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Short Communication

Role of miRNAs and their Target Genes in Diabetic Foot Ulcer Wound Healing

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MSK: Conceptualization, carried out the experiments, writing, SH: developed the theoretical formalism, performed the analytic calculations, and performed the numerical simulations. MNK: writing, TAM: contributed to sample collection and preparation and RQ: Conceptualization, performed the numerical simulations, writing, and final editing. All the authors discussed the results and contributed to the final.

Key words
***MiRNAs*, Amputation, Ulcer**

ABSTRACT

The objective of this study was to explore the correlation among the expression of miRNAs: *miR-143*, *miR-145* and *miR-146a* and their target genes: *MAP3K7*, *LOX*, and *THBD* in tissue samples of patients with type 2 diabetes mellitus (T2DM) and the onset of diabetic foot ulcer (DFU) and control samples. Fifty confirmed patients with Diabetes without having an associated complication, fifty patients of DFU, and fifty controls. To determine the expression levels of miR's and their targeted genes, qPCR was used to analyze differential expression in DFU, T2DM, and controls. We found that all the miRNAs (*miR-143*, *miR-145,* and *miR-146a*) expression levels in the DFU were lower than T2DM group and normal group while their target genes expression level in the DFU higher than T2DM as well as in the normal having significant level ($P < 0.01$). The decreased expression of miRNAs (*miR-143*, *miR-145,* and *miR-146a*) and increased expression of their target genes (*MAP3K7*, *LOX*, and *THBD*).

Diabetes mellitus (DM) is the most common disease in the entire world, the outcome of these life-threatening complications is not controlled properly with a balanced diet and exercise; one of them is diabetic foot ulcer (DFU), which is a disease that ultimately leads to amputation (Adnan and Asim, 2020). DFU is considered an adult's disease in the past but due to poor control of diabetes and a passive lifestyle it also affects the young generation, most adults are affected because of poor blood supply to the extreme ends of the body especially the lower extreme ends, poor blood supply and loss of sensation in the foot, injury or infection not judged by the body and developed chronic wounds which took more time in healing and sometimes not healed resulted into an amputation. The molecular pathology of DFU represents certain sets of genes under the control of specific miRNAs involved in the healing process of chronic wounds. If these miRNAs are in low concentration, then the genes under the control of these miRNAs do not function in the proper wound-healing process (Khan and Junaid, 2017). There are several miRNAs involved in DFU wound healing such as in inflammation *miR-143*, *145*, and *miR-146a* are directly associated with DFU disease inflammation and insulin resistance (Syed *et al*., 2019). The *miR-146a* is directly regulating the inflammatory process of *NF-κB* cytokine signalling, its expression also increased in response to *TNFα* and *IL-6* inflammatory cytokines. *MiR-143* and *miR-145* worked in combination and form a cluster, overexpression of this cluster disturbs the *TNFα* and *NF-κB* mechanism. Several *miRNAs* (*miR-16*, *-17*, *-18,* and *-21*) are associated with diabetes and diabetes-related complications. *Mirna-146a* expression level increased in the DFU individuals. *Mir-16* is involved in the inhibition of *cyclooxygenase-2* and is associated with diabetes and its complications, similarly *miR-21* (Ashraf *et al*., 2011) is associated with a decrease in diabetic foot ulcer healing. Impaired wound healing is also associated with the inhibition of angiotensin-converting enzyme (ACE) inhibition and the *miR-143* and *miR-145* cluster is involved in it. Overexpression of *miR-99a* in diabetes is associated with poor wound healing in DFU as has an activation involvement in the *Akt* pathway.

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The maturation phase also disturbs because of *miR-143* involvement and is associated with diabetic foot ulcer complication (Berbudi *et al*., 2020).

To support our hypothesis, we have selected three miRNAs (*143, 145, and 146a*) which are mostly involved in the wound healing process, and their target genes (*MAP3K7, LOX, and THBD*), therefore their effects can be checked in the diabetic patients without DFU, DFU patients and control samples. The present research is designed to study the expression of miRNAs and their target genes in DFU patients and diabetes individuals having no other complications are used as controls. DFU is an extremely complicated disease as several pathways are involved to disturb the immune system a simple infection can lead to amputation.

Materials and methods

Skin biopsy samples were obtained with consent taken from all the participants; ethical approval was already taken from the tertiary care hospitals. Demographic features of all the subjects and all the basic biochemical tests were performed in the research laboratory. RNA extraction was done with the help of a kit and following the protocol provided with the kit (Norgen Biotek Corp, Thorold, Ontario, Canada). To check the quality of extracted RNA, quantification was done using the spectrophotometer and countercheck on the gel electrophoresis system. RNA was converted to cDNA using the revert aid first strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA) following the protocol provided with the kit. The following method was used to calculate the normalization with *GAPDH*. SPSS version 23 was used for statistical analysis.

Following primers (5' →3') were used:

143-3p F: TGCTGCATCTCTGGTCAGTTG
and R: GCAGAACAACTTCTCTCTTCCTG
MAP3K7 F: GAATCTGAGAGGAAAGCGTTTATTG
and R: CCATCACAAGACACACTGGAT
MIR-145-5p F: CTCACGGTCCAGTTTTCCCAG
and R: ATGACCTCAAGAACAGTATTTCCAG
LOX F: GATTGAGTCCTGGCTGTTATGA
and R: GGGTTTACACTGACCTTTAGGAT
miR-146a F: GTTTGGCCTCTGAAATTCAGTT
and R: GTGCAGGGTCCGAGGT
THBD F: CCCAGGAGACAGTTCAAGAAA
and R: CCCAATTCCACAAGACCAGTAG
GAPDH F: CAAGGTCATCCATGACAACTTTG
and R: GTCCACCACCCTGTTGCTGTAG

Results and discussion

The expression of selected miRNAs and their target genes predicted from the mortar base database were used for analysis. Our findings show that the expression of miRNAs was decreased in the DFU samples compared to control samples. All three miRNAs were lower in DFU as compared to target genes. Expression of target genes was higher in control samples as compared to selected miRNAs. Fold change differences in selected miRNAs and their target genes are represented separately to illustrate their lower expression in miRNAs in DFU and respective target genes. Biochemical tests (Lipid profile) and basic diabetes test (HbA1c) were performed using a spectrophotometer GO system with the locally available kits. Results are shown in Table I.

Table I. Basic demographic features and biochemical test results (Mean±SD).

Parameters	Healthy	T2DM	DFU
Age	51.92 ± 9.6	52.13 ± 6.8	51.63 ± 8.2
Diabetes duration	N/A	10.81 ± 4.8	14.01 ± 5.0
HbA1c	5.8 ±2.3	7.7 ±4.5	9.9±4.2
B.P. (U)	120.54±15.8	125.3 ± 5.2	123.8 ± 7.4
B.P. (L)	79.91 ± 12.5	84.97 ± 2.7	82.48 ± 6.8
Body fats	26.72 ± 11.3	34.23 ± 4.6	32.67 ± 5.8
Visceral fats	8.85 ± 4.3	12.88 ± 7.4	10.85 ± 8.3
Cholesterol	182.06±38.9	212.56±18.92	256.24±12.69
Triglyceride	118.09±35.1	182.14±15.41	174.34 ± 11.4
HDL	32.72 ± 10.0	45.12 ± 5.7	55.39 ± 12.5
LDL	120.29±41.3	129.82±21.35	126.23 ± 22.7
VLDL	15.56 ± 2.34	23.67 ± 7.11	28.44 ± 8.43
Sex			
Male	27 (54%)	20 (40%)	28 (56%)
Female	23 (46%)	30 (60%)	22 (44%)
Smoking			
No	11 (55.16%)	13 (55.16%)	19 (55.16%)
Yes	39 (44.84%)	37 (44.84%)	31 (44.84%)
BMI			
Normal weight	3 (6%)	5 (10%)	4 (8%)
Overweight	24 (48%)	24 (48%)	28 (56%)
Obese	23 (46%)	21 (42%)	18 (36%)

SD, standard deviation; HDL; high density lipoprotein; LDL, low density lipoprotein; VLDL, very low-density lipoprotein, BMI, basic mass index.

Expression analysis reveals that all the miRNAs were downregulated, and their target genes were upregulated in our study. *Hsa-mir-143-3p* and *has-mir-145-5p* always worked in combination which also proved in our study that both these miRNAs were downregulated and the third miRNA, *has-mir-146a* also downregulated. We have done a comparison of disease DFU samples with Diabetic patients without having any other complication as control and then compare diseased DFU samples compared with healthy individuals of the same age groups without having any disease as control. *Hsa-mir-143-*

3p expression revealed that 0.53-fold decrease in DM patients and 5.41-fold decrease in healthy individuals. The has-mir-145-5p expression also decreased 0.3-fold in DM and 1.6-fold in healthy individuals. Hsa-mir-146a expression also decreased 0.61-fold in DM and 2.68 in healthy individuals. Target genes selected from miRNA target databases miRDB were up regulated in DM and healthy individuals when compared to DFU patients. The target gene of *has-mir-143-3p*, *MAP3K7* gene up-regulated 3.8-fold in DM and 5.4-fold up-regulated in healthy individuals, *LOX* gene was the target gent of *has-mir-145-5p* was also up-regulated 1.9-fold in DM and 5.3-fold in healthy individuals, *THBD* target gene of has-mir-146a was down-regulated 0.2-fold in DM and 1.1-fold in healthy individuals. Regression analysis revealed that these results were significant (P-value < 0.05) in the case of miRs (*143, 145,* and *146a*) in DFU and healthy individuals and non-significant when compared to diseased DFU and DM as control samples. *MAP3K7* gene is significantly up-regulated in both DM and healthy individuals, while the *LOX* gene is significantly up-regulated in DFU and healthy individuals, and *THBD* no significantly upregulated in both cases of DM and healthy individuals. The results were represented in Figure 1.

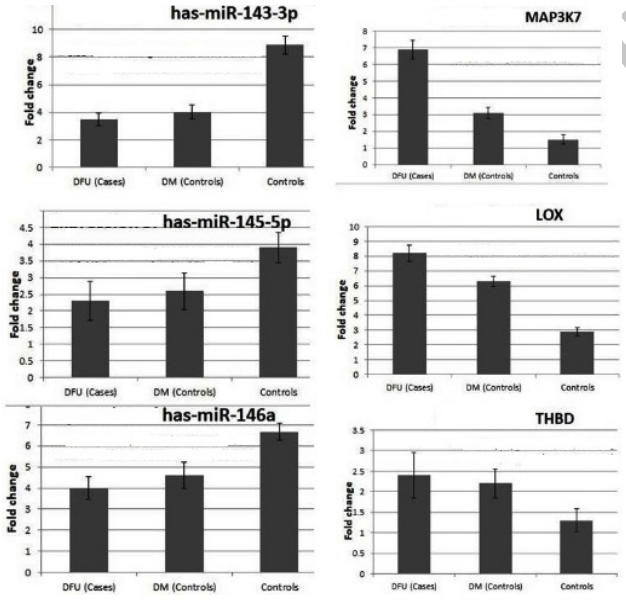

Fig. 1. miRNAs levels and expression of their target genes in T2DM and DFU patients.

Interestingly miRNAs are also recognized in previous studies and can also play a vital role in controlling these biological pathways involved in the healing phases of chronic wounds. Understanding the functional role of miRNAs and their target genes provides insight to treat DFU accurately and efficiently (Ali *et al*., 2020; Xu *et al*., 2020). *MiRNA-146a* increased in human diabetic skin and decreased in DFU has also been studied in previous studies, our results exactly proved that in Pakistani population it also decreases in DFU patients. Furthermore, its target gene *THBD* expression also increased in DFU as compared to controls (Shi *et al*., 2021). During inflammation *miR-146a* has target genes *TRAF6* (Tavasolian *et al*., 2020), *IL1* receptor-associated kinases 1 and 2 (*IRAK1* and *IRAK2*), and *IRAK2* increased in the healing phase, therefore, increasing the interleukin 6 (*IL-6*) level (Sanada *et al*., 2020). *Mirna-146* expression decreased in diabetic mice also studied previously and in human skin, its expression also decreased in our results. Toll-like receptors (*TLR*) are a major contributor to innate immunity increasing the expression of *miRNA-146a* and therefore improving the healing of the diabetic wound. *MiR-146a*, when not expressed up to their normal level linked with poor and delay healing of the diabetic wound, therefore resulting in abnormal healing, leading to amputation in the future (Akash *et al*., 2020).

MiR-143/145 both are mostly performing their function by forming clusters together and major roles in cell differentiation, proliferation, angiogenesis, and apoptosis, therefore a key player in the wound healing process (Pang *et al*., 2020). Decreased expression level in DFU indicates that they have a positive role in wound healing. Higher levels of *miR-143* in diabetes are linked with pre-diabetes as associated with the inflammation process. *TNFα* is a reported target of *miR-143* and is linked with diabetes. An increase in *TNFα* means other cytokines such as *IL-6* (Baz and Ibrahim, 2020).

MAP3K7 has an alternative name *TAK1*, it is a protein kinase that is activated by *TGFβ* (Tomás *et al*., 2016), which is an essential regulator of *NF-κB* (Wang *et al*., 2019) that is the main mediator of cell survival. Deficiency of *TAK1* persuades sensitivity of *TNFα* induced cell death. *TAK1* is also important to fight against microbes and maintain blood flow (Cuarental *et al*., 2019). In remodeling the *miR-143/145* cluster has the targets *IRS1*, *PDGFD*, and *αSMA*, therefore their role in persistent inflammation or excessive scar formation. *MiR-145* directly targets the *MYO5A* which functions in the cytoplasm to transport the vesicle and insulin-dependent *GLUT4* movement. *LOX* was also a target of *miR-145* and is direct linking with *collagen-I* and *collagen-III*, therefore leading to declining in the adipose tissue. Both *miR-143/145* down-regulations are associated with the delay in wound healing; therefore, the wound-healing contraction process is out of order and abnormal healing means losing the tissue integrity to recover from chronic wounds (Nie *et al*., 2020).

Insulin receptor substrate-1 (*IRS-1*) was also under the control of *miR-145*, therefore suppression of *miR-145* was associated with a delay in wound healing in the long run.

Similarly, this is the case with *TRAF-6* and *NF-κB*, plus cytokines circulatory levels of *TNFα* and *IL-6*. There is the involvement of *miR-143/145* cluster in the development of diabetes targeting angiotensin-converting enzyme (*ACE*), as a result, causes impaired wound healing. *ACE* inhibition by *miR-143/145* cluster also demonstrates that protection in wound healing is reduced with the downregulation of this cluster in the system (Tomás *et al*., 2016).

The significance of the *miR-146a* is very clear with their role in molecular brake on inflammation it is the authentic therapeutic agent in DFU as the fact that it is significantly downregulated in diabetic mouse wounds and our results also proved in humans their down-regulation represents a promising biomarker as well.

Conclusions

Down-regulation of selected miRNAs has-143-3p, has-mir-145-5p, and has-mir-146a and their target genes up-regulation in DFU required further validation on a large set of population, therefore these miRNAs and target genes can be used for initial diagnosis in Diabetic patients as in our results small difference in DM and DFU while the significant difference among DFU and healthy individuals.

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IRB approval

IRB number: CIIT/Bio/ERB/19/83

Ethical statement

Abide by the laws, rules, and regulations take the responsibility of the work.

Statement of conflict of interest

The authors have declared no conflict of interest.

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