontiers in Life Sciences and Related Technologies

Journal homepage: http://dergipark.org.tr/en/pub/flsrt

Research article

Effects of keratin6/16 heterodimer on diabetic wound healing treatment with topical metformin

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Abstract

Diabetes is an important public health problem, and it is well known that healing processes are impaired in diabetic wounds as one of its complications. Keratins are structural proteins found in skin cells and play a vital role in wound healing and skin integrity. While there is increasing interest in the anti-inflammatory properties of metformin, a drug commonly used for diabetes, its potential effect on wound healing and keratins is not yet fully understood. In this context, it was aimed to evaluate how metformin administration affects keratin 6 and keratin 16 expression at both mRNA and protein levels. In this study conducted on diabetic rats, the effects of topically applied metformin on keratins in wound healing were investigated. Then, protein and mRNA expression levels of keratin 6 and keratin 16 in treated wounds were compared with untreated wounds using reverse transcription polymerase chain reaction and immunohistochemistry methods. The results of the study are likely to detail changes in the expression levels of keratin 6 and keratin 16 after metformin administration. This information will shed light on how metformin affects the molecular mechanisms involved in wound healing, particularly concerning these important structural proteins. Understanding these changes may provide insight into potential therapeutic approaches to improve diabetic wound healing. By elucidating the effect of metformin on keratin expression, the study may contribute to the development of targeted therapies aimed at improving the healing process in diabetic wounds.

Keywords: *Diabetes mellitus; diabetic wound model; keratin; metformin; K6/16*

1. Introduction

Keratins that compose the major intermediate filament cytoskeleton of the epidermis are classified as type I (K9-K40) or type II (K1-K8, K70). Keratins of both types are generated by activated keratinocytes and are always co-expressed as a heterodimer between a type I and a type II keratin (Franke et al., 1981; Moll et al., 1982; Hatzfeld and Weber, 1990; Oshima, 2002; Schweizer et al., 2006; Herrman et al., 2009 Jacob et al., 2018). Keratins, which are important for organized keratinocyte proliferation and epithelial integrity, are said to be engaged in intracellular signaling pathways such as stress protection, apoptosis, and wound healing (Pan et al., 2013; Mayet et al., 2014).

Wound healing occurs in four overlapping stages: coagulation and haemostasis, inflammation, proliferation, and tissue remodelling. Keratinocytes have two roles in wound healing: they fill the wound site by proliferation and migration, and they build the epidermal layer through maturation and differentiation (Velnar et al., 2009; Mayet et al., 2014; Pastar et al., 2014). During the proliferation phase, epidermal keratinocytes re-epithelialize the wound, which is an important stage in wound healing. Promoting keratinocyte proliferation is therefore an important step in enhancing the skin wound healing process. Keratinocytes at the wound front travel across the underlying granulation tissue during re-epithelialization, finally meeting keratinocytes moving from the other edge to heal the defect (Bellavia et al., 2014). Migrating keratinocytes generate

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https://doi.org/10.51753/flsrt.1399275 Author contributions
Received 02 December 2023; Accepted 19 March 2024
Available online 30 April 2024

more K6, K16, and K17 keratins, which are hypothesized to increase migrating cells' viscoelastic properties and are regulated by growth factors found in the wound environment. Because various keratins play diverse functions in the wound-healing process, their expression changes depending on the stage of wound healing (Freedberg et al., 2001; Wong and Coulombe, 2003; Shawandi et al., 2017). Keratinocytes move from wound borders, multiply, and release cytokines to trigger tissue response in acute wounds. However, decreased wound healing, such as that seen in diabetes mellitus (DM), can result in chronic wounds in which keratinocytes fail to migrate, leaving this stage of wound repair incomplete (Krzyszczyk et al., 2018).

DM impairs immune response capability, including immune cell function reduction, and is a serious public health issue (Khanra et al., 2015). Hyperglycaemia impairs re-epithelialization processes such as protein synthesis, migration, and proliferation of keratinocytes and fibroblasts (Park et al., 2011; Andrade et al., 2017; Lima et al., 2017; Kim et al., 2018). The expression of key keratinocyte proteins involved in re-epithelialization, including cytoskeletal keratin proteins (K2, K6, and K10), which are critical for keratinocyte differentiation, is disrupted in diabetic foot ulcer patients (Blakytny and Jude, 2009).

Derived from galegin, a natural product derived from the *Galega officinalis* plant used in herbal medicine, metformin is not designed to target a specific pathway or disease mechanism. However, although it was subsequently accepted as the first-choice drug in the treatment of Type II DM worldwide, its molecular mechanisms of action are still debated (Howlett and Bailey, 2007; Graham et al., 2011). Studies on wound healing and diabetic wounds involve many complex factors, such as various growth factors, cell signalling, inflammatory process, and tissue regeneration. The specific role of K6/16 in these processes is not fully understood and information in the existing literature is limited. Furthermore, studies on the effects of metformin on K6 and K16 specifically in the context of wound healing are lacking. The treatment and healing process of diabetic wounds is often a result of multiple factors, and the effects of drugs are complex, so more scientific studies are needed to have a clear understanding of the effects of metformin on these keratin proteins. In this context, metformin was applied, the most commonly used antidiabetic agent today, topically on the wounds by creating a diabetic wound model and aimed to investigate its effects on wound healing on K6 and K16.

2. Materials and methods

2.1. Animal study

For this study, an application was made to Bezmialem Vakıf University Animal Experiments Local Ethics Committee, and it was approved with the decision numbered 2020/15. In the study, it was used 24 adult male Wistar Hannover albino rats (300 g ± 20 g, LD; 12:12). During the trial, all rats were housed in separate cages. These rats were separated into two groups: diabetic and non-diabetic rats, and then divided each group into four groups: metformin-treated and saline-applied groups, with six rats randomly assigned to each. At the beginning of the study, all animals' blood glucose levels and body weights were measured. A single intraperitoneal dose of 60 mg/kg Streptozotocin (STZ) was injected to induce diabetes, while the non-diabetic groups were injected with saline only. The glucose levels were examined in blood samples collected from the rats' tail veins at least 72 hours later. Rats with values greater than 250 mg/dL were termed diabetic, whereas rats with values less than this cut off were excluded from the experiment.

The metformin solution was prepared freshly every day at a concentration of 3 mM. It was filtered before each application and then applied it to the wound. Before each operation, rats were anesthetized with pental sodium. The dorsal region of each anaesthetized rat was shaved and made ready for the operation by providing aseptic conditions. In the dorsal region of each animal in the experimental groups, 3 circular full-thickness excisional wound models were created with a 12 mm diameter sterile punch. Based on the midline in the dorsal region of the animal, the location of the wounds was determined by placing the first wound on the upper left, the second wound on the upper right and the third wound in the middle and below both wounds opened on the top. This first procedure was accepted as day 0 and the experimental period was started. According to the stages of wound healing physiology, other biopsy days were planned as day 3 for the inflammation stage, day 7 for the proliferation

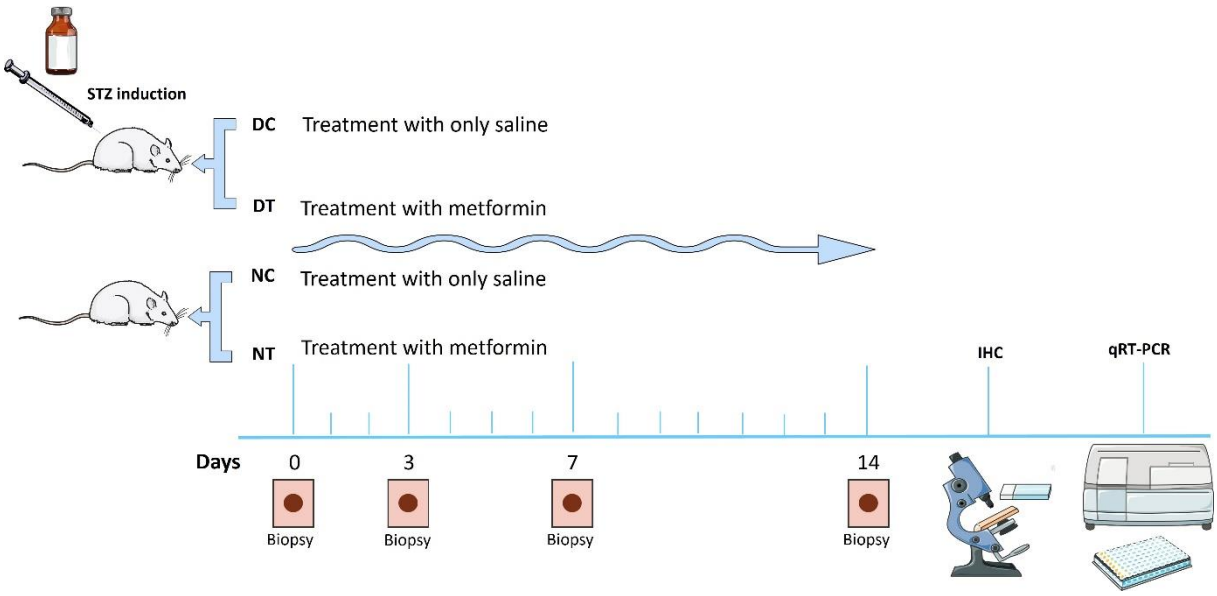

Fig. 1. The animal study is diagrammed on the figure.

stage, and day 14 for the remodelisation stage. The upper left scar tissue was removed on the 3rd day, the upper right scar tissue on the 7th day and the scar tissue in the lower and middle line of the two wounds on the 14th day under anaesthesia and under sterile conditions. On day 14, after taking the last wound biopsy, the experiment was terminated without any treatment and sacrificed all rats under high-dose anaesthesia. Every day, sterile saline was administered to the control wounds and a newly made 3 mM metformin solution to the treatment wounds. By absorbing sterile surgical sponges, both applications were supplied. the wound area was covered with a single layer of gauze to prevent any infection or physical manipulation and continued to apply the treatment at the same time every day for 14 days. The animal study is diagrammed in Fig. 1.

2.2. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

At the end of the 0th, 3rd, 7th and 14th days, wound biopsy tissues of the rats in the treated group and the control group were surgically removed under anaesthesia. Wound biopsy tissues taken using nuclease-free microtubes and zirconium beads were lysed in lysis solution by a homogenizer. The purity and quantity of total RNA extracted with a ready-to-use kit were then determined in NanoDrop Microvolume Spectrophotometer. The change in the quantity of *KRT6A* and *KRT16* mRNA was quantified using the SYBR green reverse transcription real-time polymerase chain reaction method (Table 1). The *G6PD* gene was used as a reference gene to precisely determine the mRNA levels. To check for contamination, the reaction was always run with a negative control. The system calculated the relative mRNA expression levels of *KRT6A* and *KRT16* and compared them to *G6PD*.

Table 1
Primers of genes *KRT6A*, *KRT16* and *G6PD* for performing qRT-PCR.

Gene Name (*Symbol*)	Primer Sequence (5'→ 3')
Keratin 6A (*KRT6A*)	F: ATGTGTGGGAACCATCCTG R: TCCCTCAGGAAGAGGAAATG
Keratin 16 (*KRT16*)	F: GCAGAGCCAGGAGTACAACA R: GAATAGGACTGCCCAGAGGA
Glucose 6-phosphate dehydrogenase (*G6PD*)	F: ACATCCGCAAACAGAGTGAG R: GCTGTTGAGGTGCTTGTAGG

2.3. Immunohistochemistry (IHC)

Following each operation, the tissues were fixed in a 10% neutral formalin fixative before utilizing standard light microscopy follow-up techniques to embed the tissues in paraffin. 4-5 mm-thick paraffin sections were taken using a microtome and placed on positively charged slides for immunohistochemistry. The tissue sections that had undergone standard procedures for deparaffinization, rehydration, and removal of antigenic masking shortly prior to immunostaining were then subjected to immunohistochemical staining. Using the pre-made kit (Rabbit specific HRP/DAB Detection IHC Detection Kit-Abcam), the immunohistochemical staining was carried out using rabbit polyclonal anti-cytokeratin 6 and anti-cytokeratin 16 primary antibodies to identify tissue antigens. Light microscopy was used to do semi-quantitative immunoreaction measurements in 10 randomly chosen locations at a 40X magnification. It was evaluated semi-quantitatively by accepting -:0, +:1, ++:2, and +++:3 according to the severity of immunoreactivity (Niu et al., 2014).

2.4. Statistical evaluation

The Graphpad Prism 8.0.2 package program was used to analyse the data acquired after the study. Differences between groups were assessed using the ANOVA test, and pairwise comparisons were done using the Tukey test for significant results. On day 0, the t-test was used to compare the non-diabetic and diabetic groups in pairs. As descriptive statistics, the mean standard error value was provided. $P<0.05$ was regarded as statistically significant.

3. Results

3.1. Demonstration of KRT6A expression by qRT-PCR

Pairwise comparisons were done using the t-test for each parameter and the effects of diabetes were presented as a result of analyses made on tissue samples collected from the normal groups (N0) and STZ-induced diabetic groups (D0) on the day of the initial wound (day 0). The qRT-PCR method was used to examine *KRT6A* mRNA expression levels in diabetic and non-diabetic control and treatment groups. The diabetic group had a statistically significant rise in *KRT6A* mRNA levels when compared to the non-diabetic group, according to the data ($p=0.0058$).

According to these data (Fig. 2A), when compared to the first day of treatment, there was a considerable rise on the 14th day in the non-diabetic control (NC) group. ($p=0.0199$). In the non-diabetic treatment (NT) group, wound healing improved significantly on all three treatment days as compared to the first day (respectively; $p=0.0406$, $p=0.031$, $p=0.0163$). On treatment days, there was no significant difference in the diabetic control (DC) group compared to day 0. In the diabetic treatment (DT) group, however, there was a considerable increase on the seventh day as compared to the initial day ($p=0.0392$).

3.2. Demonstration of KRT16 expression by qRT-PCR

Pairwise comparisons were done using the t-test for each parameter and the effects of diabetes were presented as a result of analyses made on tissue samples collected from the normal groups and STZ-induced diabetic groups on the day of the initial wound (day 0). According to the resulting data, it was determined that there was a statistically significant increase in *KRT16* mRNA level in the diabetic group compared to the healthy group ($p=0.0392$).

Considering these findings (Fig. 2B), the NT group showed a substantial increase on the seventh day of wound healing compared to day 0 ($p=0.0027$). There was no significant difference in the NC, DC, and DT groups on treatment days compared to the first day.

3.3. Evaluation of keratin 6 expression by IHC

Pairwise comparisons were done using the t-test for each parameter and the effects of diabetes were presented as a result of analyses made on tissue samples collected from the normal groups and STZ-induced diabetic groups on the day of the initial wound (day 0). The IHC method was used to examine the levels of K6 protein expression in all groups. According to the results, a statistically significant increase in immunopositivity was

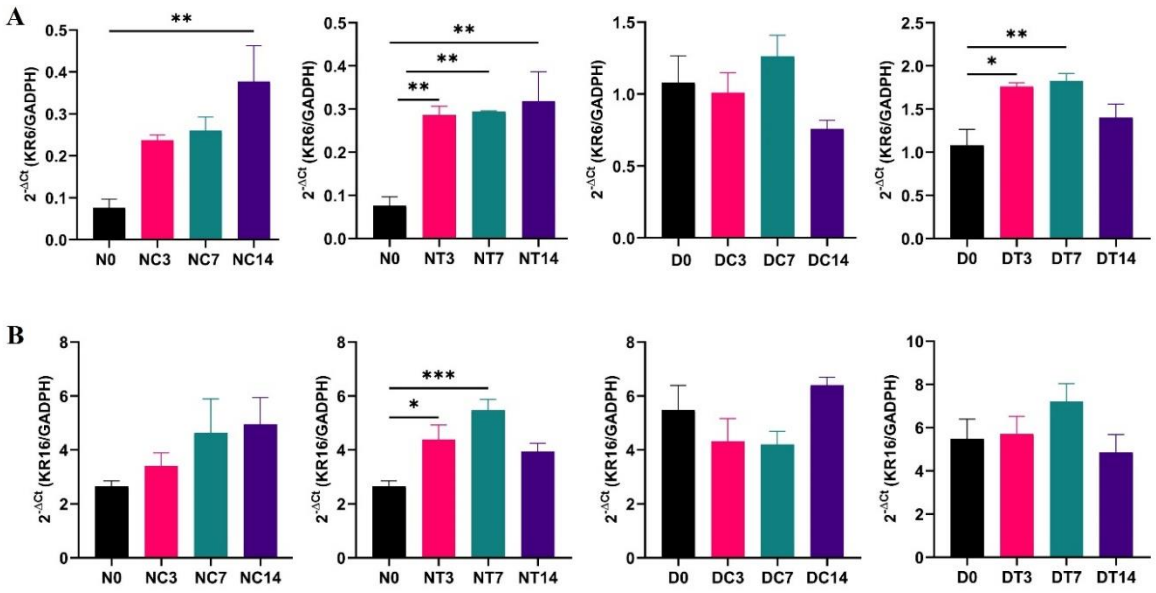

Fig. 2. KERATIN6A (A) and KERATIN16 (B) relative mRNA levels were measured using RT-qPCR for every group. All data were compared to the baseline of day 0. *p<0.05, **p<0.01, ***p<0.001. On the first day after wound opening, the groups were non-diabetic (N0), diabetic (D0); The groups are as follows: non-diabetic treatment (NT), non-diabetic control (NC), diabetic control (DC), and diabetic treatment (DT).

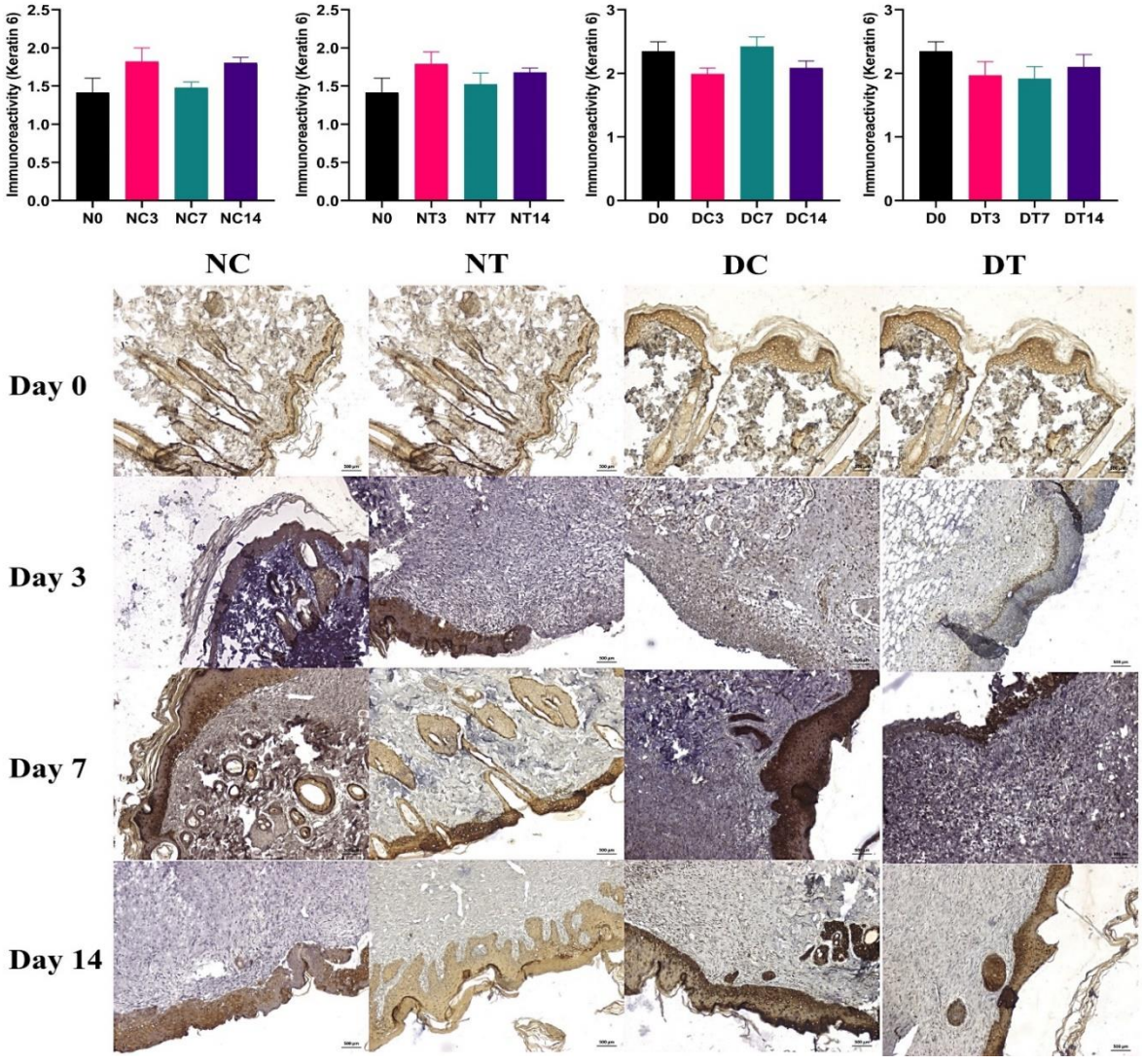

Fig. 3. Graphical representation of Keratin 6 immunoreactivity and microscopic images of wound tissue preparations after IHC staining in all control and treatment groups. Type II IF Keratin 6 was labelled in brown in the wound tissue preparations, and nuclei were labelled in blue with Mayer's haematoxylin.

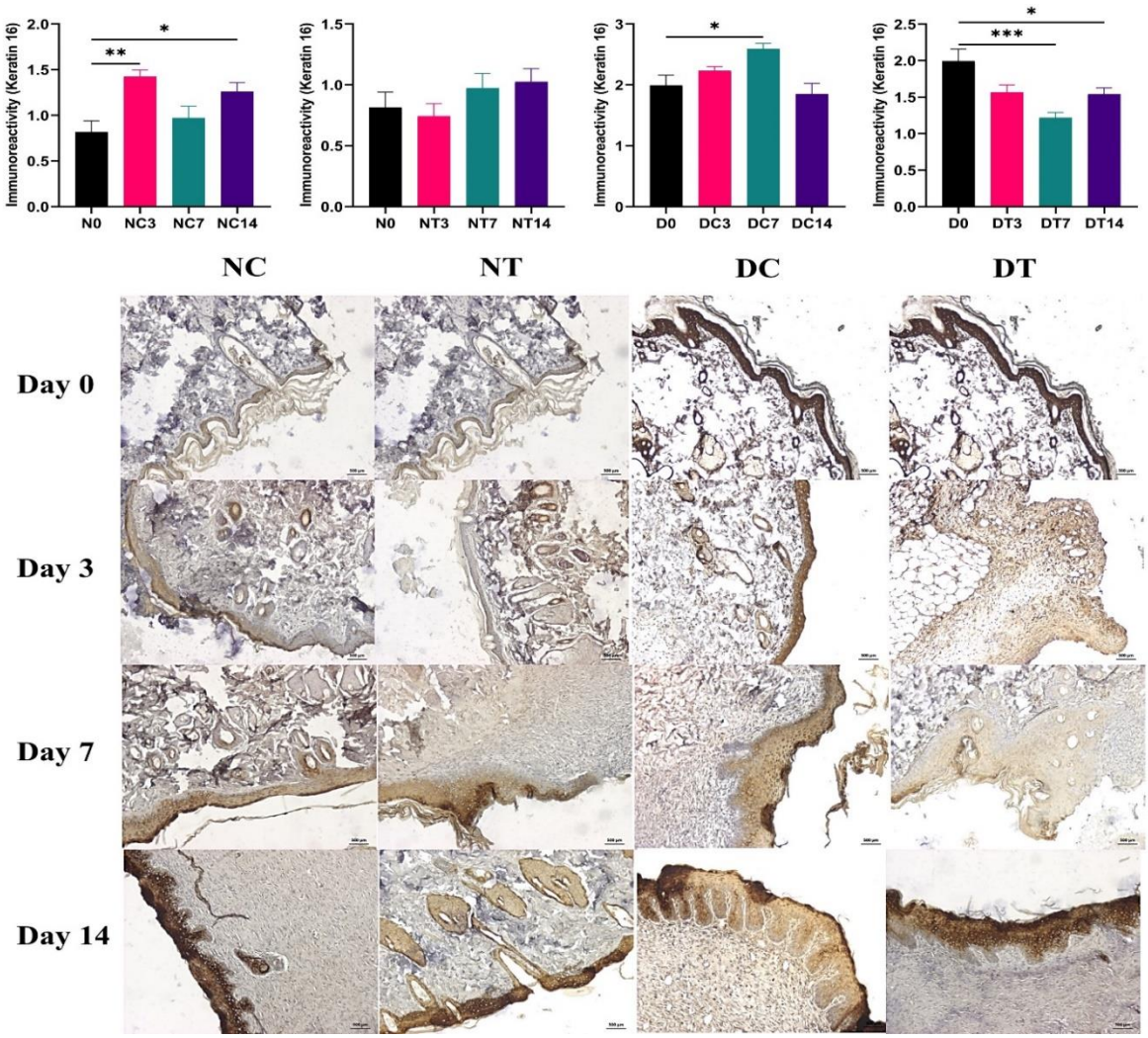

Fig. 4. Graphical representation of Keratin 16 immunoreactivity and microscopic images of wound tissue preparations after IHC staining for every group. Type I IF Keratin 16 was labelled in brown in the wound tissue preparations, and nuclei were labelled in blue with Mayer's haematoxylin. *p<0.05, **p<0.01, ***p<0.001.

detected in the diabetic group compared to the normal group in the assessment of K6 immunohistochemical staining (p=0.0044). According to these findings (Fig. 3); When intragroup comparison was made according to days in all four groups, no significant difference was seen compared to day 0.

3.4. Evaluation of keratin 16 expression by IHC

Pairwise comparisons were done using the t-test for each parameter and the effects of diabetes were presented as a result of analyses made on tissue samples collected from the normal groups and STZ-induced diabetic groups on the day of the initial wound (day 0). The IHC method was used to examine the levels of K16 protein expression in diabetic and non-diabetic control and treatment groups. According to the findings, a statistically significant increase in immunopositivity was identified in the diabetic group compared to the normal group in the assessment of K16 immunohistochemical staining (p=0.0004).

According to these data (Fig. 4), a substantial increase was seen in the NC group on the third and fourteenth days as compared to the initial day. (respectively; p=0.0051, p=0.0451). Analysing intragroup comparisons based on days compared to the first day showed that there was no significant difference in the NT group. While the DC group had a substantial increase on the seventh treatment day compared to the first day, the DT group saw a significant decline on the seventh day (respectively; p=0.0253, p=0.0021).

4. Discussion

The function of the skin epidermis, which protects our bodies from environmental pathogens and attacks by forming a physical and immunological barrier, is highly dependent on the presence and integrity of the keratin network, the epidermis' dominant cell type. In keratinocytes, keratins are the most prominent cytoskeletal proteins (Schweizer et al., 2006; Raja et al., 2007; Jacob et al., 2018). When the skin is physically injured, resident keratinocytes, fibroblasts, and other inflammatory immune cells contribute to a cellular and molecular wound-healing process that includes carefully regulated stages of haemostasis, inflammation, proliferation, and remodelling. Keratinocytes are rapidly activated in response to damage and create a range of "alarmins" to guard against a danger signal. Wound proximal keratinocytes momentarily delay terminal differentiation during wound healing to prepare for active migration and proliferation (Martin, 1997; Wikramanayake et al., 2014). Keratinocytes migrate and multiply from wound edges in acute wounds, but in delayed wound healing, such as DM, the inability of keratinocytes to migrate may contribute to the establishment of chronic wounds and delay wound recovery

(Krzyszczyk et al., 2018). Keratinocytes on the borders of chronic wounds vary from those on the edges of intact epidermis or acute wounds. In contrast to normal skin, which only has mitotically active keratinocytes at the basal layer, keratinocytes in chronic wounds divide along the suprabasal layers (Stojadinovic et al., 2005).

Under homeostatic conditions, suprabasal keratinocytes in the intact epidermis express differentiation-specific keratins K1 and K10, whereas the interfollicular epidermis does not normally express K6, 16, or K17, even though these keratins are considered barrier alarmins that are rapidly induced in keratinocytes upon wounding. Suprabasal keratinocytes, on the other hand, quickly downregulate K1/K10 expression after damage. Transient wound-specific keratins Type II IFs K6a/K6b isoforms are rapidly and significantly elevated in stressed keratinocytes in the suprabasal layers of the epidermis within hours after injury (Usui et al., 2005; Patel et al., 2006; Savatin et al., 2014; Gravino et al., 2017). This induction occurs mostly in the postmitotic compartment of the wound edge epidermis, making it easier to stimulate proliferation rather than differentiation (Paladini et al., 1996; McGowan and Coulombe, 1998; Takahashi et al., 1998; Hobbs et al., 2012). K6, 16, and 17 expression is maintained during epithelial remodelling until barrier function is restored, demonstrating that keratins play critical physiological roles during healing.

Furthermore, K16/17-K6 expression in keratinocytes suggests a highly active and proliferative phase in pathological conditions (Sun et al., 1983; Mansbridge and Knapp, 1987; Takahashi et al., 1998; Koch and Roop, 2004 Zhang, 2018). These keratin couples form a flexible scale that allows keratinocytes to tolerate physical stress and govern a range of activities, including apoptosis protection and immunological homeostasis management. Thus, activation of these genes signifies keratinocyte hyper-proliferation in response to injury or inflammation, and they have been widely used as wound-activated keratinocyte indicators in both human and mouse skin (Paladini et al., 1996; Wong and Coulombe, 2003). Following this knowledge, metformin was used as a topical therapy on the model of a full-thickness excisional wound that it was generated in STZ-induced diabetic and non-diabetic rats. K6 and K16 mRNA and protein expression levels were evaluated in wound biopsy samples collected on days 0, 3, 7, and 14. When hyperglycaemia, a clinical indication in DM that is considered to be an inflammatory condition, was compared to the non-diabetic group, K6/16 levels were found to be considerably higher in wound biopsy samples obtained on day 0. This finding suggests that these two forms of keratin, also known as alarmin, might be used as a biomarker in diabetic wounds.

The induction of K16 by interfollicular keratinocytes is a critical response to epidermal injury. K16 promotes the rearrangement of cytoplasmic keratin filaments at a wound site. Moreover, it is stated that the induction and increased amount of K16 protein helps the keratinocyte activation process and therefore has a positive effect on epithelialization (Paladini et al., 1996). However, contrary to these data, a study conducted with transgenic mice showed that high K16 protein levels had the opposite effect, thus high K16 protein levels partially impaired keratinocyte migration, and K16 overexpression delayed the closure of full-thickness skin wounds *in vivo* (Wawersik et al., 2001). The result of the same study group's study in mouse keratinocytes revealed that forced expression of K16 caused a decrease in cell adhesion while not changing cell proliferation (Wawersik and Coulombe, 2000). Whey protein therapy was used for full-thickness excisional wounds generated in STZ-induced diabetic rats in a study shared similarities with this study. As a result of the therapy, K16 reactivity was found to be low and intense in diabetic-injured and diabetic-injured treated animals on the fourth day, whereas it was moderate in both normal-injured untreated and normal-injured treated groups. On the eighth day, they have reported that the normal injured group has low reactivity, the treated normal injured group has nil reactivity, the diabetes injured group has medium reactivity, and the treated diabetic injured group has low reactivity (Ahmed et al., 2015). Data from the same study, on the other hand, also report that experimentally tested diabetic wounds are unable to properly regulate the healing phases when K16 expression is delayed to day 8. This study showed that after topical metformin treatment was applied to the wounds, the K16 protein level in the non-diabetic (healthy) and diabetic groups was lower than in the controls. The rise on the third day in the untreated non-diabetic group and the seventh day in the diabetic group was linked to a delay in wound healing. These findings can be explained by the fact that K16 activation was turned on earlier in the therapy. Alternatively, metformin may be able to manage the appropriate time of K16 activation by regulating it, particularly in diabetic wounds. Especially in the diabetic treatment group, the decrease compared to day 0 and the fact that the protein level is parallel to normal wound healing on day 3 suggest that these proteins are expressed at earlier stages and initiate the molecular cascade for wound healing at an earlier stage.

K16 gene expression results on the 7th day were higher in the treated groups compared to the controls, at the protein level. However, considering the gene expression level results within the group, it is concluded that based on the standard wound healing process, metformin induces gene expression until the 7th day, but this level decreases on the 14th day, and there is an irregular expression in untreated diabetic wounds, which negatively affects wound healing. In this regard, a clinical investigation on adult participants used coarse-grit microdermabrasion, discovered that 6 and 24 hours after the treatment, there was an 11-fold increase in K16 gene expression. While K16 was not originally detected, it was discovered in the suprabasal layers of the epidermis 24 and 48 hours after treatment, and samples with increased K16 expression also had the largest increase in type I collagen expression (Karimipour et al., 2009). When looking at the profile of K6, which forms a heterodimer with K16, previous studies also show that complex results are obtained. K6a does not play a substantial function in keratinocyte proliferation or migration, although it may play a role in keratinocyte activation following damage, according to a study in knockout mice (Wojcik et al., 2000). Another study employing an *ex vivo* skin explant culture model to highlight the significance of K6 loss during wound healing discovered that despite normal *KRT6A* mRNA levels in the *KRT6A/KRT6B* null skin segment, there was a lower concentration of K16, resulting in K16 forming K6/16 heteropolymers. They discovered that K6 is essential for keratinocyte stability and that these proteins are required to improve keratinocyte wound epithelialization potential (Wong and Coulombe, 2003).

Moreover, the role of keratins is very critical not only in skin defects caused by diabetes but also in skin damage induced by many different factors (Baek et al., 2024; Blumer, 2024; Majid, 2024). For instance, in clinical studies on Pachyonychiacongenita syndrome, which is an autosomal dominant disease and has a prevalence of one in a million

worldwide, they showed that there is a mutation in keratin 6/16 proteins and that the disease exhibits a serious condition in its course (Cheng et al., 2023; Chu et al., 2023). Recent studies also demonstrate the significant role of keratin 6/16 in wound healing (Ghatak et al., 2023; Groh and Magin, 2023; Cohen et al., 2024; Michalak-Micka et al., 2024). One of these studies showed that the extract of *Tarantula cubensis*, a spider species, increased cytokeratin, and collagen in both epithelial and connective tissue in rats with buccal mucosa lesions and had a significant healing effect (Simsek and Ozmen, 2024). Studies show not only the change in the amount of keratin in the wound as a result of the applied treatment, but also the angiogenic, proliferative, and antimicrobial effects of keratin-based treatment approaches (Ramey-Ward et al., 2023; Sun et al., 2023; Tavakoli et al., 2023; Sellappan et al., 2024; Sun et al., 2024; Winkfield et al., 2024).

These findings demonstrate that there is no significant difference in protein quantity across groups when comparing K6, however, the diabetic group exhibited an increase compared to the non-diabetic group on the 7th day. While the gene expression levels in the diabetic control group were irregular, it was found that metformin administration induced gene expression relative to day 0 in both the non-diabetic and treatment groups. These findings show that the K6/16 keratin pair is necessary to maintain the flexibility required to endure the rigors of a wound site at the expense of a delay in epithelialization, and they highlight the keratins' involvement in collective cell migration.

5. Conclusion

DM is a chronic metabolic condition that can interfere with wound healing owing to malfunctioning of the many cell types involved. Diabetic patients' wounds typically heal more slowly. Keratins are structural proteins present in the skin that play a crucial function in tissue integrity. K6 and K16 are forms of keratin that are only found in the epidermis. K6/16 expression has been demonstrated in studies to be changed in diabetic wounds. In conclusion, poor wound healing is a typical consequence of diabetes, and alterations in K6 and K16 expression may contribute to this issue. These findings suggest that targeting K6/16 expression or activity with metformin might be a promising therapeutic method for improving wound healing in diabetics. Nonetheless, there has been little study on the precise effects of metformin on K6 and K16 in the context of wound healing. It is plausible, however, that metformin's ability to enhance glycaemic control indirectly aids wound healing by lowering inflammation and enhancing total tissue repair. K6/16 dimers are also thought to be involved in the modulation of inflammatory responses, which are required for successful wound healing. More research is needed, however, to completely comprehend the impact of metformin in diabetic wound healing, especially on K6/16 dimers, and to investigate new therapeutic approaches.

Acknowledgments: The author declares that she has not known competing financial interests or personal relationships.

Conflict of interest: The author declares that she has no conflict of interests.

Informed consent: Bezmialem Vakıf University Animal Experiments Local Ethics Committee approved this study, with the decision numbered 2020/15.

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Cite as: Tombulturk, F. K. (2024). Effects of keratin6/16 heterodimer on diabetic wound healing treatment with topical metformin. *Front Life Sci RT*, *5*(1), 65-73.