



The Effects of *Lucilia sericata* Larval Secretions on The Expressions of MicroRNAs that are Suggested to be Related with Wound Healing in Experimental Diabetic Rat Wound Model

Deneysel Diyabetik Sıçan Yara Modelinde *Lucilia sericata* Larva Salgılarının Yara İyileşme Süreci ile İlişkilendirilmiş MikroRNA'ların Ekspresyon Seviyeleri Üzerine Etkisi

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ABSTRACT

Objective: Normal wound healing is achieved by a cascade of many cellular activities. This process is affected by some of the metabolic diseases like Diabetes Mellitus (DM). DM causes bad prognosis and is one of the major contributors to chronic wound healing problems. Recently, *Lucilia sericata* larvae are used for wound healing as they are very effective agents in wound healing process. It's still unclear that how the larvae affect the molecular mechanisms and signaling pathways of chronic wound healing. MicroRNAs (miRNAs) can induce gene expression in post-transcriptional mechanisms. In this study, our aim was to determine whether the larvae secretions could change the expression patterns of selected miRNAs on the diabetic microenvironment.

Methods: Wistar Albino rats were classified as diabetic and non-diabetic, then full thickness cutaneous wounds were created at the dorsal region of rats. Wound tissues were collected on days 0, 3, 7 and 14 post wounding. The association between *miR21*, *miR146a*, *miR146b* and *miR29a* gene expression profiles and wound healing

ÖZ

Amaç: Normal yara iyileşmesi bir çok hücrel faaliyetin zincirleme tepkimeleri ile gerçekleşmektedir. Diabetes Mellitus gibi metabolik hastalığa sahip kişilerde ise bu süreç olumsuz etkilenmekte, kötü iyileşen ya da iyileşmeyen kronik yaraların meydana gelmesine neden olmaktadır. *Lucilia sericata* larvalarının yara iyileşmesinde etkin rol oynamakta olduğu bildirilmiştir ancak kronik yara iyileşmesinin moleküler mekanizmasında ve ilgili yollardaki etkileri hala bilinmemektedir. RNA türlerinden biri olan mikroRNA'ların (miRNA) bir bölümünün yüksek glukoz düzeylerinde ekspresyonlarının değiştiği gösterilmiştir. Bu sebeple çalışmamızda; yara iyileştirmesinde rolü olduğu bildirilen bazı miRNA'ların ekspresyonlarının diyabetik şartlarda larva salgısı uygulaması ile deri iyileşmesi süresince ne yönde etkilendiği incelenmiştir.

Yöntemler: Deneysel çalışmamızda kullanılan Wistar Albino türü sıçanlar diyabetik ve diyabetik olmayanlar olarak gruplandırıldıktan sonra sırt bölgesinde tam kat yara oluşturulmuştur. Oluşturulan yara bölgelerinden 0., 3., 7. ve 14. günlerde biyopsi örneği alınmıştır. Çalışmamızda literatür taraması yapılarak yara iyileşmesi ile ilişkilendirilmiş *miR21*, *miR146a*, *miR146b* ve *miR29a* genlerinin

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process was investigated by using Real Time polymerase chain reaction method.

Results: The increase in expression of *miR21* gene in diabetics treatment group was seen most on 3rd day compared with diabetics control group. An increase in expression profiles of *miR146a*, *miR146b* and *miR29* genes was seen most in larval treatment group compared with diabetics control group.

Conclusion: Our results have shown that larval secretion in wounds formed in diabetic rats affects the expression of miRNAs associated with wound healing process. Larval secretion therapy has been shown to increase wound healing and reduce inflammatory response by altering the expression of miR146a in diabetic rats

Keywords: Normal wound healing, diabetic wound healing, maggot debridement therapy, *Lucilia sericata*, microRNA expression

ifade analizleri, gerçek zamanlı polimeraz zincir reaksiyonu ile değerlendirilmiştir.

Bulgular: *miR21* geninin diyabetik kontrole göre diyabetik tedavi grubundaki ifade artışı en fazla 3. günde görülürken, *miR146b*, *miR146a* ve *miR29a* genlerindeki ifade artışı diyabetik kontrol grubuna göre kıyaslandığında en fazla larva salgısı tedavisi uygulanan gruplarda olduğu görülmüştür.

Sonuç: Sonuçlarımız ile diyabetik sıçanlarda oluşturulmuş yaralarda larva salgısının yara iyileşme süreci ile ilişkilendirilmiş miRNA'ların ifadelerini etkilediği bulgulanmıştır. Larva salgısı tedavisinin diyabetiklerde miR146a'nın ifadesini değiştirerek yara iyileşmesini arttırdığı ve enflamatuvar yanıtı azalttığı gösterilmiştir.

Anahtar Sözcükler: Normal yara iyileşmesi, diyabetik yara iyileşmesi, larva debridman tedavisi, *Lucilia sericata*, mikroRNA ekspresyonu

Introduction

Although inadequate wound healing in diabetic patients is not fully understood, several studies in various diabetic human and animal models have shown that some disruptions have occurred in various stages of the wound healing process. It has been shown that the diabetic microenvironment significantly affects keratinocyte and fibroblast functions, and can alter extracellular matrix (ECM) structure and function (1-3).

MicroRNAs (miRNAs), known as small non-coding RNAs, are single-stranded RNA molecules that act negatively on genes at the translational level. In various studies, it has been observed that the release of cytokines and interleukins in chronic wounds contributes to various molecular stages such as angiogenesis and cell proliferation (4-6). The wound healing is delayed as a result of the negative factors that occur in the healing process of chronic wounds. Treatments applied to shorten this process are effective. The studies have revealed that larval secretion treatment is successful in debridement of chronic wounds (7,8).

Considering that miRNAs are required for the various phases of chronic wound healing and that miRNA levels change as a result of various treatments that accelerate chronic wound healing, larval secretions can affect miRNAs during the period of acceleration of healing by affecting the various phases of wound healing in diabetic microenvironment (9). However, there is no study concerning the effect of larval secretions on the expression of miRNAs associated with wound healing in diabetic subjects. In our study; it is predicted that the expression of miRNAs associated with wound healing may also change. For this reason, we aimed to determine whether or not *Lucilia sericata* larval secretions change the expression of miRNAs, miR21, miR146a, miR146b and miR29a, which were shown in bioinformatics studies to have a critical potential in effective healing (10).

Methods

Animal Experiments and Tissue Preparation

In our study, 20 male Wistar Albino rats weighing 300-350 g were obtained. Male rats were preferred in our study, as processes such

as breeding period, birth, nursing care, lactation period in the female rats would affect the results of the experiment. Our study was approved by the İstanbul University Animal Experiments Ethics Committee (decision no: 2013/44). Under standard conditions, the rats were given free access to water and food, and 4 groups of 5 animals were separated. The first two groups were separated into treatment groups while the other two groups were divided into control groups. Pre-experimentally, the rats were fasted overnight and were injected 60 mg/kg streptozotocin (STZ) intraperitoneally, pH=4.5 in 0.1 M citrate buffer, to form diabetes. Diabetes Mellitus formation was checked 3 days after the injection of STZ by measuring the blood glucose level in the rat's tail vein. Those with blood glucose levels above 250 mg/dL were considered diabetic and included in the study (11,12). The rats to be used in the experiment were anesthetized with intraperitoneal (i.p.) Pentobarbitone sodium (Pental sodium) (IU Ulgay Medicine Industry Co. Inc., İstanbul, Turkey) with a dose of 40 mg/kg and then dorsal fur was shaved. The biopsy "Punch" tool was used to create a full-layer excisional wound model. Four 12 mm-diameter tissues of epidermis and dermis were excised at equal distances of about 1 cm from each other on dorsal thoracic region. The day of wound formation was accepted as day 0. Both PBS and larval secretions were absorbed into the surgical sponges and placed on the wound. Biopsy materials were taken in the 3rd, 7th and 14th days after the wound was formed. Tissue fragments were homogenized with a homogenizer (Next advance, USA) by placing 500 µL of Qiazol lysis buffer (Qiagen, USA) and 3 times of tissue weight of zirconium beads (Next advance, USA) for use in RNA isolation method and kept at -80 °C.

Real-time PCR (RT-PCR)

After isolation of the RNA from the homogenized tissue, the purity and quantity of the samples were measured on a nanodrop spectrophotometer. Following cDNA synthesis, samples were used for real time polymerase chain reaction (PCR) procedures to detect the expression levels of miRNAs that were previously associated with wound healing. In our study, four genes that were found to be effective in the wound healing process in the literature were identified as miR21, mir29a, mir146a, mir146b.

A real-time PCR run was made for each of the 70 cDNA samples and was repeated 2 times. Inconsistent results were not evaluated. Coefficients calculated by the $2^{-\Delta\Delta CT}$ method, and values less than 2 and greater than -2 were considered to be insignificant.

Results

Results from biopsy samples taken at days 0, 3, 7, and 14 days of wound healing were evaluated separately for *miR21*, *miR146a*, *miR29a* and *miR146b* genes. For the 3rd, 7th and 14th days, fold changes of the treatment group compared with the control group were calculated in both diabetic and normal groups (Figure 1).

According to the findings obtained by real-time PCR study, fold changes of *miR146a*, *miR21*, *miR29a* and *miR146b* genes in diabetic rat skins compared to normal rat skins were examined separately in day 0. Accordingly, expression of *miR146a* gene decreased 3.75 fold, *miR29a* gene decreased 4.43 fold, *miR21* gene decreased 9.37 fold, and *miR146b* gene decreased 12.57 fold in diabetic group compared to normal group. When four genes were compared among themselves, it was seen that fold change value was highest in *miR146b* gene (Figure 2).

The expression of *miR21* gene in the 3rd day increased 42.75-fold in diabetic therapy (DT) group compared with diabetic

control (DC) group, whereas it increased 12.78-fold in the 7th day. In the DT group, the *miR21* expression increased 2.92-fold more than the DC group in the 14th day. In the healthy group, the expression of *miR21* in the 3rd day was 2.40-fold higher in the normal therapy (NT) group than in the normal control (NC) group, whereas it was 1.40-fold in the 7th day and 1.06-fold in the 14th day (Figure 3).

While the *miR146a* gene showed a 9.79-fold increase in DT compared to the DC group in the 3rd day, it increased 4.11-fold in the 7th day. The DT group showed a 7.45-fold increase in expression of *miR146a* in day 14 compared to the DC group. In the diabetic group, it was seen that the increase of folds was highest in 3rd day, while it was lowest in 7th day, when the 3rd, 7th and 14th days were compared within themselves. In the healthy group, the expression of *miR146a* gene in the 3rd day increased 3.19-fold. The expression of the *146a* gene in the 7th day decreased 1.51-fold in NT compared to the NC group, but decreased 4.22-fold in the 14th day. When the days 3, 7 and 14 were evaluated in the normal group, it was seen that the expression of *miR146a* showed the highest increase in the third day (Figure 4).

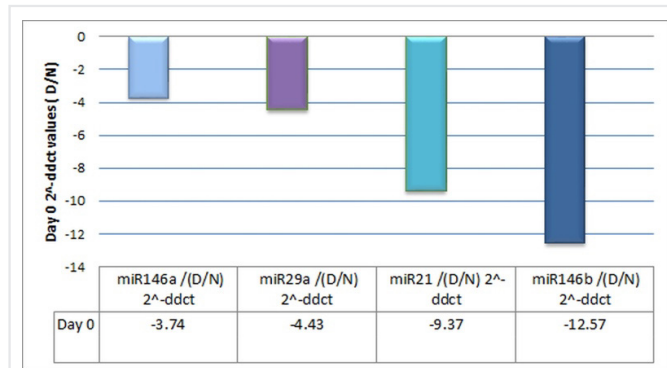


Figure 1. Representation of *miR146a*, *miR146b*, *miR21*, and *miR29a* gene expression in the diabetic skin and the normal skin. Expressions of related genes are shown as fold change

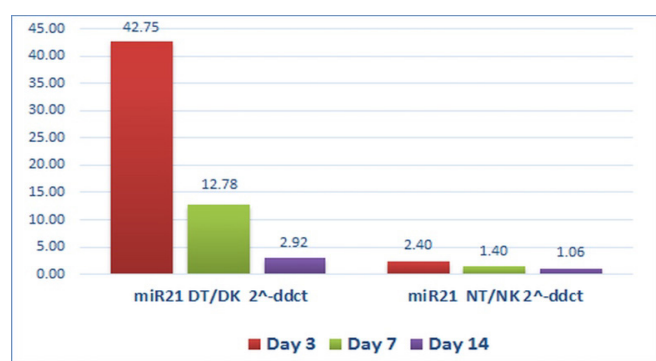


Figure 2. Changes in expression of *miR21* gene in the larval secretion applied wound tissues in 3rd, 7th and 14th days, compared to the control group. Expression of the genes involved is shown as fold change, and diabetic and normal groups are evaluated within themselves

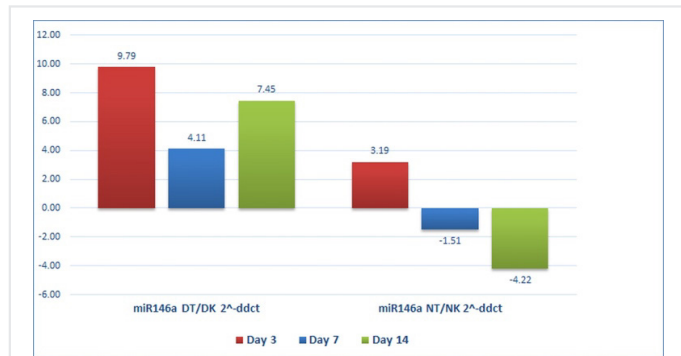


Figure 3. Changes in expression of *miR146a* gene in the larval secretion applied wound tissue in the 3rd, 7th and 14th days, compared to the control group. Expression of related genes is shown as fold change, and diabetic and normal groups are evaluated within themselves
DT: Diabetic therapy, NT: Normal therapy

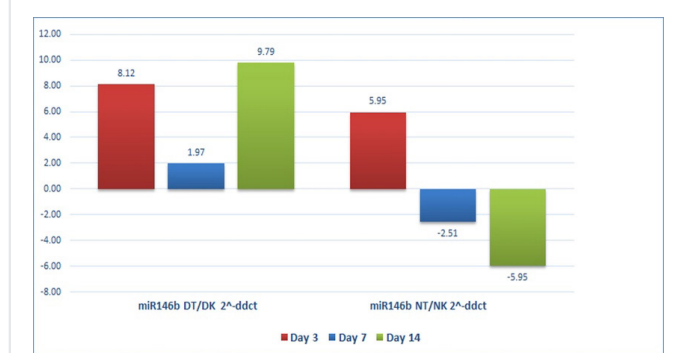


Figure 4. Changes in expression of *miR146b* gene in the larval secretion applied wound tissue in the 3rd, 7th and 14th days, compared to the control group. Expression of related genes is shown as fold change, and diabetic and normal groups are evaluated within themselves
DT: Diabetic therapy, NT: Normal therapy

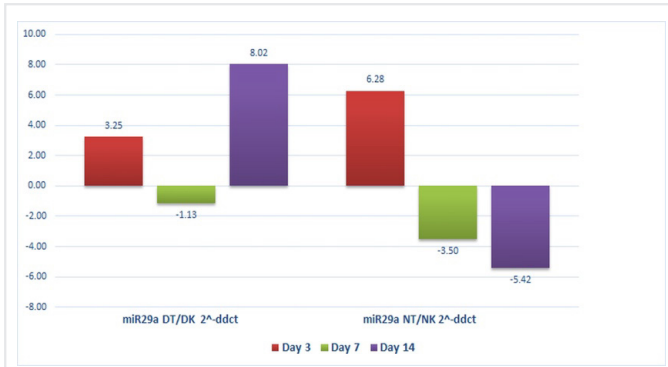


Figure 5. Changes in expression of *miR29a* gene in the larval secretion applied wound tissue in the 3rd, 7th and 14th days, compared to the control group. Expression of related genes is shown as fold change, and diabetic and normal groups are evaluated within themselves

DT: Diabetic therapy, NT: Normal therapy

When the *miR146b* gene was compared with the DC group in the 3rd day, the increase in the DT group was 8.12-fold, while the increase in the 7th day was 1.97-fold and 9.79-fold in the 14th day. It was seen that the fold increase was the highest in 14th day and the lowest in 7th day. In the healthy group, the expression of *miR146b* in the 3rd day increased 5.95-fold in the NT group compared to the NC group, whereas it decreased 5.91-fold in the 14th day in the NT group compared to the NC group. It decreased 2.51-fold in the NT group compared to the NC group in the 7th day. When the days 3, 7 and 14 were evaluated in the normal group, the expression of *miR146b* increased the most in the 3rd day, whereas in the 14th day it showed down regulation and the expression decreased 5.95-fold (Figure 5).

The expression of *miR29a* showed a 3.25-fold increase in the DT group compared to the DC group in the 3rd day, a 1.13-fold decrease in the 7th day and 8.02-fold increase in the 14th day. It was observed that the maximum fold increase was in the 14th day. In the healthy group, the expression of *miR29a* in the 3rd day increased 6.28-fold in the NT group compared to the NC group, decreased 3.50-fold in NT group compared to the NC group in the 7th day, and decreased 5.42-fold in the 14th day. When the 3rd, 7th, and 14th days in the normal group were evaluated within themselves, the expression of *miR29a* increased the most in day 3, whereas in day 14 it decreased by 5.42-fold with down regulation.

Discussion

It is well known that the wound healing process is a highly orchestrated series of mechanisms and biological cascades, although its complex molecular mechanisms are not yet discovered completely (13,14). Recent studies indicate that miRNA levels are altered during normal skin wound healing and these alterations lead to wound healing defects in pathological stages such as diabetes (3). Although there are a large number of miRNA species expressed in wound microenvironment during different phases of wound healing, bioinformatics studies showed that *miR21*, *miR146a*, *miR146b* and *miR29a* target the

mRNAs encoding many wound healing-related proteins, all of which show critical potential in effective healing (10,15-18). Also we know that high glucose levels significantly affects the expression of a large set of miRNAs (19). Since the larvae of *Lucilia sericata* improve the healing process, the study of the changes in the larvae secretions-induced miRNA expression in wound microenvironment may yield insights into understanding the molecular mechanisms that regulate wound healing and may provide new and more efficient treatment for wounds (20). Therefore, the present study was conducted to investigate the effects of the larvae secretions on wound healing in diabetic rats in relation to the expression levels of *miR21*, *miR146a*, *miR146b* and *miR29a*.

For this purpose, it was aimed to observe the effect of larval secretion on open wounds in diabetic and normal Wistar albino rats, and to represent the relatively open wound as the wound type, a full layer excisional wound model was used. Three pieces of 12 mm-diameter tissues including dermis and epidermis, each with an equal distance of about 1 cm were taken from each rat on dorsal thoracic region with a punch biopsy instrument. Scar tissue was removed in days 0, 3, 7, and 14 after wound formation. Some of the tissues underwent real-time PCR for the purpose of carrying out expression assays of *miR146a*, *miR146b*, *miR29a* and *miR21*.

The migration of fibroblasts to the wound site facilitates the synthesis of growth factors in ECM which leads migration of other cell types to the wound site. In a study by Madhyastha et al. (21), it was investigated that how different miRNAs associated with cell growth and proliferation contributed to the healing of diabetic wounds. The differences in expression of the selected miRNAs in diabetic and normal wound healing were compared. It was shown that *miR21* expressed by fibroblasts, keratinocytes, melanocytes and inflammatory cells, had significant effects on the migration of fibroblasts to the wound area. In the same study, it was also reported that the expression of the *miR21* gene varied in wound healing process. In our study, it was observed that the expression of *miR21* increased 42.75-fold in the group treated with larval secretion compared to the untreated diabetic group in the 3rd day. When the role of fibroblasts in wound healing is taken into account, observing a good course of wound healing in the diabetic group on which the larval secretion is applied is possible. In the same study, it was stated that the expression of *miR21* gene changed during different days of wound healing. In our study, results supporting this finding were obtained, whereas fold change values of the expression of *miR21* gene differed in days 3, 7, and 14.

Yang and colleagues investigated the HaCaT cell line to monitor the effect of *miR21* on keratinocyte migration during wound healing and observed the change of expression of the *miR21* gene in the presence of Transforming growth factor beta1 (TGFβ1), which stimulates the cellular uptake of growth factors by facilitating cellular movements of monocytes, lymphocytes, macrophages, keratinocytes. The expression of *miR21* increased as a result of treatment with the HaCaT cell line, which caused

in vitro TGF β mediated keratinocyte migration. The same study also showed that the cause of delay in re-epithelialization was related to the down regulation of miR21. These findings suggest that the increase in expression of *miR21* gene in diabetic treatment group compared to DC group may occur as a result of TGF β increase in the environment with the therapeutic effect of larval treatment when in the third day of our study (22).

Studies have shown that miR29a is down-regulated in systemic sclerosis dermal fibroblasts, which leads to many forms of fibrosis, compared with the normal group (23).

Collagen synthesis and regulation are very important events occurring at the maturation stage of wound healing. In healthy skin fibroblasts, miR29a directly affects collagen synthesis in the post transcriptional stage. It is also known that the synthesis of miR29a is in control of TGF β , platelet derived growth factor-B and interleukin (IL)-4 in healthy skin (24). Some studies showed that the miR29 family has an effect on the wound-specific cellular functions and the cytokine network (25,26). Ramachandran noted that miR29a is crucial in the regulation of fibrosis, which is down-regulated in the final stage of ECM synthesis. In the same study, it was mentioned that miR29a is in the target position for many proteins required for ECM synthesis. (27).

In studies conducted by Wang et al. (28), TGF β increased angiogenesis by upregulating miR29a. In our study, expression of the *miR29a* gene was observed to decrease 5.42-fold in day 14 in normal treatment group compared to NC group. This indicates that the angiogenesis, which is now a feature of the proliferative phase, has completed and the wound healing has begun to terminate in the 14th day in the normal treatment group compared with the NC group. In day 7, similarly to day 14, the *miR29a* gene expression decreased 5.42-fold in normal treatment group compared to NC group.

Although the expression of the *miR146a* and *miR146b* genes, members of the *miR146* gene family, involved in regulation of immune and inflammatory responses, has been reported to change in many pathological conditions; miR146a dysregulation has also been associated with many chronic inflammatory diseases such as psoriasis and rheumatoid arthritis (25,29). In our study, in the diabetic group treated with larvae compared to DC group, the expression of *miR146a* gene was observed to increase 9.79-fold in the 3rd day, 4.11-fold in the 7th day, and 7.45-fold in the 14th day. This upregulation has been shown to be associated with downregulation of *IRAK1*, *TRAF6*, and other pathways associated with these genes and *NF κ B*, *IL-6* and *MIP2*, the target genes of *miR146a* (30). These data suggest that secretion of larvae in the treatment group promotes wound healing in diabetics and changes the miR146a expression and appears to reduce the inflammatory response.

Conclusion

In summary, our results have shown that larval secretion in wounds formed in diabetic rats affects the expression of miRNAs associated with wound healing process.

Larval secretion therapy has been shown to increase wound healing and may reduce inflammatory response by altering the expression of miR146a in diabetics.

Ethics

Ethics Committee Approval: Our study was approved by the İstanbul University Animal Experiments Ethics Committee (decision no: 2013/44).

Informed Consent: Patient approval has not been obtained as it is performed on animals.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: A.Ö., F.A., E.P., İ.O., Design: A.Ö., İ.O., E.P., Data Collection or Processing: Ö.K., F.A., M.T., Analysis or Interpretation: A.Ö., M.T., F.A., H.A., Literature Search: Ö.K., H.A., Writing: A.Ö.

Conflict of Interest: There is no conflict of interest between the authors.

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