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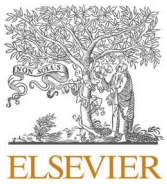

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Epigenetic regulation of Nrf2-Mediated angiogenesis in diabetic foot ulcer progression: Role of histone deacetylases

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ABSTRACT

Nuclear factor E2-related factor 2 (Nrf2), a redox-sensitive transcription factor, regulates proangiogenic mediators, and antioxidant and detoxification enzymes. However, hitherto its regulation in the progression of DFU was poorly examined. The regulation of Nrf2 has been reported to be affected by various factors, including histone deacetylase (HDACs) and DNA methylation. The present study aimed to profile all classes of HDACs and correlate them with Nrf2 and angiogenic markers in the tissue biopsies of different grades of DFU patients (n = 20 in each grade). The gene expression profile of Nrf2 and its downstream targets, angiogenic markers, and all classes of HDACs were assessed using qPCR. Spearman's correlation was performed to analyze the correlation of HDACs with Nrf2 and its downstream targets along with angiogenic markers. We observed a progressive decrease in the gene expression of Nrf2 and angiogenic markers such as VEGF, HIF-1α, and SDF-1α and also an increase in the TSP-2 expression in different grades of DFU. In parallel, a significant downregulation of HDAC2/8 and SIRT1/2/4 has been observed in various grades of DFU subjects. On the other hand, HDAC1/3/4/11 and SIRT3/5/6/7 showed upregulation in different grades of DFU and the maximum increase was observed in Grade 3 patients. A significant negative correlation between Nrf2 and HDAC4, angiogenic markers, and HDAC4 suggested the pivotal role of the HDAC4-regulated Nrf2-mediated angiogenesis among DFU subjects. We have generated a first line of evidence on the epigenetic regulation of Nrf2 and its correlation with angiogenesis in the progression of diabetic foot ulcers.

1. Introduction

More than 537 million people are estimated to have diabetes mellitus (DM) worldwide this number is predicted to rise to 643 million by 2030 and 783 million by 2045 [1]. DM individuals tend to develop comorbidities in their lifetime, putting them at a greater risk of vascular complications [2]. The majority of them develop peripheral arterial disease (PAD) and diabetic peripheral neuropathy (DPN) due to uncontrolled diabetes, which causes a lack of sensation in the foot and symptomatically leads to open sore with recurrent infection, a condition called diabetic foot ulcer (DFU) [3]. These ulcers require keen and multi-disciplinary attention as they lack self-healing abilities. Treatments like hyperbaric oxygen (HBO) therapy, ozone therapy along with hydrogel, and antimicrobial-based dressing are widely followed. Although these therapies are in use, they often require prolonged treatment to effectively enhance wound healing, posing challenges for individuals with DFU. Current treatment strategies primarily focus on mitigating damage through tissue debridement, rather than addressing the underlying pathophysiology of disease progression. Therefore, developing alternative approaches, such as modulators, could enhance the activity of molecular targets, including transcription factors related to angiogenesis, which are crucial for healing diabetic wounds. However, molecular factors that foresee the progression of foot ulcers are merely known [4,5].

Cutaneous wound healing is a complex process where angiogenesis plays a crucial role in forming new blood vessels and helps in wound vascularisation [6]. Vascular endothelial growth factor (VEGF) is a proangiogenic factor that promotes the migration of cells and penetration of blood capillaries which is partly controlled by hypoxia-inducible factor (HIF)-1α [7]. Accumulated evidence highlights the involvement

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of various factors in facilitating angiogenesis in individuals with DFU, including fibroblast-mediated neo-angiogenesis, regenerative medicine-based approaches, and the action of microRNAs. Among these, fibroblast-related wound healing has been extensively studied due to its ability to regulate granulation tissue formation and neo-angiogenesis by modulating the extracellular matrix (ECM) network and matrix protein secretion. This, in turn, regulates endothelial cell function and promotes wound closure [8]. However, chronic inflammation and a hyperglycemic environment can adversely affect fibroblast heterogeneity and activation [9]. On the other hand, mesenchymal stem cells (MSCs) have been shown to promote angiogenesis by mobilizing various angiogenic markers such as VEGF, IGF-1, MMP-9, and Ang-1 into the wound environment. This process facilitates the proliferation and differentiation of endothelial progenitor cells, aiding in the healing of DFU wounds [10,11]. Another approach to facilitating angiogenesis in wound healing is through microRNA-mediated therapy. Numerous microRNAs have been identified as pro-angiogenic, with delivery methods including exosomes, microvesicles, or nanoparticles that target the wound site directly. However, instead of focusing solely on delivering pro-angiogenic microRNAs, future studies could explore the inhibition of anti-angiogenic microRNAs as a strategy to enhance angiogenesis [12]. Impaired angiogenesis associated with chronic oxidative stress is one of the hallmarks in people with DFU and thus hastens wound healing.

Previously we reported that Nrf2, as a critical transcription factor, helps combat oxidative stress and aids in regulating angiogenesis during the wound-healing process. Nrf2 regulates redox homeostasis and bolsters array of antioxidant enzymes like catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) and a set of drug-metabolizing enzymes, such as glutathione S-transferase (GST), NAD(P)H: quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1) [13–15]. Few reports highlighted that the levels of Nrf2 and its downstream targets are low in DFU [16], and its underlying mechanism is not completely elucidated. We studied the Single Nucleotide Polymorphism of Nrf2 in individuals with different grades of DFU [17], and also have documented the role of Nrf2 in the lncRNA MALAT1/HIF-1α axis that regulates angiogenesis in DFU individuals and further validated through CRISPR/Cas9-mediated knockout of Nrf2 in endothelial cells [18]. In addition, we have reported the function of miR-23c and miR-27b in regulating angiogenesis through Nrf2 signaling [19,20]. On a therapeutic note, we demonstrated the healing of wounds along with an increase in Nrf2-driven angiogenesis in DFU individuals who underwent hyperbaric oxygen (HBO) therapy [21].

Many hypotheses have been designed and established for the dysregulation of Nrf2 signaling. However, epigenetic alternations, like aberrant DNA methylation and histone modifications, have been crucial for regulating the Nrf2 signaling pathway under stress conditions [22]. Indeed, recent discoveries highlight the importance of "molecular erasers," the histone deacetylases (HDACs), in disease progression due to their ability to regulate gene expression. HDACs are critical enzymes involved in essential cellular functions, including the regulation of angiogenesis in many diseases. Recruitment of HDACs independent of DNA methylation consists of the role of transcription factors and nuclear receptors. The association of HDACs being recently studied and its function in regulating Nrf2-mediated angiogenesis in DFU is still unknown [23]. While a few studies have reported the epigenetic regulation of Nrf2 by DNA methylation, additional epigenetic changes that control Nrf2 in cancer patients, the function of HDACs in regulating Nrf2 expression in people with DFU remains unknown [24–26].

In contrast to earlier research by Teena et al. that used peripheral blood, we used tissue samples to examine molecular alterations at the wound site in a more direct manner [16]. In the present study, we aimed to comprehensively profile the diverse classes of HDACs within tissue biopsies obtained from different grades of DFU. We sought to pinpoint specific HDACs that disrupt the Nrf2-mediated angiogenic pathway in order to illuminate a promising target for therapeutic intervention. Furthermore, we conducted a thorough validation of the observed expression patterns of HDACs and Nrf2 in tissue biopsies using an *in vitro* model system.

2. Materials and methods

2.1. Enrollment of study subjects

A cross-sectional observation study with a total of 80 participants from south Indian population with different grades of DFU were recruited in this study and classified according to University of Texas (The University of Texas Staging System for Diabetic Foot Ulcers with Associated Interventions). The participants with uninfected epithelialized wounds were considered grade 0 (n = 20; Male 10/Female 10) with an average age of 55.87 ± 9.37. Superficial wounds not involving tendons were classified as grade 1 (n = 20; Male 15/Female 5) with an average age of 59.26 ± 11.40, whereas wounds penetrating tendons were classified as grade 2 (n = 20; Male 12/Female 8) with an average age of 57.67 ± 10.83. Grade 3 (n = 20; Male 14/Female 6) with an average age of 60.35 ± 12.57 includes DFU individuals with wounds penetrating to bone or into a joint. The grades of each individual were determined by a well-trained podiatrist in a tertiary care centre for diabetes in Chennai, South India. Individuals with DFU and with 5–10 years duration of diabetes with no other complications were included. Informed consent was obtained from the study participants willing to participate in this study. The study was performed in line with the *Declaration of Helsinki*, with an Institutional Ethical clearance (IEC/N-005/05/2022) from MV Hospital for Diabetes and Prof M. Viswanathan diabetes research centre, Chennai. Individuals with type 1 diabetes, pneumonia, gestational diabetes, inflammatory bowel disease, sepsis, meningitis, or hematologic diseases and those who underwent surgery in the past 2–3 weeks were excluded from this study. A pilot study was first carried out using 5 participants per group. Based on the preliminary results, with a confidence interval of 95 %, an estimated p value < 0.05, and a power of 80 %, the present sample size n = 20 was derived.

The anthropometric and demographic parameters like BMI, age, weight, height, and duration of diabetes were recorded for all the study participants. Biochemical analyses were carried out on a fully automated analyzer. Estimation of FPG, serum cholesterol, serum triglycerides (TGL), HDL cholesterol (HDL-c), and LDL cholesterol (LDL-c) were analyzed using standard methods as described earlier [27], triplicate readings were obtained on the first metatarsal at an increasing voltage in both legs. The collected tissue biopsies were cleaned with PBS and stored in an RNA stabilization reagent (Qiagen, USA) at −80 °C till further use.

2.2. In vitro model system conditions

For *in vitro* model system, human endothelial cells (EA.hy929) were cultured in Dulbecco Modified Eagle Medium (DMEM) medium at 37 °C in 5 % CO2 humidified incubator supplemented with 10 % FBS (GIBCO, Life Technologies, Carlsbad, CA). Once the cells reached 70 % confluency, trypsinized and passaged. To create the diabetes environment, the cells were serum-starved in DMEM with 1 % FBS for 3 h before treatment, followed by the exposure in a hyperglycemic microenvironment (HGM) with the final concentration of 33.3 mM of glucose with 20 ng/mL of IL-1β, TNF-α and IFN-γ for a period of 72h, mimic in alignment with people with diabetes. Further, the cells were then washed with 1X PBS and harvested for total RNA isolation.

2.3. Total RNA extraction

Total RNA from cells and tissue biopsies was carried out using TRIzol reagent. The tissues were transferred to a clean mortar and pistil and crushed into pieces using liquid nitrogen under aseptic conditions. About 300 L of trizol was added to the cells and tissue powder, collected in a sterile Eppendorf tube. The homogenate was then mixed

by vortex for about 30 min. After which, 200 μL of chloroform was added to the homogenate and mixed thoroughly. The contents were then briefly centrifuged at 12000 rpm for 20 min at 4 °C. The separated aqueous layer was mixed with an equal volume of isopropanol and incubated for overnight at −20 °C and centrifuged at 12000 rpm for 20 min at 4 °C. The resultant pellet was washed twice with 70 % ethanol and suspended in nuclease-free water.

2.4. Quantitative RT-PCR (q-RT-PCR) analysis

The concentration and purity of obtained RNA were measured using a Nano-quant spectrophotometer (Thermo Scientific). About 1 μg of RNA with purity 2.0 was selectively reverse transcribed using PrimeScirpt RT reagent (Takara). The cDNA obtained was used to assess target gene expression levels using SSO Advanced SYBR premix (Bio-Rad) on a CFX connect RT-PCR instrument (Bio-Rad). The gene expression of Nrf2, its downstream targets (NQO1, CAT, SOD, HO-1), angiogenic markers (HIF-1α, SDF-1α, VEGF), anti-angiogenic marker TSP-2 and all HDACs (class I– IV) was calculated using $2-\Delta\Delta Ct$, normalized to GAPDH housekeeping gene.

2.5. SDS- PAGE and immunoblotting

Briefly, 30 mg of tissue was weighed, and 1X 10^5 cells were taken for protein extraction using RIPA Buffer and the mixture were homogenized with zirconium beads for approximately 8 cycles or until the sample is completely homogenized. The mixture was then centrifuged at 12000 RPM for 20 min at 4 °C. Carefully transfer the supernatant into fresh tubes, and the protein concentration was determined using Bradford protein estimation assay at 595 nm. About 35 g of the protein was loaded onto SDS-PAGE and transferred to the nitrocellulose membrane. Once transferred, the membrane was blocked with 3 % BSA, followed by blotting using primary antibodies, Nrf2 (1:1000 dilution, Abcam, USA; Abclonal Canada), SOD2 (1:500 dilution, Sc-30080, Santa Cruz, USA) and β-actin (1:500, Sc-47778, Santa Cruz, USA), and the secondary antibody anti-rabbit and anti-mouse IgG HRP conjugated corresponding to primary was added. The protein expression levels were detected by using enhanced chemiluminescence using (ECL) kit (Bio-Rad, PA, USA), and the bands were captured using ChemiDoc (Fusion SL, Vilber Lourmat).

2.6. Statistical analysis

The listed biochemical parameters were analyzed using one-way ANOVA. Comparable variables between the groups were analyzed using Student's t-test and represented as mean ± SEM. All analysis for graphical plots was performed on GraphPad Prism software (v.8.4.2). Pearson's correlation analysis was used to determine the correlation between HDACs and angiogenic targets used in this study. This correlation analysis was performed on SPSS software (v.20.0).

3. Results

3.1. Anthropometric measurements and biochemical parameters

Table 1 provides an overview of the clinical signs, biochemical parameters, and anthropometric measurements of the 80 study participants from the South Indian population. It is important to note that there were no significant differences in the age and BMI of participants across the various study groups. Furthermore, elevated levels of HbA1c and postprandial glucose (PPG) were observed in the G1 to G3 DFU groups, in addition to higher levels of urea and creatinine, as compared to the G0 DFU group. It is worth mentioning that there were no significant differences in the levels of total cholesterol, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) among individuals with different grades of DFU. Further, all the tissue biopsies were subjected to RNA isolation followed by qRT-PCR and the samples were stored at −80 °C till further use.

Table 1
Details of Clinical and biochemical characteristics in the study groups.

Clinical Parameters	G0 DFU (n = 20)	G1 DFU (n = 20)	G2 DFU (n = 20)	G3 DFU (n = 20)
Gender (M/F)	10 M/10F	15 M/5F	12 M/8F	14 M/6F
Age (Years)	55.87 ± 9.37	59.26 ± 11.40	57.67 ± 10.83	60.35 ± 12.57
BMI (kg/m^2)	28.46 ± 5.13	28.70 ± 5.49	29.61 ± 3.57	29.66 ± 3.97
SBP (mm Hg)	127.36 ± 16.28	124 ± 19.03	126.56 ± 18.72	125.03 ± 11.31
DBP (mm Hg)	76.53 ± 10.53	72.64 ± 7.53	77.16 ± 11.63	77 ± 9.10
FPG (mg/dL)	147.61 ± 44.76	188.35 ± 60.15*	165.95 ± 66.38	147.72 ± 37.10
PPG (mg/dL)	239.91 ± 76.39	276.85 ± 80.41	253.54 ± 40.47	266.33 ± 61.20
HbA1c (%)	9.1 ± 2.08	9.16 ±	9.46 ± 2.13	9.82 ± 2
Total cholesterol (mg/dL)	148.32 ± 38.73	156.08 ± 54.59	143.85 ± 51.16	134.19 ± 42.12
HDL-c (mg/dL)	48.09 ± 8.11	49.57 ± 10.01	38.47 ± 12.75*	39.76 ± 10.03*
LDL-c (mg/dL)	76.13 ±	75.42 ± 29.07	75.15 ± 36.20	71 ± 27.70
Urea (mg/dL)	27.85 ± 12.21	25.85 ± 9.98	29.80 ± 14.81	32.2 ± 15.66
Creatinine (mg/dL)	1.05 ± 0.28	1.11 ± 0.28	1.31 ±	1.36 ± 0.75
WBC (10^9/L)	8.32 ±	9.25 ± 2.34	12.03 ± 5.9	11.24 ± 4.5

*p < 0.05, **p < 0.01, ***p < 0.001; ###p < 0.001; Data represented as Mean ± SEM.
* Represents comparison between G0 DFU and experimental groups. G0 - Patients with uninfected epithelialized wounds (n = 20); G1- Superficial wounds not involving tendons (n = 20); G2 - Wounds penetrating tendons (n = 20); G3 - Patients with wounds penetrating to bone or into a joint (n = 20).

3.2. Nrf2 expression in tissue biopsies of study population

As depicted in Fig. 1, a stepwise reduction in the expression of Nrf2 was evident across the various grades of DFU. Notably, a nearly 3-fold decrease in Nrf2 expression was observed in G3 DFU, accompanied by a 1.8-fold reduction in G2 DFU and a 1.2-fold decrease in G1 DFU when compared to G0 DFU (Fig. 1a). Furthermore, the decline in Nrf2 expression was reflected at the protein level, as illustrated in Fig. 1b. Relative to individuals with G0 DFU, those with G1 DFU exhibited a 0.6-fold decrease in Nrf2 expression, while G2 DFU showed a 0.47-fold reduction, and G3 DFU displayed a 0.375-fold decrease, with a p-value <0.05.

3.3. Gene expression of Nrf2 downstream targets in tissue biopsies of the study population

The downstream targets of Nrf2, namely NQO1, SOD, CAT, and HO-1 Fig. 2, exhibited a consistent expression pattern paralleling that of Nrf2. In G1 DFU cases, a noticeable decrease in mRNA expression of these Nrf2 downstream targets, NQO1, SOD, HO-1, and CAT (Fig. 2a–d) was observed, with an average fold change of 1.5 when compared to the G0 DFU study population. In G2 DFU, a more pronounced reduction was observed, with these target genes showing a two-fold decrease relative to G0 DFU. This downregulation was further exacerbated in G3 DFU cases when compared to G2 DFU. Furthermore, a reduction in the protein expression level of SOD2 (Fig. 2e) was observed as the disease progressed. Compared to G0 DFU, individuals with G1 DFU exhibited a 0.65-fold decrease, those with G2 DFU showed a 0.34-fold decrease, and this reduction was even more pronounced in G3 DFU, with a 0.07-fold change (p-value <0.001). Notably, the downregulation of both Nrf2 and its downstream target genes was consistently observed in the tissue

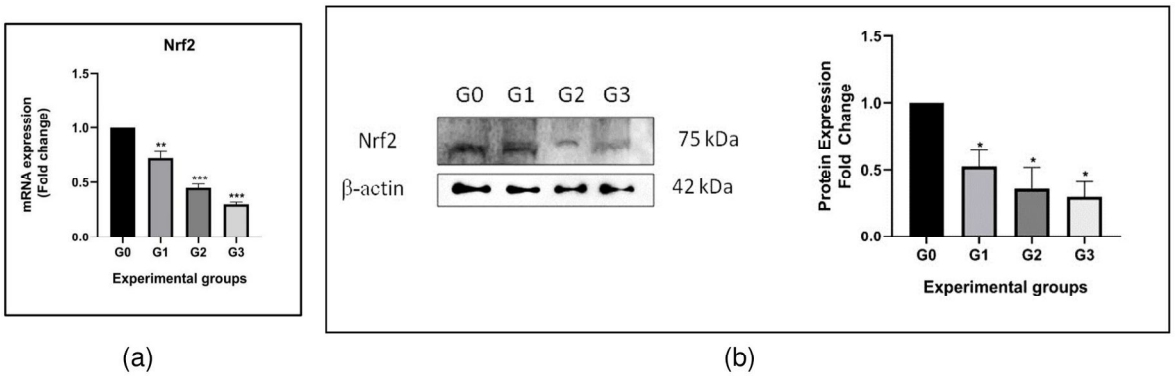

Fig. 1. Gene expression (a) and protein expression (b) of Nrf2 in tissue biopsies of different grades of DFU patients as assessed by qPCR and Western blot analysis. G0 - Patients with uninfected epithelialized wounds (n = 20); G1- Superficial wounds not involving tendons (n = 20); G2 - Wounds penetrating tendons (n = 20); G3 - Patients with wounds penetrating to bone or into a joint (n = 20). Data are represented as mean ± SEM; *p < 0.05, **P < 0.01, ***P < 0.001.

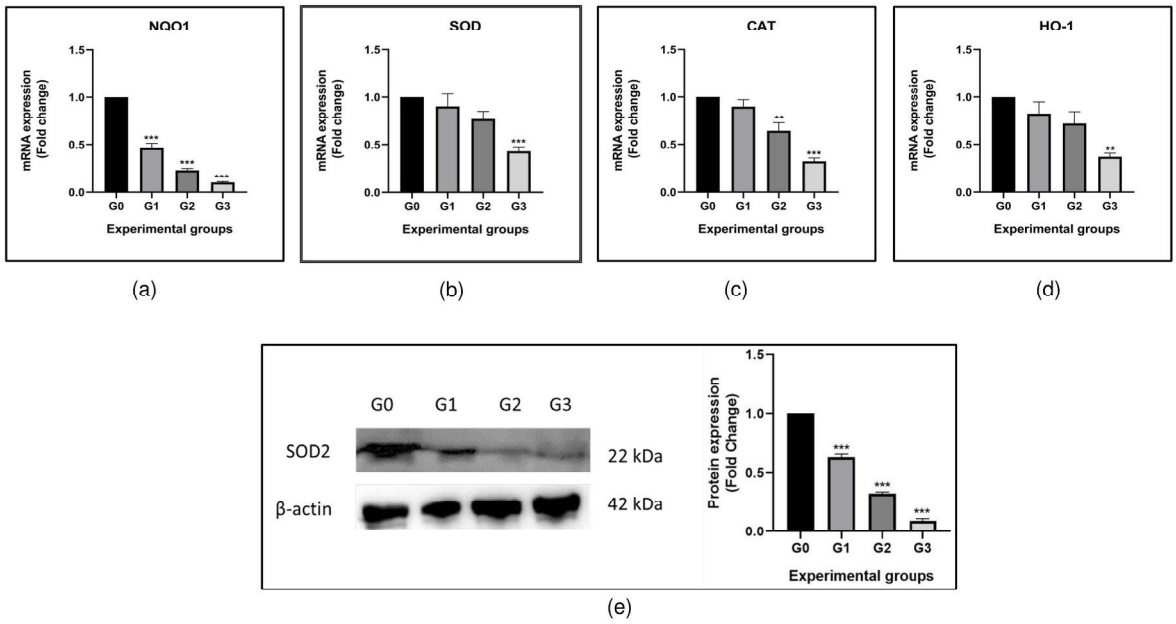

Fig. 2. Gene expression of Nrf2 downstream targets NQO1 (a), SOD-1 (b), CAT (c) and HO-1(d) in tissue biopsies of different grades of DFU patients as assessed by qPCR and Protein expression of SOD2 (e) assessed by Western blot analysis. G0 - Patients with uninfected epithelialized wounds (n = 20); G1- Superficial wounds not involving tendons (n = 20); G2 - Wounds penetrating tendons (n = 20); G3 - Patients with wounds penetrating to bone or into a joint (n = 20). Data are represented as mean ± SEM; *p < 0.05, **P < 0.01, ***P < 0.001.

biopsies of individuals as the progression of DFU.

3.4. Gene expression analysis of angiogenic and anti-angiogenic markers among the study population

The expression of angiogenic markers within the study population is depicted in Fig. 3. Notably, G1 DFU individuals displayed a decrease in the expression of angiogenic markers, including VEGF, HIF-1α, and SDF-1α, in comparison to G0 DFU. Moreover, G2 DFU participants exhibited a more significant reduction, with a 1.7-fold decrease in the expression of these angiogenic markers when compared to G0 DFU. The most substantial reduction in expression levels was observed in G3 DFU individuals when compared to G0 DFU participants. In contrast, the anti-angiogenic marker TSP-2 showed an increased expression in G1 DFU compared to G0 DFU. This upregulation continued in G2 DFU, with an even more pronounced increase observed in G3 DFU individuals compared to G0 DFU.

3.5. Analysis of the gene expression of HDACs among the study population

3.5.1. Class I HDACs

The relative gene expression of all classes of HDACs was evaluated using q-RT-PCR and is illustrated in Fig. 4. Among the class-I HDACs, HDAC1 (Fig. 4a) and HDAC3 (Fig. 4b) exhibited increased expression levels when compared to G0 DFU cases. Notably, there was a progressive increase in their expression in G2 DFU, and in G3 DFU cases, these HDACs displayed even more pronounced upregulation, with an average fold change of 1.8 compared to G0 DFU. Conversely, a significant downregulation was observed in HDAC2 (Fig. 4c) and HDAC8 (Fig. 4d) of the class-I HDACs. Furthermore, both of these HDACs demonstrated a decrease in their expression in G2 DFU when compared to G0 DFU. This downregulation was further accentuated, with an average 3-fold reduction in expression in G3 DFU when compared to G0 DFU.

3.5.2. Class IV and class II HDACs

As depicted in Fig. 5a, HDAC11 from class-IV exhibited an

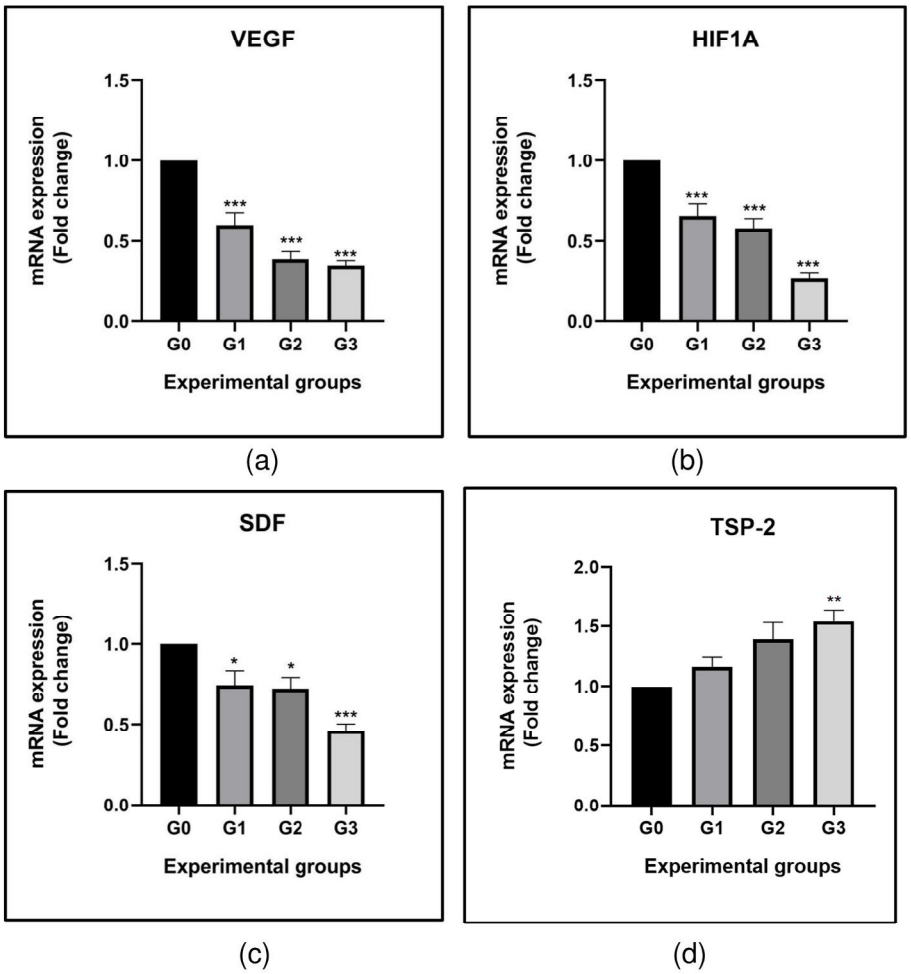

Fig. 3. Gene expression of angiogenic markers VEGF (a), HIF1A (b), and SDF (c) and anti-angiogenic marker TSP-2 (d) in tissue biopsies of different grades of DFU patients as assessed by qPCR. G0 - Patients with uninfected epithelialized wounds (n = 20); G1- Superficial wounds not involving tendons (n = 20); G2 - Wounds penetrating tendons (n = 20); G3 - Patients with wounds penetrating to bone or into a joint (n = 20). Data are represented as mean ± SEM; *p < 0.05, **P < 0.01, ***P < 0.001.

upregulation that correlated with the disease's progression, with fold changes ranging from 1.85 in G1 DFU to 2.5 in G3 DFU compared to G0 DFU. In Fig. 5b, among class-II HDACs, HDAC4 showed an increased expression, with a substantial 2.8-fold increase in G2 DFU and a remarkable 3.91-fold increase in G3 DFU when compared to G0 DFU.

Conversely, HDAC6, HDAC9, and HDAC10 displayed decreased expression patterns that paralleled the advancement of the disease. In G1 DFU, the fold changes ranged from 1.2 to 1.7 compared to G0 DFU. This downregulation intensified with disease progression, particularly in G2 DFU, where the expression of these HDACs was further reduced. Notably, in G3 DFU, the levels of HDAC5, HDAC6, HDAC9, and HDAC10 exhibited a considerable decrease, with an average fold change of 3 when compared to G0 DFU.

3.5.3. *Class III HDACs (sirtuins)*

Within class III HDACs represented in Fig. 6, SIRT1 (Fig. 6a), SIRT2 (Fig. 6b), and SIRT4 (Fig. 6c) exhibited significant downregulation with an increase in the grade of DFU. SIRT1 displayed a remarkable up to 2-fold decrease in expression across G1 to G3 DFU in comparison to G0 DFU. Similarly, SIRT2 and SIRT4 were downregulated in both G1 and G2 DFU, with an average fold change of 1.5 compared to G0 DFU. Furthermore, these HDACs displayed a 2-fold reduction in expression in G3 DFU when compared to G0 DFU.

Conversely, SIRT5 (Fig. 6d), SIRT6 (Fig. 6e), and SIRT7 (Fig. 6f) exhibited upregulation in G3 DFU, with an average fold change of 1.5, without significant increases in G1 and G2 DFU individuals when compared to G0 DFU. In contrast, SIRT3 (Fig. 6g) displayed increased expression of up to 1.75-fold in G2, and G3 cases in comparison to G0 cases. The heat map in Fig. 7a provides a concise summary of the differential expression of HDACs analyzed in the study.

Additionally, we validated the association of 18 isoforms of HDACs with Nrf2 using the String Map analysis tool (version 12.0). As depicted in Fig. 7b, the analysis predicted a direct interaction of HDAC1, 2, and 3 with Nrf2, based on data retrieved from text mining, experiments, and databases. The interaction of HDAC4 was also indicated, and it was suggested that the dysregulation of Nrf2 by HDAC4 was mediated through HDAC3 and HDAC1, which exhibited a strong affinity towards Nrf2.

3.6. *Correlation of HDACs with the expression of Nrf2 and angiogenic markers*

Table 2 shows Pearson's correlation of angiogenic markers (HIF-1α, SDF-1α, VEGF) with G3 DFUs mRNA expression of HDACs. As represented in Table 2, the HIF-1α showed a significant negative correlation with HDAC1 (r = -0.823, p = 0.003), HDAC3(r = -0.896, p = 0.014), HDAC4 (r = -0.911, p = 0.002), HDAC11 (r = -0.894, p = 0.045), SIRT5 (r = -0.802, p = 0.005) and SIRT6 (r = -0.789, p = 0.007). Also, the VEGF showed a significant negative correlation with HDAC1 (r = -0.835, p = 0.015), HDAC3 (r = -0.827, p = 0.041), HDAC4 (r = -0.940,

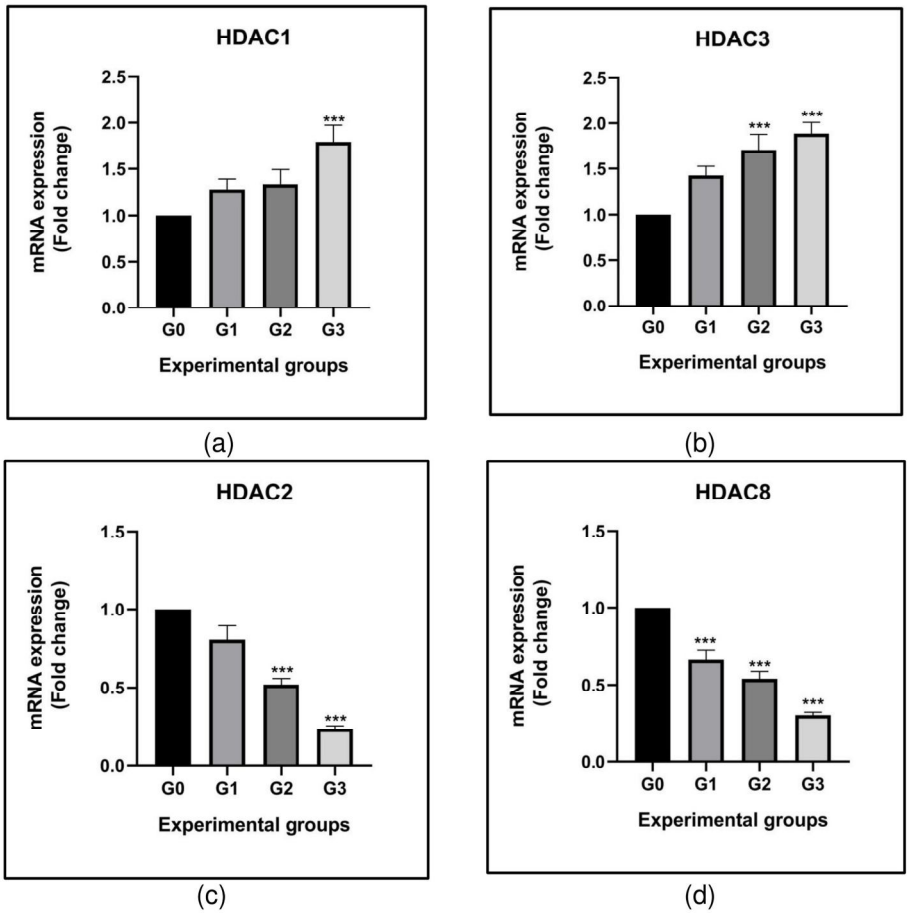

Fig. 4. Gene expression of class I HDACs. HDAC 1 (a), HDAC3 (b), HDAC2 (c), and HDAC8 (d) in tissue biopsies of different grades of DFU patients as assessed by qPCR. G0 - Patients with uninfected epithelialized wounds (n = 20); G1- Superficial wounds not involving tendons (n = 20); G2 - Wounds penetrating tendons (n = 20); G3 - Patients with wounds penetrating to bone or into a joint (n = 20). Data are represented as mean ± SEM; *p < 0.05, **P < 0.01, ***P < 0.001.

p = 0.003), HDAC11 (r = -0.862, p = 0.041), SIRT5 (r = -0.729, p = 0.017) and SIRT6 (r = -0.844, p = 0.002). Whereas the SDF-1α depicted a negative correlation with HDAC1 (r = -0.668, p = 0.035), HDAC4 (r = -0.766, p = 0.010), HDAC11 (r = -0.660, p = 0.038), SIRT3 (r = -0.664, p = 0.036), SIRT5(r = -0.742, p = 0.014), and SIRT6 (r = -0.821, p = 0.004).

Table 3 shows the correlation of HDAC levels with the expression of Nrf2 among G3 DFU participants using Pearson's correlation analysis. Among 18 HDACs, HDAC1 (r = −0.877, p = 0.001), HDAC3 (r = -0.895, p = 0.006), HDAC4 (r = -0.911, p = 0.001), HDAC11 (r = -0.797, p = 0.002), SIRT3 (r = -0.700, p = 0.011), SIRT5 (r = -0.679, p = 0.031) and SIRT6 (r = -0.708, p = 0.022) showed negative correlation and other HDACs showed positive correlation when compared with G3 DFUs Nrf2 mRNA expression.

3.7. Validation of Nrf2 and its downstream targets expressions in the hyperglycaemic microenvironment (HGM)-induced endothelial cells in vitro

To validate our findings obtained from tissue biopsies, we utilized an *in vitro* model system involving human endothelial cells exposed to an HGM. Firstly, we assessed the percentage viability of endothelial cells at various time points following exposure to high glucose and HGM containing 10 and 20 ng/mL of a cytokine cocktail. Based on the data, we selected 72 h of HGM exposure for further experiments, as this time point showed a significant decrease in cell viability (Supplementary Fig. 3). Next, we examined the mRNA expression of Nrf2 and its downstream targets. As illustrated in Fig. 8, a significant decrease in the expression of Nrf2 (Fig. 8a) was observed, with a 3.5-fold reduction ($p < 0.001$), as well as a corresponding decrease in the expression of its downstream targets, including NQO1 (2-fold, $p < 0.05$), SOD-1 (1.5-fold, $p < 0.001$), CAT (2.5-fold, $p < 0.01$), and HO-1 (2.54-fold, $p < 0.01$) (Fig. 8c), in the HGM-induced endothelial cells when compared to the control group. Furthermore, the protein expression levels of Nrf2 and SOD2 were significantly reduced in cells treated with HGM, showing fold changes of 0.39 ($p < 0.05$) and 0.34 ($p < 0.001$), respectively, compared to control cells (Fig. 8b & d). These results validate and support the clinical data obtained in our study.

3.8. Validation of HDACs expression in hyperglycaemic microenvironment-induced endothelial cells in vitro

To validate the expression of HDACs obtained from the clinical data, we employed an *in vitro* model system. As shown in Fig. 8e, HDACs 1, 3, 4, and 11 exhibited an increase in their mRNA expression in HGM-induced E9 cells. Likewise, members of the sirtuin family HDACs, specifically SIRT 5, 6, and 7, displayed increased expression when compared to control cells without HGM induction. Furthermore, HDAC isoforms, such as HDAC2, 6, 7, 8, 9, 10, and SIRT 1, 2, and 4, were found to be decreased in cells exposed to HGM compared to cells without any treatment, consistent with the data obtained from tissue biopsies of individuals diagnosed with DFU.

4. Discussion

The antioxidant defense system is deficient in people with chronic

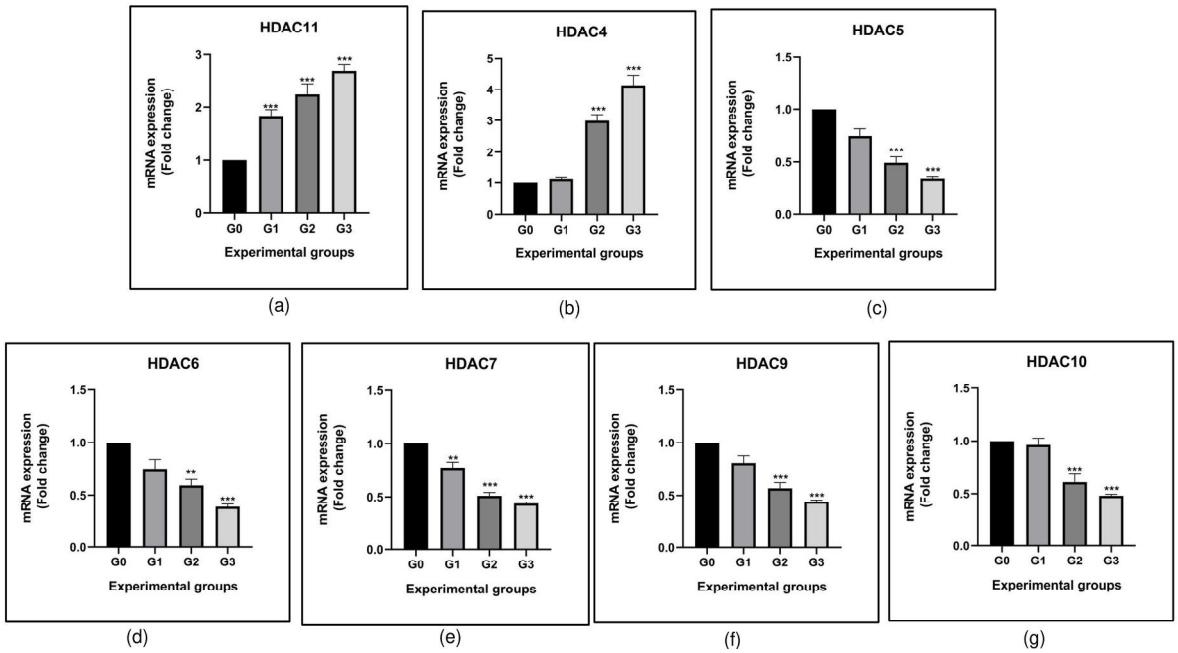

Fig. 5. Gene expression of class IV and II HDACs. HDAC11(a), HDAC4 (b), HDAC5(c), HDAC6(d), HDAC7(e), HDAC9(f) and HDAC10 (g) in tissue biopsies of different grades of DFU patients as assessed by qPCR. G0 - Patients with uninfected epithelialized wounds (n = 20); G1- Superficial wounds not involving tendons (n = 20); G2 - Wounds penetrating tendons (n = 20); G3 - Patients with wounds penetrating to bone or into a joint (n = 20). Data are represented as mean ± SEM; *p < 0.05, **P < 0.01, ***P < 0.001.

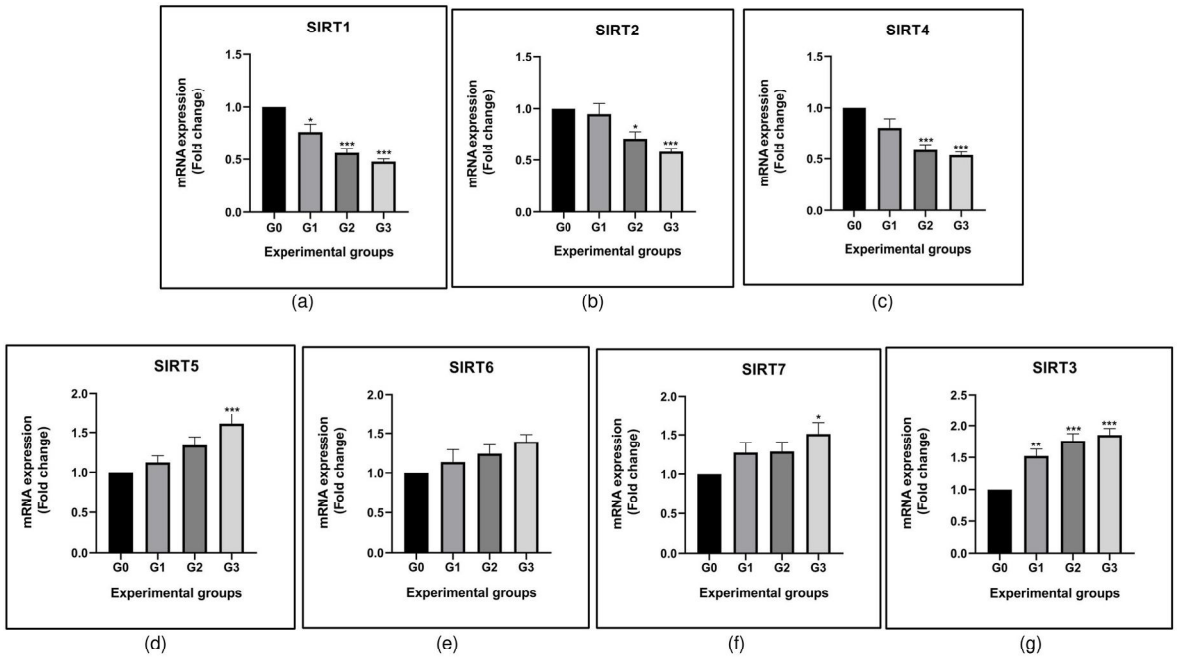

Fig. 6. Gene expression of class III HDACs (a) SIRT1, (b) SIRT2, (c) SIRT4, (d) SIRT5, (e) SIRT6, (f) SIRT7 and (g) SIRT3 in tissue biopsies of different grades of DFU patients as assessed by qPCR. G0 - Patients with uninfected epithelialized wounds (n = 20); G1- Superficial wounds not involving tendons (n = 20); G2 - Wounds penetrating tendons (n = 20); G3 - Patients with wounds penetrating to bone or into a joint (n = 20). Data are represented as mean ± SEM; *p < 0.05, **P < 0.01, ***P < 0.001.

hyperglycemic conditions, which induces vascular complications. To tackle such a stressful environment, boosting the worsened levels of a master target Nrf2 in hyperglycemic states has been identified widely with fruitful outcomes [28]. But the molecular factors that regulate the expression of Nrf2 in DFU are still unknown. In this line, we determined the expression of different HDACs in regulation Nrf2-mediated angiogenesis in people with DFU. In our study, we observed a reduced mRNA and protein expression of Nrf2, and SOD2 and reduced mRNA expression of its downstream targets in DFU participants in a grade-wise manner. Reduced growth factors like VEGF and chemokines like SDF-1α in DFU individuals in circulation and tissue have been reported [29]. In parallel, our current study reported a reduced expression of angiogenic

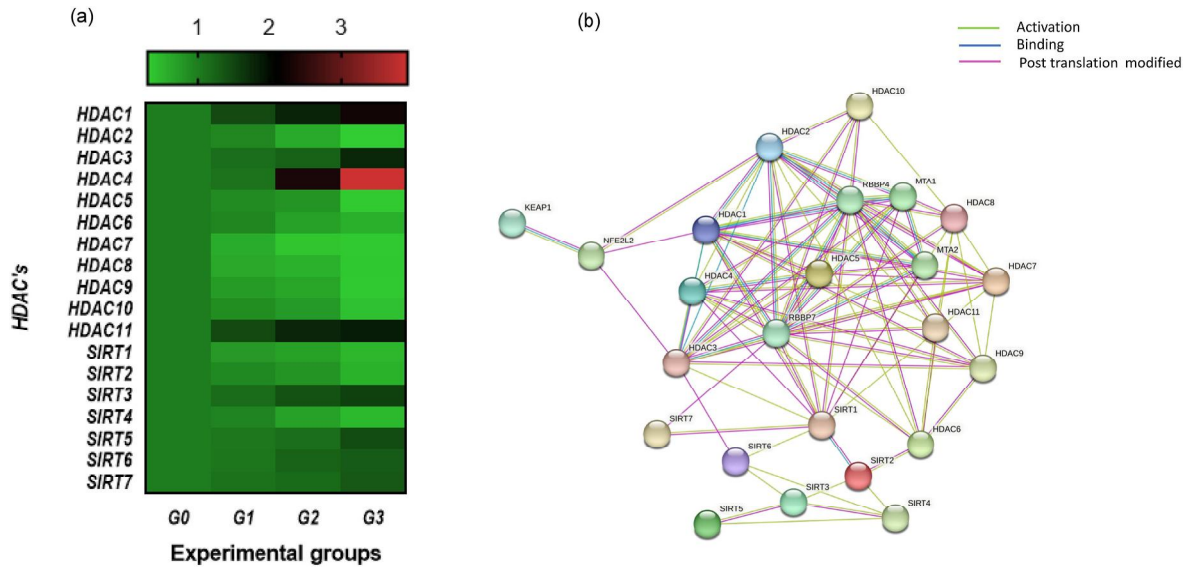

Fig. 7. Heatmap showing the differential expression of *HDAC* s (Class I–IV) in tissue biopsies of DFU study subjects created using GraphPad Prism 8.4.0. G0 - Patients with uninfected epithelialized wounds (n = 20); G1- Superficial wounds not involving tendons (n = 20); G2 - Wounds penetrating tendons (n = 20); G3 - Patients with wounds penetrating to bone or into a joint (n = 20). (b) String map analysis depicting the association between Nrf2 and different isoforms of HDACs; Interaction colours are based on published experimental results, with Green representing activation, blue represents binding and purple represents post translation modified.

Table 2
Pearson's correlation coefficient of Angiogenic markers with HDACs among G3 DFU study group.

Variables	HIF1α		VEGF		SDF-1α	
	r value	p value	r value	p value	r value	p value
HDAC1	–	0.003	−0.835	0.015	–	0.035
HDAC2	0.821		0.666	0.035	0.879	0.001
HDAC3	−0. 896	0.014	−0.827	0.041	–	0.079
HDAC4	−0.911	0.002	−0.940	0.003	−0. 766	0.010
HDAC5	0.701		0.629	0.051		0.069
HDAC6	0.731		0.602	0.065		0.079
HDAC7		0.003	0.856	0.002		0.004
HDAC8	0.851		0.869	0.001	0.882	0.001
HDAC9		0.025	0.649	0.042	0.653	0.041
HDACI0		0.030		0.026	0.710	0.021
HDAC11	–	0.045	−0.862	0.041	–	0.038
SIRT1	0.680	0.031		0.018		0.010
SIRT2	0.630	0.051	0.592	0.071		0.085
SIRT3	–	0.052	–	0.054	–	0.036
SIRT4	0.741		0.705	0.023		0.034
SIRT5	–	0.005	–	0.017	−0. 742	0.014
SIRT6	−0. 789	0.007	−0.844	0.002	−0.821	0.004
SIRT7	0.869	0.001	0.864	0.001	0.860	0.001

*p < 0.05, **p < 0.01, ***p < 0.001; r represents Pearson's coefficient and p represents significance.

Table 3
Pearson's correlation coefficient of Nrf2 with HDACs among G3 DFU study group.

HDACs	r value	p value
HDAC1	−0.877	0.001
HDAC2		0.027
HDAC3	−0.895	0.006
HDAC4	–	0.001
HDAC5		0.024
HDAC6		0.002
HDAC7		0.025
HDAC8		0.033
HDAC9		0.012
HDAC10	0.810	0.001
HDAC11	–	0.002
SIRT1		0.007
SIRT2		0.004
SIRT3	−0.700	0.011
SIRT4		0.031
SIRT5	−0.679	0.031
SIRT6	–	0.022
SIRT7	–	0.025

*p < 0.05, **p < 0.01, ***p < 0.001.The statistical significance was analyzed using SPSS software (v.20.0); r represents Pearson's coefficient and p represents significance.

markers such as VEGF, HIF-1α, and SDF-1α in a grade-wise manner, which signifies impaired angiogenesis in the DFU study population. Li et al. have identified the interplay between Nrf2 and angiogenesis through VEGF in the brain tissue of humans with venous hypertension [30]. In contrast, the expression of the anti-angiogenic factor TSP-2 is induced in a diabetic environment, contributing to impaired wound healing [31]. Consistent with this, our investigation showed that TSP-2 expression increased progressively with the severity of DFU grades. Indeed, one of our recent reports has demonstrated the role of Nrf2 in regulating angiogenesis through the MALAT1/HIF-1α loop in the tissues of DFU individuals [18]. This evidence strongly suggests Nrf2 as a chief angiogenesis regulator; thus, we assessed the upstream regulators of Nrf2 associated with its inhibition in DFU participants.

We have previously identified single nucleotide polymorphisms on the Nrf2 gene in the circulation of participants with type 2 diabetes and DFU individuals compared to controls. Through this research, a few deleterious mutations in the Nrf2 gene have been reported. However, we could not identify the causative factors of Nrf2 dysregulation in DFU [32]. Though some genetic alterations were reported initially as a potential cause for Nrf2 dysregulation, recent reports on epigenetic machinery that alter the expression of Nrf2 have given a degree of fine-tuning [33]. DNA methylation is one of the epigenetic mechanisms where most CpG-rich areas in human gene promoters are found to be hypo-methylated in the diseased state [34]. However, abnormal methylation can also be seen in cases of cancers. Another epigenetic signature, the HDACs, helps in gene silencing by removing acetyl groups from lysine residues in histone and non-histone proteins, causing alternations in the shape of chromatin [35]. It is crucial to track the molecular event of each HDACs involved in the regulation of Nrf2 to solve the puzzle of this metabolic disorder.

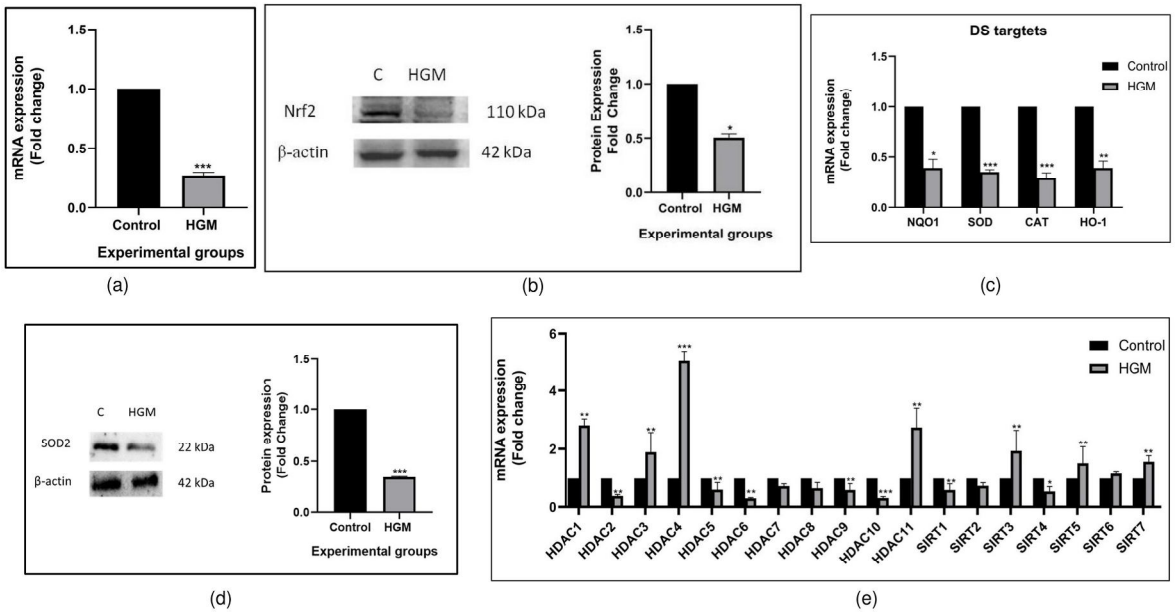

Fig. 8. Gene expression of Nrf2 (a) and protein expression of Nrf2 (b), its downstream targets (c), protein expression of SOD2 (d), and all classes of HDACs (e) in hyperglycemic microenvironment exposed endothelial cells. Hyperglycemic microenvironment, i.e. 33.3 mM of glucose with 20 ng/mL of IL-1β, TNF-α, and IFN-γ for a period of 72h, which mimics diabetic patients. Data are represented as mean ± SEM; *p < 0.05, **P < 0.01, ***P < 0.001.

Our study represented a progressive increase in the expressions of HDAC-1 and 3 under the class-I category of HDACs. The translocated Nrf2 makes a complex with small mafs, binds to the ARE site, and helps in the transcription of antioxidant genes. However, the HDAC-1 binds competitively to maf once it makes a complex with basic leucine zipper transcription factor 1 (Bach1), thus inhibiting the Nrf2-ARE-mediated antioxidant signaling. A negative correlation observed in our study between Nrf2 and HDAC-1 in the DFU study population further strengthens the deficit of antioxidant genes among DFU grades compared to controls [36]. HDAC3 also negatively regulates Nrf2 by inducing hypoacetylation, binding to the ARE site, and repressing ARE-dependent gene transcription [37]. Among class-I HDACs, HDAC-2 and 8 showed a progressive decrease in different grades of DFU. Studies have identified the negative regulation of HDAC-1 over HDAC-2. Knocking down HDAC-1 resulted in overexpression of HDAC-2 and vice versa [38]. This exciting finding by Jeong et al. further supported our data, where HDAC-1 and 2 are associated with the expression of Nrf2 in a negative and positive manner, respectively. Furthermore, a study from Mercado et al. has proved a positive correlation between HDAC-2 and Nrf2 by silencing HDAC-2, which reduced Nrf2 stability and antioxidant signaling in human bronchial epithelial cells [39]. HDAC-8 is positively associated with poor glycemic control and insulin resistance. As reported in this study, DFU participants also had poor glycemic control and hence, decreased expressions of HDAC-8 in different grades of DFU [40].

Considering class II, HDAC-4 is increased, as observed in our study. As per the finding of Wang et al., HDAC-4 activates NF-kB by increasing ROS accumulation. It is not surprising that NF-kB suppresses Nrf2, and activation of NK-kB signaling through upregulation of HDAC-4 arrests the action of Nrf2 [41], as seen in our current study. In other HDACs of class II, the HDAC-5,7,9 is positively correlated to the expression of Nrf2 in DFU, especially among the grade 3 cases compared to the different grades. To the extent of our knowledge, our current study is the first to provide evidence of the regulation of HDACs-5,7 and 9 in DFU.

Sirtuins (SIRTs) are widely classified under class-III HDACs. Their role in maintaining homeostasis in cellular functions is important, as seen in other classes of HDACs. SIRT1, whose expression is relatively low in DFU cases compared to controls, has been reported to have a direct association with HIF-1α and VEGF. Apart from Nrf2, a direct regulation of angiogenesis is also controlled by SIRT1. This was confirmed by Laemmle and the group, where silencing SIRT1 was observed to further down the expression of angiogenic markers hepatocellular carcinoma cells *in vitro* [42]. The Nrf2-specific role of SIRT1 was reported by Huang et al. where SIRT-1 was negatively correlated with IL-6, a pro-inflammatory cytokine that disturbs Nrf2 signaling [43].

Moreover, this research group also suggested a positive association between Nrf2 and SIRT-1, similar to our study. One other potential sirtuin, the SIRT-2, was observed to be comparatively low in DFU participants. This data was supported by a few reports where SIRT2 was reported to help in the dissociation of the Nrf2-Keap1 complex and facilitate Nrf2 nuclear translocation [44–47]. Nrf2-mediated SIRT3 induction has been discussed by Kim et al. in one of their earlier reports. More than the association between Nrf2 and SIRT3, this research has identified the ARE binding site in the SIRT3 gene and its role against endoplasmic reticulum stress in individuals liver disease [48].

In the same way, SIRT6 was also identified as a transcriptional coactivator of Nrf2 that protects against oxidative injury in acetaminophen-induced hepatotoxicity [49]. And SIRT7 was identified to mitigate renal fibrosis through KLF15/Nrf2 signaling in hypertension and hypertensive-mediated renal injured mice [47]. In our study, we highlight SIRT3, 6, 7 as the upstreams of Nrf2, whose dysregulation caused lowered expression of Nrf2 in the progression of DFU. Alternatively, SIRT4 was downregulated with decreased levels of Nrf2, suggesting a positive correlation among DFU individuals in a grade-wise manner. To our knowledge, we did not find any line of evidence for SIRT 4 and 5-mediated Nrf2 regulation.

The study conducted contributes to our knowledge of the complex epigenetic mechanisms controlling angiogenesis in diabetic foot ulcers (DFU). An in-depth knowledge of the regulation of all 18 kinds of HDACs is obtained their thorough profile, which notably indicates a strong negative association between Nrf2 and HDACs. Also, we have highlighted the association of HDAC-mediated dysregulation of Nrf2-impaired angiogenesis, which eventually leads to difficulty in the closure of wounds. Together HDACs are one of the critical elements for the regulation of gene expression like Nrf2 that is involved in cellular homeostatic mechanisms. Altered expression of HDACs plays an active role in disease progression, as seen in DFU, and targeting them would help foresee defined homeostasis in disease conditions like DFU.

The expression data from the clinical samples was validated by analyzing the expression pattern in hyperglycemic microenvironment-induced endothelial cells as an *in vitro* model. While there exists evidence for HDAC4-mediated podocyte injury in diabetes-induced mice kidneys [50] also, a report by Huang et al. states that HDAC3 inhibition may contribute to the activation of Nrf2 signaling thereby protecting ROS accumulation [43], we also put forth HDAC1/3 and 4 as signature epigenetic candidates in the progression of DFU associated with Nrf2 dysregulation in the advancement of DFU in humans. However, inhibition of such potent HDAC may result in a widespread alternation of Nrf2 gene regulation and help gain homeostasis for a controlled cellular response, which will be considered further. This crucial discovery suggests a paradigm shift in the management of DFU by highlighting the possibility of HDACs-regulated Nrf2-mediated angiogenesis as a focal area for focused therapeutic approaches. Though this study provides insight into the regulation of Nrf2 mediated through HDACs in the progression of DFU, this study limits the boundaries among the south Indian Population. Further studies in other populations would provide validity for the present findings.

5. Conclusion

Our research is the first to investigate the dysregulation of HDACs in the progression of DFU. While we have previously discussed the mechanisms of Nrf2 inhibition, it is important to acknowledge that the exact roles of some HDACs remain to be fully understood. Despite this, our study provides compelling evidence that HDAC1, 3, and 4 significantly contribute to the dysregulation of Nrf2, thereby playing a role in the progression of DFU. These findings suggest new therapeutic avenues, particularly the use of HDAC inhibitors (HDACi) targeted at wound sites to regulate Nrf2 epigenetically and promote proper angiogenesis in DFU. This approach holds promise for improving DFU treatment and enhancing the healing process.

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Conflicts of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Kannan Harithpriya: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ravichandran Jayasuriya:** Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **K.L. Milan:** Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Udyama Juttada:** Writing – review & editing, Validation, Software, Methodology, Investigation, Formal analysis. **Satyavani Kumpatla:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Conceptualization. **Vijay Viswanathan:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Conceptualization. **Kunka Mohanram Ramkumar:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Data curation, Conceptualization.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2024.110133.

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