Identification of Gene Targets of Activated Sirtuins in Diabetic Wound Healing

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I **Aqsa Mazhar** hereby state that my MS thesis titled "**Identification of Gene Targets of Activated Sirtuins in Diabetic Wound Healing**" is my own work and has not been submitted previously by me for taking any degree from National University of Sciences and Technology, Islamabad or anywhere else in the country/ world.

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DEDICATION

This dissertation is dedicated to my beloved late father whom I owe all my success, and whose teachings and guidance always enlighten my path.

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SC	Stratum Corneum
IL-1β	Interleukin-1β
TNFα	tumor necrosis factor α
PDGF	Platelet-derived growth factor
EGF	Epidermal growth factor
TGF	Transforming growth factor
ECs	Endothelial cells
VEGF	Vascular endothelial growth factor
EMT	Epithelial-mesenchymal transition
AGEs	Advanced glycation end products
RAGE	Advanced glycation end product
PAD	Peripheral artery disease
HDAC	Histone deacetylase activity
EMT	Epithelial-mesenchymal transition
ROS	Reactive oxygen species

SIRT1	Sirtuin 1
SIRT3	Sirtuin 3
SIRT6	Sirtuin 6
NF-κB	Nuclear factor kappa B
MM-9	Matrix metalloproteinase-9
PARP-1	Poly(ADP-ribose) polymerase 1
DNMT1	DNA-methyltransferase 1
H&E	Hematoxylin and Eosin
KEGG	Kyoto Encyclopedia of Genes and Genomes
TF	Transcription Factor
FOXO	Forkhead box transcription factors
ETC	Electron transport chain
TFAM	Mitochondrial Transcription Factor A
AMPK	AMP-activated protein kinase
NFAT	Nuclear factor of activated T-cells
VEGF	Vascular endothelial growth factor

ECM	Extracellular Matrix
XRCC6	X-Ray Repair Cross Complementing 6
NHEJ	Non-homologous end joining
BAX	Bcl-2 Associated X-protein
HUVEC	Human umbilical vein endothelial cells
HIF1α	Hypoxia-inducible factor-1A
JNK	c-Jun N-terminal kinases
PGC1α	Peroxisome proliferator-activated receptor-γ coactivator 1-α
mtTFA	Mitochondrial transcription factor A
NFR	Nuclear factor erythroid 2–related factor
NCoR	Nuclear receptor co-repressor
SMRT	Silencing mediator of retinoid and thyroid hormone receptors
VECs	Vascular endothelial cells
GLUT1	Glucose transporter 1
NNT	Nicotinamide nucleotide transhydrogenase
NMNAT3	Mononucleotide Adenyltransferase 3

MYC	Myelocytomatosis oncogene
RELA	REL-associated protein
TATAF1B	TATA-box Binding Protein Associated Factor 1B
MYOD1	Myoblast determination protein 1
KAT5	Lysine Acetyltransferase 5
PDHA1	Pyruvate Dehydrogenase E1 Alpha 1 Subunit
SOD2	Superoxide Dismutase 2
SDHA	Succinate Dehydrogenase Complex Flavoprotein Subunit A
CERS	Ceramide Synthases
KAT2A	Lysine acetyltransferase 2A
NCOA	Nuclear Receptor Coactivator 2
PKM	Pyruvate Kinase M
SMARCC2	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin subfamily C member 2

ABSTRACT

One of the complications of diabetes is a delay in the healing of wounds, which, if not treated timely, might result in the amputation of lower limbs. Sirtuins have significant functions in regulating a wide range of biological processes, including metabolic diseases and inflammation. Sirtuins 1, 3, and 6 are promising therapeutic targets in treating diabetic wounds. However, the target genes of these situations and their effects on gene expression are yet to be fully understood in diabetic wound healing. This study used lapachol to increase sirtuin levels in diabetic mice. Lapachol is a naphthoquinone and exhibits a broad range of therapeutics. In the *in-vivo* experiment, a wound was created in diabetic mice, and lapachol was applied topically on the mice skin. Wound contraction was monitored from days 1 to 10. Mice treated with 0.1% lapachol showed the most accelerated wound closure compared to other groups, signifying lapachol healing capability. An *in-silico* analysis was conducted to find key gene targets of the activated sirtuins involved in diabetic wound healing, and several functional studies were performed using tools and databases such as ShinyGO, Ingenuity Pathway Analysis and Kyoto Encyclopedia of Genes Genomes pathway (KEGG), and Cytoscape analysis. XRCC6, FOXO3, HIF1A, P53, NNT, PPARGC1A, PPARγ, PPARα, and GLUT1 are potential key gene targets of activated sirtuin 1, 3, and 6, which are probably involved in diabetic wound regulation. This can offer therapeutic potential for improving the healing of diabetic wounds.

Keywords: Sirtuins, diabetic wound healing, lapachol, gene targets

CHAPTER 1: INTRODUCTION

Skin, the largest organ in humans, is essential for nourishment and protection of the outer layer of our body from different toxins and infections. Skin plays a vital role in maintaining the biological equilibrium of the human body (Sorg et al., 2017). The human body has intricate nerve networks encompassing various sensory receptors. These receptors are present in each layer of the skin, including the epidermis, dermis, and hypodermis (Wang et al., 2021), which help maintain the structure and health of our skin through a variety of molecular and cellular activities. Skin injury initiates a complex wound-healing process, including hemostasis, inflammation, cell proliferation, and remodelling. These processes aim to restore and repair the skin's protective structure and function. The skin is affected by some internal factors such as diabetes mellitus, age, and heredity and by external factors such as environmental exposure and nutrition. Advances in understanding the physiological concepts behind skin and wound healing have led in the development of novel therapeutic techniques with the promise to enhance wound healing.

1.1 Structure and Function of Skin

Human skin contains three major layers known as epidermis, dermis and hypodermis. The role of these layers is explained below:

1.1.1 Epidermis

It includes physical, biochemical, chemical, and adaptive immunological barriers. The stratum corneum (SC) is the main element of the physical barrier, the nucleated epidermis, which includes cell-cell junctions and associated cytoskeletal proteins. It makes a substantial contribution. As a defense mechanism against pathogens and a supporter of innate immunity, the chemical/biochemical barrier comprises acids, macrophages, hydrolytic enzymes, lipids, antimicrobial peptides (Proksch et al., 2008).

1.1.2 Dermis

The membrane that connects the dermis to the epidermis. It comprises two layers of connective tissue, primarily the reticular and papillary layers. Collagen fiber bundles are embedded in a much-closed compact in the dermis. Sweat glands, muscles, hair follicles, blood vessels, sensory neurons, and hair are all found in the dermis.

1.1.3 Hypodermis

It is the deepest layer of the skin, present below the dermis. The hypodermis is situated beneath the dermis in Figure 1.1 The skin's lowest layer comprises adipose lobules, sensory neurons, blood arteries, and a few skin appendages, including hair follicles (Yousef et al., 2017).

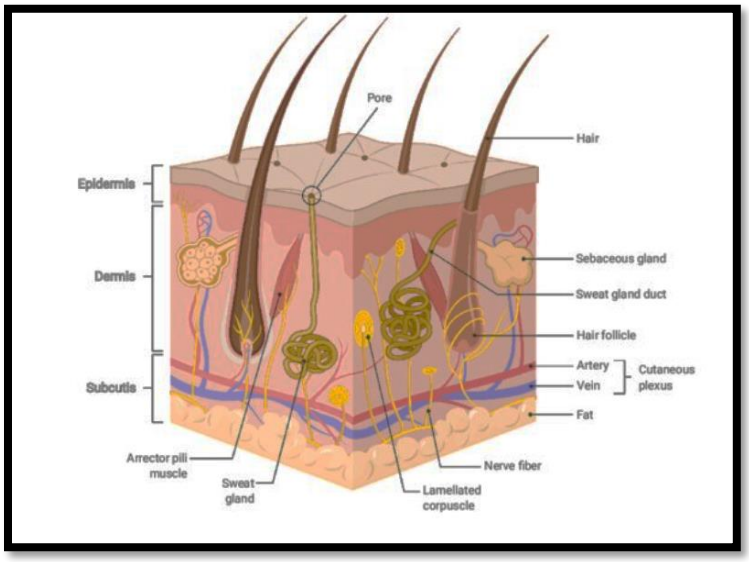

Figure 1.1 Skin anatomy detailed vector image

1.2 Wound and Classification of Wound

A wound results from injury to biological tissues such as skin and organs. Various types of injuries can cause wounds. It is crucial to thoroughly cleanse and treat the wounds in order to avoid infections or damage (Herman & Bordoni, 2020). A wound can occur due

to pathogenic mechanisms originating outside or internally inside the affected organ. They can arise from either accidental or they might be related to an underlying illness. Regardless of origin and form, injury harms the tissue and disturbs the surrounding environment (Bischoff et al., 1999). A harmful stimulus triggers a series of physiological reactions, including bleeding, constriction of blood vessels accompanied by blood clotting, activation of the complement system, and an inflammatory response.

A fully healed wound is characterized as one that has been wholly restored of the tissue's anatomical structure, function, and appearance within a suitable timeframe. Most wounds occur due to minor injuries; nonetheless, certain wound does not heal promptly and in an organized manner. Various systemic and local variables can halt the progress of wound healing by disrupting the balance of the repair processes, leading to chronic, non-healing wounds.

1.2.1 Acute wounds

Acute wounds are categorized as wounds that undergo a natural healing process, following a specific timeline and order, resulting in both functional and anatomical restoration. The typical duration of the healing process often varies from 5 to 10 days or up to 30 days (Ubbink et al., 2015). Acute wounds refer to injuries that result from environmental conditions leading to traumatic damage. These wounds exhibit a meticulous equilibrium between the generation and breakdown of cells and extracellular matrix (ECM), resulting in a methodical healing process. Acute wounds are categorized into many groups depending on the specific environmental elements that cause the damage. In

general, acute wounds may be categorized into two groups: (i) Traumatic Wounds and (ii) Surgical Wounds (Monaco & Lawrence, 2003).

1.2.2 Chronic wounds

Wounds are classified as chronic when their healing process differs from the expected path or does not fall within a standard healing path. The procedure is lengthy, and wounds identified or classified as chronic frequently resist treatment (Whitney, 2005). It is an abnormal healing process and can be primarily categorized as vascular ulcers, diabetic ulcers, and pressure ulcers (Alven et al., 2022). Chronic wounds display a continuous phase of inflammation, which leads to the attraction of microorganisms, biofilms, and platelet-derived factors, such as transforming growth factor-beta (TGF-β) or molecules produced from the extracellular matrix (ECM). The cascade of pro-inflammatory cytokines, such as IL-1β (interleukin-1β) and TNFα (tumor necrosis factor α), persists for an extended duration, resulting in significant amounts of protease in the wound bed (Pakyari et al., 2013). Protease levels in chronic wounds surpass those of inhibitors, leading to ECM (extracellular matrix) degradation and enhancing the proliferative and inflammatory stages. ROS (reactive oxygen species) are elevated at the site of a chronic lesion due to the formation of inflammatory cells, affecting ECM proteins and accelerating cellular senescence (Gushiken et al., 2021). Chronic injuries are identified by abnormalities in the cells and dermis, including lower density of growth factor receptors and diminished ability to respond to signals that promote wound healing. In contrast, proteases in acute injuries are subject to strict regulation by their inhibitors, which prevents the degradation of ECM and promotes the proliferative phase (Burgess et al., 2021).

1.3 Skin Wound Healing

The skin, the human body's largest organ, is a protective barrier against the infiltration of detrimental exterior elements, safeguarding the inside tissues and organs. A skin wound disrupts the standard anatomical structure and function of the skin. In addition, it would disturb the microenvironment of the nearby tissue (Wang et al., 2021). Wound healing is an intricate yet well-coordinated physiological process, including various cells and chemical mediators. Cutaneous wounds are characterized by the disruption of the integrity of the skin due to external or internal sources. External causes, also known as external components, can harm the skin, such as accidental wounds. On the other hand, internal components disrupt metabolic pathways, such as diabetic wounds (Irfan-Maqsood & Cells, 2018).

1.4 The Stages of Wound Healing

1.4.1 Homeostasis

Hemostasis is a crucial stage to start and maintain the healing process. The primary components of hemostasis are vasoconstriction, platelet degranulation and aggregation, and fibrin deposition, which have been widely recognised. Collectively, these occurrences lead to the creation of a blood clot and the stop of bleeding.

1.4.2 Inflammation

Macrophages remove debris and bacteria from the region and secrete many crucial cytokines and factors, including EGF, PDGF, FGF, and TGF-β, which subsequently trigger the development of granulation tissue. Macrophages function as APCs (antigen-presenting

cells) as well. IL-8 acts as a chemical signal that attracts fibroblasts and neutrophils, stimulating the cell division of keratinocytes (Pakyari et al., 2013). The wound healing involves a complex and delicate balance between causing and preventing inflammation, which must be controlled by the unique circumstances within the wound's immediate surrounding environment. Because inflammation and scarring are closely related, it's important to properly control inflammation (Baron et al., 2020). Membrane-bound receptors draw in leukocytes and other cells during this stage. Cell migration, differentiation, and proliferation are all triggered by cellular signaling pathways (Schreml et al., 2010). The first cells that enter the wound site are neutrophils, which are then followed by monocytes and lymphocytes (Behm et al., 2012).

1.4.3 Proliferation

Wound healing involves a complex and delicate balance between causing and preventing inflammation, which must be controlled in accordance with the unique circumstances in the wound's surrounding environment. Because inflammation and scarring are closely related, it's important to properly control inflammation (Baron et al., 2020). Membrane-bound receptors draw in leukocytes and other cells during this stage. Cell migration, differentiation, and proliferation are all triggered by cellular signaling pathways (Schreml et al., 2010). The first cells to enter the wound site are neutrophils, which are then followed by monocytes and lymphocytes. The body repairs and regenerates the damaged tissue during the proliferative stage of wound healing. This phase usually begins within a few days after the injury. Depending on the extent and size of the wound, it may take a few weeks or more to heal. The processes of fibroplasia, angiogenesis, and re-epithelization are involved in this stage of wound healing. Increased keratin production results from

erythropoietin's stimulation of keratinocyte proliferation and specialization. Additionally, it promotes keratinocyte migration, which is essential for re-epithelialization (Cheah et al., 2021). The proliferative phase's primary actions include:

1.4.3 Angiogenesis

Angiogenesis begins after the hemostatic plug matures into a fibroblast growth factor; platelets generate TGF-β and PDGF (platelet-derived growth factor). The body releases VEGF (vascular endothelial growth factor) in hypoxic conditions or low oxygen levels. This and other cytokines encourage endothelial cells to form new blood vessels (neovascularization) and mend damaged ones. During the progression of angiogenesis, a complex system of small blood vessels called capillaries is developed in the wound area, originating from branches of existing healthy blood vessels. Initially, the capillaries are thin and easily penetrated, causing tissue edema and the development of healing granulation tissue (Cheah et al., 2021).

1.4.4 Fibroblast Proliferation and Collagen Production

After an injury, fibroblasts and myofibroblasts in the nearby tissue are stimulated to undergo rapid cell division for three days (Li et al., 2007). Subsequently, they move toward the wound, drawn by molecules like PDGF and TGF-β secreted by inflammatory cells and platelets. Fibroblasts emerge in the wound three days after damage. Upon entering the wound, they rapidly multiply and generate the matrix proteins proteoglycans, fibronectin, hyaluronan, and type 1 and type 3 pro-collagen (Harper et al., 2014).

1.4.5 Granulation tissue formation

Granulation tissue is essential for wound healing during the proliferative phase. The newly developed connective tissue is distinguished by its reddish or pinkish color, caused by freshly created capillaries that provide vital nutrients and oxygen to the healing area. Granulation tissue plays a crucial role in the latter phases of wound healing by offering a supporting ECM rich in collagen and other structural components.

1.4.6 Re- epithelialization

Epithelial cells rapidly migrate from the wound borders shortly after the initial injury, forming a cohesive layer that covers the wound and adheres to the underlying matrix. EMT (epithelial-mesenchymal transition) is a biological process that enables epithelial cells to acquire the ability to move and migrate over the surface of a wound. This phase can be finished within 24 hours in wounds that are essentially closed. Changes in cytokine levels induce a transition of epithelial cells from a mobile state to cell division, facilitating the replenishment of epithelial cell populations and the wound-healing process (Harper et al., 2014).

1.4.7 Wound contraction

Myofibroblasts, specialized cells capable of contraction, contribute to wound healing by reducing the size of the wound by contracting and bringing the wound edges closer together. This procedure reduces the surface area that requires coverage by new tissue.

1.4.8 Remodeling

It is the last phase of wound healing and persists for a period of 6-24 months following the initial damage. This process encompasses the regression of blood vessels, the remodeling of granulation tissue, and the production of new extracellular matrix (ECM) components (Ramasastry, 2005). This stage entails a delicate equilibrium between combining elements to form new substances and breaking down substances as the collagen and other proteins deposited in the wound gradually become more structured and orderly (George Broughton et al., 2006).

1.5 Diabetes Mellitus

It is a chronic metabolic disease that is becoming more frequent in society. It is defined by sustained hyperglycemia, which is increased blood glucose levels, and this condition can have significant, long-lasting medical implications (Burgess et al., 2021). An autoimmune disorder, type 1 diabetes (5% prevalence), and type 2 diabetes (95%) that are related to obesity and is the most prevalent forms of diabetes. Diabetes other forms are exceedingly rare, and result from a single gene mutation; gestational diabetes is a form of diabetes that progresses during pregnancy (Dwivedi & Pandey, 2020). Diabetes is an incurable condition. Elevated blood glucose levels result in symptoms such as frequent urination, heightened thirst, and increased appetite (Kumar et al., 2020).

According to 2019 statistics, a total of 135.6 million people aged between 65 and 99 years old worldwide were expected to have diabetes; by 2030, this number is projected to increase to 195.2 million, making diabetes the most prevalent global epidemic of the twenty-first century (Deng et al., 2023). Diabetes is anticipated to impact 26.7% of the

adult population in Pakistan in 2022, with a cumulative incidence of around 33 million cases, as reported by the International Diabetes Federation. Moreover, this number continues to rise annually, a cause for concern. Additionally, it is plausible to suggest that a considerable number of patients remain undiagnosed, thereby substantially increasing the true prevalence and potential risks associated with untreated complications (Azeem et al., 2022).

Left untreated, diabetes can lead to numerous consequences. Diabetic individuals commonly experience persistent injuries, including diabetic foot ulcers (DFU). These wounds exhibit characteristics indicating an extended wound-healing trajectory, leading to the need for extensive treatment and subsequent amputation of limbs (Alven et al., 2022). Foot ulcer complications include functional impairment, infection, hospitalization, amputation, and even loss of life. The collective risk of having a foot ulcer ranges from 19 to 34 percent, and it increases as the medical complexity and longevity of diabetic patients rise. Increase morbidity and mortality rates is related to incident ulceration: 65% recurrence rates at 3–5 years, 20% lifetime lower extremity amputation incidence, and 50–70 percent five year mortality. Following a prolonged period of decline, new data indicate that the incidence of amputation has increased by up to 50 percent in some regions over the past several years, particularly among young and racial and ethnic minority populations (McDermott et al., 2023).

The effective management of wounds in individuals with diabetes has emerged as a substantial concern for the worldwide healthcare community, resulting in significant effects on the quality of life suffered by people with diabetes (J. Liu et al., 2022). Chronic diabetic wounds present a significant consequence associated with diabetes, a condition

characterized by persisting inflammation, reduced epithelialization motility, and poor healing of the wound (Y. Liu et al., 2022).

1.6 Pathophysiological Factors of Diabetic Wound Healing

1.6.1 Impaired Angiogenesis

Diabetic wounds experience reduced blood flow caused by vascular problems and decreased formation of new blood vessels, resulting in long-term oxygen depletion and inadequate nutrient supply. This leads to chronic tissue damage (Peppa et al., 2009). The process of angiogenesis associated with diabetic wounds is intricate, involving the disruption of proteins by AGEs (advanced glycation end products), the impairment of ECs (endothelial cells) function due to micro-environmental imbalances, and epigenetic changes. Furthermore, all of these factors are interrelated, leading to the development of angiogenesis conditions (Chen et al., 2023). Figure 1.2 shows the pathophysiological aspects of diabetic wounds.

1.6.2 Hyperglycemia:

Prolonged hyperglycemia in poorly controlled diabetes can cause the glycation of basement membrane proteins in small and medium-sized blood vessels. It results in a partial blockage and damage to the blood vessel wall, which induces ischemia in tissues, particularly in the peripheral appendages (lower limb and toes) where venous congestion impairs circulation. Foot ulcers of the lower limbs are prevalent in individuals with improper diabetes management. Additionally, the presence of diabetes-related neuropathy and paresthesia in the lower extremities may increase the risk of developing ulcers in response to minor

injuries. Inadequate blood flow to these tissues contributes to ulcer development and hinders healing (Bolajoko et al., 2020).

The following four primary mechanisms, including the production of AGEs (advanced glycation end products), hexosamine activation and polyol pathways, mitochondria dysfunction, and elevated oxidative stress (OS), all have been linked to cell damage in hyperglycemia. The oxidative stress and prolonged hyperglycemia accelerate the formation of advanced glycation end products, which deteriorate matrix rigidity and cause fibrosis via the receptor for RAGE (advanced glycation end products). Furthermore, inflammation, including endothelial activation induced by diabetes, is accompanied by an extensive range of GF and inflammatory cytokines, damages the cells and activates second messengers by binding to their corresponding receptors (Wan et al., 2022).

1.6.3 Peripheral Neuropathy

Diabetes mellitus results in inflammatory dysregulation, characterized by an imbalance in the pro- and anti-inflammatory cytokine response and a decrease in neuropeptide activity. Leukocytes are directly affected by neuropeptides, which contribute to cytokine dysregulation. Furthermore, keratinocytes, endothelial cells, and fibroblasts are all affected directly by cytokines and neuropeptides, which inhibit their proliferation and cause aberrant angiogenesis, re-epithelialization, and ECM synthesis. Impairment of cutaneous wound healing results from dysregulation in re-modeling and granulation tissue deposition, decreased neovascularization, and re-epithelialization, all of which are influenced by the abnormal cytokine expression profile (Theocharidis & Veves, 2020).

1.6.4 Peripheral Artery Disease

PAD (peripheral artery disease) refers to the complete or partial blockage of one or more peripheral arteries, excluding intracranial and non-cardiac arteries, in the upper and lower extremities, which can result in compromised blood circulation or the loss of tissue (Soyoye et al., 2021). Peripheral artery disease is a risk factor for delayed wound healing, amputation (including major and minor lower-extremity amputations), and infection. It can cause mortality in both people with type 1 and 2 diabetes and contributes to 50 to 70% of diabetic foot ulcers (McDermott et al., 2023).

1.6.5 Hypoxia

A hypoxic environment is a typical result of DFU (diabetic foot ulcers) in diabetic patients, which happens because of impaired circulation (Burgess et al., 2021). In diabetic conditions, the impaired vasculature obstructs oxygen delivery to the wound, resulting in a hypoxic environment surrounding the wound right after the injury (Catrina & Zheng, 2021). This hypoxic condition is further increased by the recruitment of inflammatory cells. While acute hypoxia initiates tissue repair and promotes cell proliferation, long-term oxygen deprivation in chronic wounds impairs healing by inhibiting extracellular matrix (ECM) synthesis, re-epithelialization, and angiogenesis. Consequently, increased wound tissue oxygenation is essential for chronic wound healing (Guan et al., 2021).

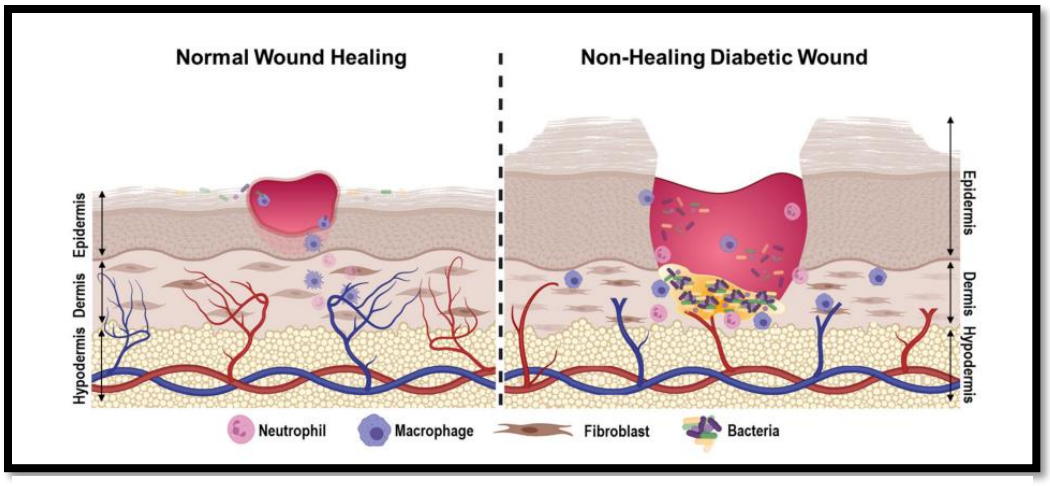

Figure 1.2: The pathophysiology of diabetic wounds.

Diabetic wounds demonstrate disrupted formation of new blood vessels, consistently poor inflammatory response, elevated levels of ROS, and persistent bacterial colonization that frequently transforms into a difficult-to-treat biofilm (Burgess et al., 2021)

1.6 Sirtuins the Histone Deacetylase Superfamily

Eukaryotic histone deacetylases are members of an evolutionary protein family called the sirtuins family, which is divided into two subfamilies with varying HDAC (histone deacetylase) activity: the sirtuins family and the classical HDAC family. The eleven members of the classical HDAC family are classified into three groups based on whether they are Zn2+-dependent enzymes with a Zn2+ ion in their catalytic pocket: class II (HDAC4, 5, 6, 7, 9, and 10) resembles the yeast Hda1 and class IV (HDAC11), whose function is still unclear. Class I (HDAC1, 2, 3, and 8) is closely connected to the yeast transcriptional regulator Rpd3. 5. Class III HDACs include sirtuins (Fiorino et al., 2014).

Sirtuins are a diverse group of deacetylases known by their need for NAD+ as a cofactor for enzymatic activity (Beegum et al., 2022). They were initially classified as class III Histone deacetylases but are now recognized as class III Lysine deacetylases (Edatt et al.,

2020). It plays a vital role in cellular processes such as DNA repair, transcription, stress resistance, and metabolism (Feldman et al., 2012). Unlike other kinds of HDACs, which bind to a water molecule to acetylated lysine via the Zn2+ active site, the sirtuins transfer an acetyl group from the lysine side chain of the substrate to the NAD+ cofactor, producing a deacetylated substrate, nicotinamide, and 2′-O-acetyl-ADP-ribose. Within the intracellular milieu, it is observed that SIRTs 1, 6, and 7 predominantly reside within the nucleus, while SIRTs 3, 4, and 5 exhibit localization within the mitochondria. Additionally, SIRT2 is primarily present within the cytoplasm (Ezhilarasan et al., 2022). Sirtuins are a family of evolutionarily conserved proteins, and they have been identified as correlated to the pathological and physiological mechanisms of the skin. Their sub-cellular localization and phylogenetic classification is mentioned in table 1.1 (Beegum et al., 2022).

Table 1.1: Sub-cellular localization and phylogenetic classification of sirtuins (Beegum et al., 2022).

Category	**Classification**	**Sub-cellular Localization**
Category 1	Sirtuin 1	Nucleus, Cytosol
Category 2	Sirtuin II	Cytoplasm
Category 3	Sirtuin III	Mitochondria
Category 4	Sirtuin IV	Mitochondria
Category 5	Sirtuin V	Mitochondria
Category 6	Sirtuin VI	Nucleus
Category 7	Sirtuin VII	Nucleus

1.6.1 The Crucial Role of Sirtuins in Wound Healing

SIRT 1, 3, and 6 (sirtuin 1), (sirtuin 3), (sirtuin 6) are not only important targets, but also have enormous potential to promote diabetic wound healing (Beegum et al., 2022). SIRT1 and SIRT3 are two of the most essential sirtuin proteins, playing critical roles in cell regeneration and in cell longevity (Bibi et al., 2021). SIRT1's important role in controlling cellular differentiation, senescence, as well as its ability to modify metabolic pathways in response to nutritional availability, have been proven in a variety of tissues (Potente et al., 2007). Recent research also indicates that pharmacologically increasing SIRT1 activation enhances vasculature and wound healing in diabetes by reducing the level

of reactive oxygen species (ROS) production (Prabhakar et al., 2020). SIRT3 additionally, is significant for physiology because it modulates mitochondrial oxidative pathways, hence limiting the rate of reactive oxygen species generation (Ansari et al., 2017). Figure 1.3 illustrates the roles of sirtuins 1, 3, and 6 in healing of diabetic wounds. It explains that diabetes causes a drop in sirtuin 1 levels, which inhibits the recruitment of macrophages, neutrophils, fibroblasts, and mast cells. It also reduces the MMP pathway, limiting angiogenesis. The sirtuin 3 down-regulation decreases VEGF (vascular endothelial growth factor) production and decreases the anti-oxidative capabilities of cells, resulting in a delay in wound healing. This behavior is connected with impaired mitochondrial function and increased necroptotic activity (Zhang et al., 2022). One important protein involved in controlling glucose homeostasis is sirtuin 6. When it is absent, insulin synthesis in response to glucose stimulation is impeded and glucose intolerance develops. Figure 1.3 illustrates how improper wound closure has been linked to SIRT 6 downregulation in cutaneous wounds. Phosphorylated NFKB expression is indicative of a delayed healing process (Beegum et al.2022).

SIRT1 and SIRT3 work together to maintain cellular metabolism by deacetylating various proteins that promote catabolic activities while concurrently blocking anabolic activities, thereby facilitating the accumulation of cellular energy reserves. Various metabolic diseases can arise due to impairments in this pathway regulated by SIRT1 and SIRT3 (Nogueiras et al., 2012). The deacetylation of FOXO1 by SIRT1, which is a negative regulator of angiogenesis, was observed to be disrupted in endothelial cells lacking SIRT1. Consequently, these SIRT1-deficient endothelial cells exhibited atypical angiogenic behavior, which can be attributed to the increased activity of FOXO1

(Prabhakar et al., 2020). SIRT1 activation prevents diabetes-induced mitochondrial and vascular damage by inhibiting the activation of nuclear factor kappa B (NF-κB), Matrix metalloproteinase (MMP)-9, and PARP-1 (poly(ADP-ribose) polymerase 1), as well as lowering histone acetylation of the DNMT1 promoter. Diabetes-induced decreased liver X receptor signaling (RXL) leads to dysregulated cholesterol metabolism and increased production of pro-inflammatory cytokines. SIRT1 is necessary for angiogenesis. SIRT1 levels decrease with the start of macro-vascular diseases, although they are maintained to prevent harm. In a similar vein, lower SIRT1 levels is related to the development of microvascular problems (Hammer et al., 2021). SIRT3 deficiency reduced blood circulation surrounding the specific wound area, elevated oxidative stress levels, and delayed wound healing. This study's findings indicate that SIRT3 restricts the production of ROS, enhances angiogenic responses, and accelerates wound healing in patients with diabetes (Yang et al., 2020). While data suggests that sirtuins play a role in various diabetes-related health concerns, it is yet to understand the detailed functions and mechanisms of Sirtuin 1, 3 and 6 gene targets in diabetic wound healing. Understanding this data is vital to develop specific and novel treatments to promote wound healing.

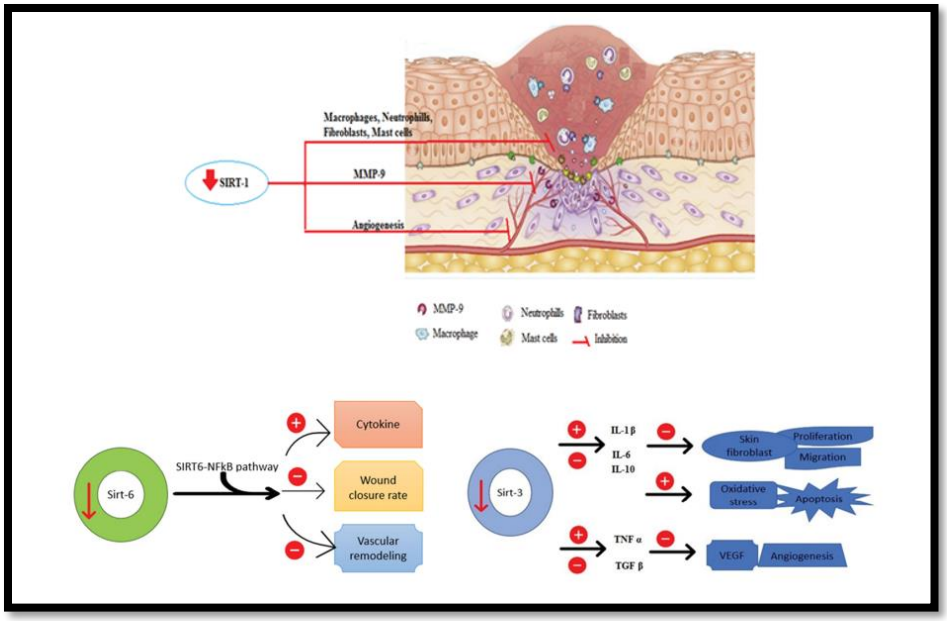

Figure 1.3 Role of SIRT1, 3, and 6 in diabetic wound healing.

(Beegum et al., 2022)

1.7 Lapachol

A naphthoquinone exhibits a broad range of therapeutic activity, including but not limited to anti-inflammatory, anticancer, anti-ulcer, antiseptic, anti-abscess, antiedemic, and bactericidal effects (Hussain et al., 2007). The biological activity of topoisomerases, enzymes crucial to DNA replication, can be disrupted by natural and synthetic naphthoquinone variants. The quinone derivatives are being utilized as pharmaceutical agents treating several tropical diseases. The grains of certain lumber trees include a yellow-colored substance called lapachol, which is obtained by the extraction from plants classified under the Bignoniaceae botanical family (Bibi et al., 2021). The laboratory-based investigation shows the efficacy of lapachol and its analogs in treating psoriasis was demonstrated through their ability to inhibit the proliferation of HaCaT, a human

keratinocyte cell line, and mitigate inflammatory responses (Hussain et al., 2007). Additionally, several lapachol analogs have been developed and used to treat a variety of cancers, including kidney, colon, melanoma, lung, breast, glioblastoma, prostate, ovarian, and leukemia. Furthermore, it has been documented that certain lapachol analogs have anticancer effects ten times stronger than the parent substance. Lapachol (1) has been shown to have several biological actions, including antibacterial, antimetastatic, antileishmanial, antiviral, antiviral, antiparasitic, anticancer, and analgesic. Anti-inflammatory, acaricidal, bactericidal, pesticidal, schistosomicidal, termiticidal, fungicidal, anti-abscess, molluscicidal, anti-epidemic, anti-Trypanosoma cruzi, and insecticidal (Hussain & Green, 2017).

When lapachol (1) is combined with bismuth (III), it has been demonstrated to have antiangiogenic properties. Recently, it has been demonstrated that T. impetiginosa's β-Lapachone (2) and α-lapachone (3), isolated from the plant, also have intriguing biological activity. Moreover, β-lapachone (2) has a wide range of physiological and pharmacological properties, including DNA damage, antifungal, anti-inflammatory, anticancer, and antibacterial properties (Guiraud et al., 1994). Interestingly, β-lapachone (2) can destroy non-transformed cells while selectively inducing cell death in different human cancer cells (Boothman et al., 1989).

1.8 Rationale

One of the biggest challenges reported in individuals with diabetes is delayed wound healing, distinguished by a persistent pro-inflammatory response and dysregulations in the processes of angiogenesis and collagen deposition. The sirtuin

enzyme family regulates various pathophysiological processes, some of which are involved in improving life expectancy, glycolysis, inflammation, and DNA repair (Dzidek et al., 2023). In this study, we will focus on finding molecular targets of sirtuins to better understand their effects by using compound lapachol in diabetic wound healing and how they influence critical processes involved in diabetic wound repair and tissue regeneration.

1.9 Objectives

These are the two main objectives of this study.

1. Identify the sirtuin gene targets involved in diabetic wound healing.

2. To further understand the biology of sirtuins to diabetic wound healing.

CHAPTER 2: MATERIAL AND METHODS

2.1 Ethics Statement

All animal procedures were conducted strictly according to the Principles of Animal Laboratory Care. The Institutional Review Board of the National University of Sciences and Technology (IRB-NUST) reviewed and approved the experiment, ensuring that all procedures followed ethical guidelines.

2.2 Grouping of Mice

The chemical lapachol was bought from a company (Sigma Aldrich). Swiss albino BALB/c mice, aged 5-6 weeks and weighing 25-35 grams, were purchased from the National Institute of Health, Islamabad. The mice were then randomly allocated to one of three groups:

I. A control group (untreated diabetic group)

II. A vehicle group

III. A lapachol 0.1% group

Only 10 mice were assigned to each group. The mice were kept under acclimatization for one week at controlled temperatures and environments.

2.3 Diabetes Induction

To induce diabetes, the mice were starved overnight. The next day, they were weighed, and a single intraperitoneal injection of alloxan monohydrate (Sigma Aldrich)

solution freshly dissolved in normal saline solution at a dose of 200mg/kg of body weight was given. Following the injection, the food supply was replenished, and 20% sucrose solution was provided in each cage to avoid the risk of mortality by hypoglycemic shock. After 12 hours, the sucrose water was substituted with regular water. Glucose levels were monitored using an (Accu-Check Instant S, gluco-meter) before and 24 hours after discontinuing sucrose water. Mice that exhibited a blood glucose level of 300 mg/dL a few days after receiving an injection were classified as diabetic.

2.4 Incisional wound model

After diabetes was confirmed in all mice, the animals were anesthetized using ketamine (1 mg/kg weight) administered through intraperitoneal injection. Hair was removed from the back of each mouse using hair removal cream and distilled water. Before wound incision, the mice's skin was cleaned with alcohol (70% ethanol). Two parallel full-thickness wounds were created on the dorsal posterior region of mice by using a 6mm biopsy punch.

2.5 Treatment

Mice treated with 200 μL of 0.1% solutions of lapachol prepared in a mixture of (Propane diol, Ethanol, and Distilled water) in ratio of 5:3:2. The mixture was topically applied once daily for 10 days post-wounding. Mice in the vehicle group were treated with solvent only.

2.6 Wound Contraction Measurement

The area of the wound in mice was photographed using a digital camera from day 0 to day 10 post-wounding, and the data was analyzed using "ImageJ software." The percentage of wound closure was calculated from the results of wound measurements taken on different days. The initial wound area (day 0) and the wound area on the 3^{rd}, 7^{th}, and 10^{th} were used to calculate the wound closure percentage using the following equation: (A0 - AX)/A0 multiplied by 100. A0 represents the initial wound area on Day 0, and AX represents the wound area on Day X after the incision.

2.7 Sample Collection

On days 3rd, 7th, and 10th post-wounding, 3 mice from each group were euthanized by using chloroform. The central wound area from each wound was collected and stored.

2.8 Histological Evaluation

For histology, tissue samples of the wound were embedded in paraffin wax after being fixed in a 10% formalin solution. The five-meter thick sections were cut and stained with hematoxylin and eosin (H&E), and Mason's trichome as per standard method (Bibi et al., 2021).

2.9 Microscopic Examination

H&E staining was used to analyze tissue granulation epidermal regeneration, and Mason's trichome staining was used to examine collagen deposition. Both stainings were analyzed and visualized under a light microscope at 10x.

2.10 Computational Analysis of Sirtuin-Related Gene Targets Involved in Diabetic Wound Healing

2.10.1 Data Collection and Predicting Diabetes wound associated Gene Target

For gene selection, the first set of gene targets linked to SIRT1, SIRT3, and SIRT6 was selected by a thorough analysis of clinical literature and open databases, emphasizing genes linked to wound healing and diabetes-related metabolic processes. The NCBI Gene database, GeneCards, and PubMed were the primary resources.

2.10.2 Tools for Prediction

To find potential pathways, gene targets were anticipated through databases and online tools such as the QIAGEN Ingenuity Pathway Analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG). The STRING pathway analysis was also used for PPI network interactions. The database (ShinyGO 8.0) provides information on pathway enrichment folds, practical annotation, and protein-protein interactions (PPI). These tools help identify potential targets based on known interactions and biological significance.

2.10.3 Pathway Enrichment Tools

ShinyGO and KEGG (Kyoto Encyclopedia of Genes and Genomes) were tools used to complete the pathway enrichment evaluation. Using these systems, the expected gene targets were analyzed and mapped in ShinyGo providing gene enrichment pathways of target genes, with particular attention paid to those involved in wound healing, metabolism, and mobile strain responses.

2.10.4 Network Analysis of Protein-Protein Interactions (PPI)

PPI Network Construction: The STRING database was utilized to build the PPI networks for the SIRT1, SIRT3, and SIRT6 gene targets. This study helped to clarify the intricate biological networks involving those sirtuins by providing an observable and quantitative evaluation of the relationships among the identified gene targets.

2.11 Essential Hub Genes Identification

The Cytoscape analysis with the cytohubba plugin was performed to identify essential hub genes in the common SIRT,1, 3 and, 6 biological networks. Through analyzing protein interactions it helped to identify potential drug targets.

CHAPTER 3: RESULTS

Our study has shown that the healing process substantially increased in the 0.1% lapachol-treated group compared to the wounds in the vehicle and control groups. The wounds treated with lapachol showed quicker and more significant closure throughout the 10-day observation period. Over time, the treated group showed an apparent reduction in the wound size, indicating that lapachol has a high potential to speed up tissue healing. Photographs taken from days 0-10 provide visible proof of this progress, most likely demonstrating quicker and more distinct healing of the wounds in the lapachol group. The vehicle group, on the other hand, did not show drastic wound closure or wound healing in diabetic mice. This implies that lapachol promotes wound closure and that the solvent alone does not much aid the healing process. The slowest rate of wound closure was seen in the control group, which did not receive any drug, confirming the efficacy of lapachol in accelerating healing shown in Figure 3.1.

The statistical significance shows that the rapid wound healing was caused by lapachol. These findings indicate that 0.1% lapachol is a potentially useful substance for promoting wound healing, especially in situations when wound repair is impaired, such as diabetes.

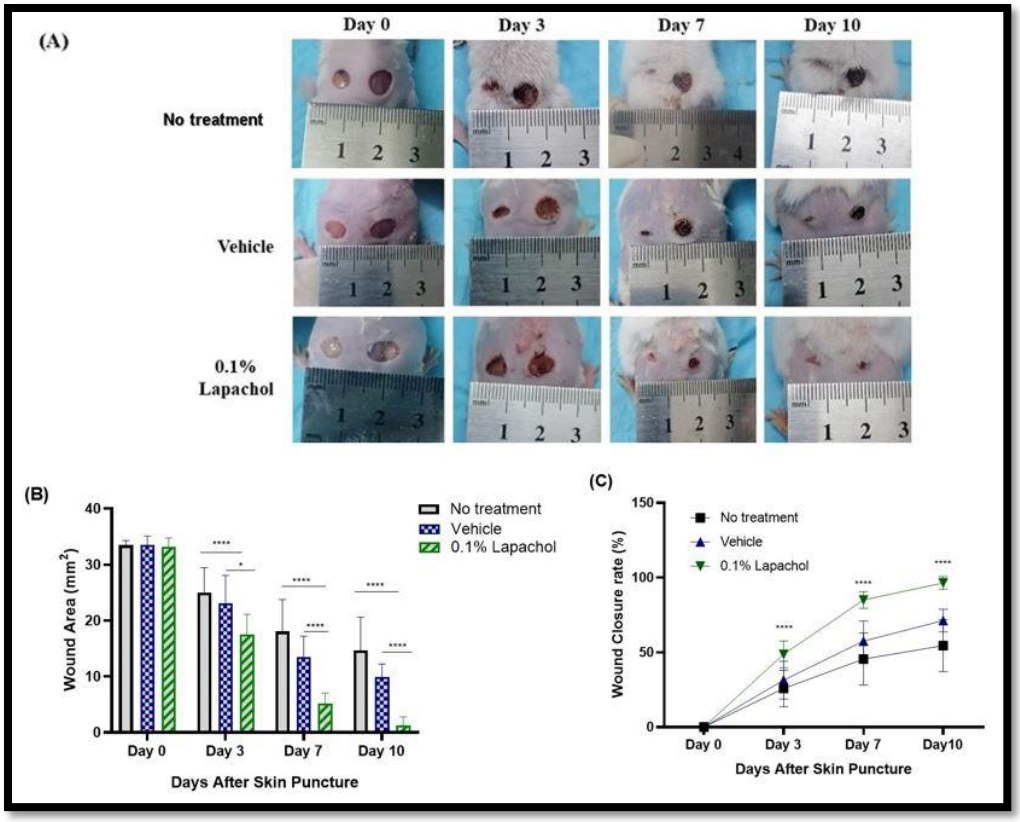

Figure 3.1: Effect of lapachol to enhance wound healing.

(A) Representative images of diabetic mice skin wounds from the lapachol-treated, vehicle-treated, and untreated groups on 0, 3rd, 7th, and 10th-period post-skin puncture. (B) Graphical representation of average wound area in different groups on different time points post-wound. (C) Graphical representation of wound closure (%) on different time points post-wound. The values were statistically analyzed by one-way ANOVA (n = 3) and represented as means ± SD. ∗p 0.0116, ∗∗∗p < 0.001 and ∗∗∗∗p < 0.0001 vs. no treatment group.

3.1 Histological Analysis

Hematoxylin and Eosin (H&E) staining was performed on skin samples obtained from 3 mice per group on the third, seventh, and tenth day of the experiment to examine the impact of lapachol on the production of granulation tissue, neo-epithelium, and skin regeneration. On days 3, 7, and 10, lapachol-treated mice showed higher tissue regeneration and granulation tissue production than the vehicle-treated group. The

epidermal and dermal layers were both well-developed and well-defined. The vehicle group that was given solvent treatment did not exhibit notable tissue regeneration or well-characterized dermal and epidermal layer development shown in Figure 3.2.

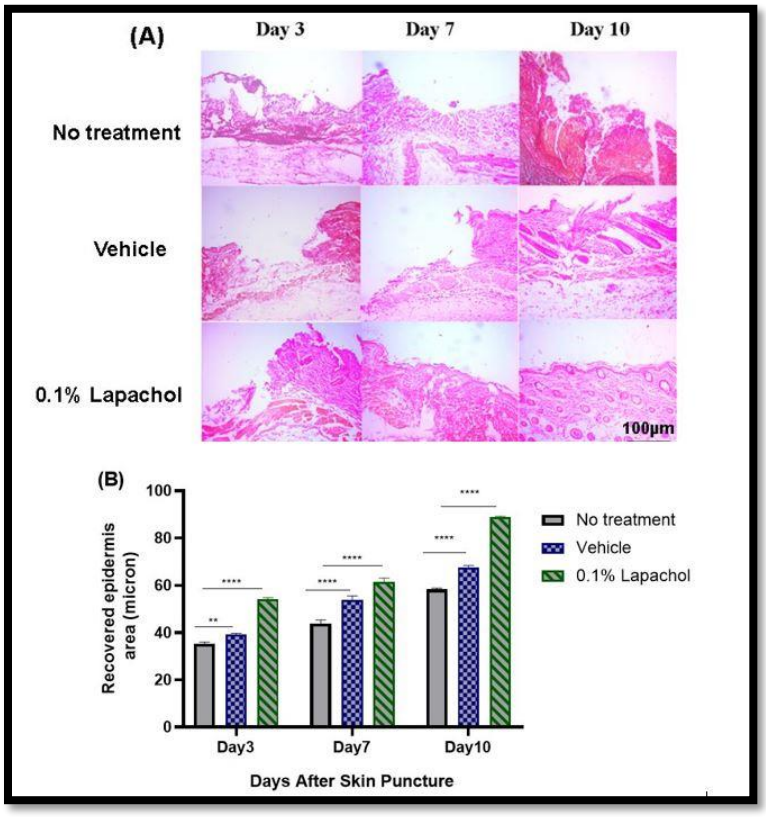

Figure 3.2: Effect of lapachol on epidermis formation.

H&E-stained skin tissue sections on days 3, 7, and day 10 -post-wounding to compare neo-epithelium generation. Microscopic visualization at 10x. (B) Graphical representation of recovered epidermis area in different groups on different periods post-wound. The values were statistically analyzed by one-way ANOVA (n = 6) and represented as means ± SD. $**p < 0.01$ and $****p < 0.0001$ vs. no treatment group.

Additionally, we analyzed the collagen deposition and organization of 0.1% lapachol treated, vehicle group, and untreated control group. Mice skin samples from each of the 3 groups were gathered and stained with Masson's trichrome dye specifically for this purpose. Interestingly, the previously mentioned outcomes were attained. Compared to the

mice treated with vehicle alone, the lapachol-treated diabetic mice exhibited a more significant amount of blue-coloured collagen deposition with homogeneous and well-organized collagen. On days 7 and 10 after the wound, the collagen in the vehicle-treated group was lower and arranged erratically. On the seventh and tenth days after injury, there was a noticeable difference in the amount of collagen deposited in the skin sample treated with lapachol compared to the solvent-treated group shown in Figure 3.3. The control group also shows very low and poorly arranged collagen production and organization.

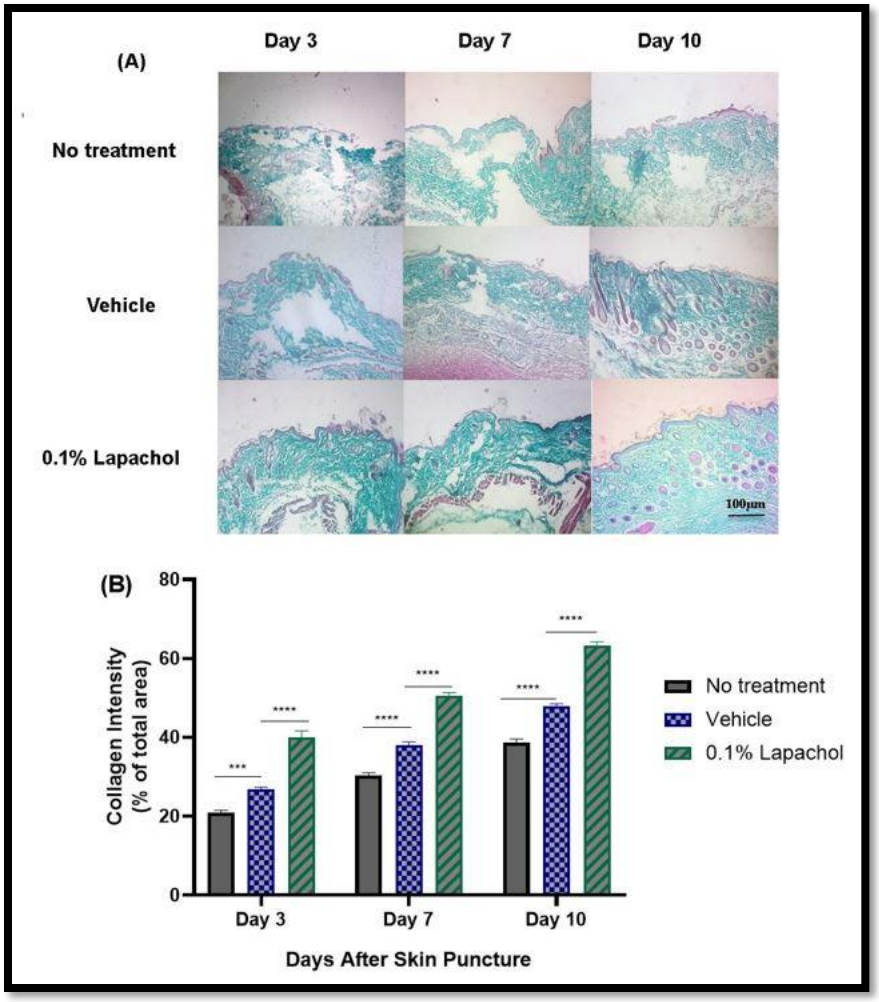

Figure 3.3: Effect of lapachol on collagen deposition.

(A) Masson's trichrome-stained sections show more collagen deposition in mouse skin treated with lapachol on days 3, 7, and 10 -post-wounding than in vehicle-treated mice. (B) Graphical representation of collagen deposition in mouse skin on day 3, day 7, and day 10 -post-injury. Values are means ± SD. and ∗∗∗$p < 0.001$ and ****$p < 0.0001$ vs. vehicle/control group.

3.2 *In-Silico* Gene Expression Analysis of Sirtuins 1, 3, and 6 in Diabetic Wound Healing

The *in-silico* study aimed to determine how SIRT1, SIRT3, and SIRT6 gene expression profiles related to healing diabetic wounds. A class of NAD+-dependent deacetylases known as sirtuins is involved in wound healing, particularly in diabetic situations. They are also important in cellular metabolism, stress resistance, and ageing.

3.2.1 Gene Targets of SIRT 1

The identified SIRT1-related gene targets are NR1H3, TAF1B, MYC, FOXO3, PPARGC1A, MEF20, NNT, RELA, MYOD1, and KAT5. These targets are known to be involved in numerous biological processes related to diabetic wound healing and have a significant impact on the metabolism, tissue regeneration and management of inflammation. SIRT1's modulation highlights the protein's significant function in the regeneration process, showing SIRT1's role in wound healing. MYC (Myelocytomatosis oncogene) controls cell proliferation, PPARGC1A (PPARG Coactivator 1alpha) improves energy metabolism and mitochondrial biogenesis, and RELA (REL-associated protein) is involved in oxidative stress response, FOXO3 plays a role in cell survival. The NF-κB pathway, which includes NF-κB, affects the immune system and inflammation. TAF1B (TATA-box Binding Protein Associated Factor 1B) is a TF (Transcription Factor) initiation complex required for gene expression regulation. MYOD1 (Myoblast determination protein 1) aids in muscle regeneration and KAT5 (Lysine Acetyltransferase 5) controls DNA repair and chromatin remodelling, NNT (Nicotinamide nucleotide transhydrogenase) activation boosts antioxidant defences.

3.2.2 Gene Targets of SIRT3

Numerous SIRT3-related gene targets, including ATP5O, ACSS1, PDHA1, SOD2, SDHA, CERS2, CERS6, and CERS1, have been found. The primary function of these genes is related to mitochondria. regulating oxidative stress and fat oxidation. They are crucial for regulating cellular health and the equilibrium of energy. Particularly when it comes to the healing of diabetic wounds. ATP5O increases the generation of mitochondrial energy upon SIRT3 activation. In diabetic wounds, it supplies the energy required for tissue regeneration and healing. PDHA1 (Pyruvate Dehydrogenase E1 Alpha 1 Subunit) enhances the generation of energy by turning on SIRT3, which effectively aids in the restoration of damaged tissues. Antioxidant defence mechanism is strengthened when SOD2 (Superoxide Dismutase 2) activates SIRT3 it protects diabetic wounds from oxidative damage. By enhancing energy metabolism and mitochondrial efficiency during wound healing, SIRT3 activation enhances SDHA (Succinate Dehydrogenase Complex Flavoprotein Subunit A) activity. Ceramides are required for membrane integrity and lipid metabolism. Ceramides are synthesized by the enzymes CERS2, CERS6, and CERS1 (Ceramide Synthases 2, 6, and 1). SIRT3 activation helps in ceramide level regulation, supporting cellular signalling, and enhancing cellular integrity.

3.2.3 Gene Targets of Activated Sirtuin 6

SIRT6 has been shown to target the following genes: TRIM28, NNT, FOXO1, FOXO3, KAT2A, NCOA2, PPARGC1A, PKM, SMARCC2, and HIF1A. These genes have significant roles in several biological processes. KAT2A (Lysine acetyltransferase 2A) is involved in chromatin remodelling and control gene expression. By deacetylating

KAT2A, stimulating SIRT6 modifies the expression of genes linked to DNA repair, inflammation, and metabolism. A transcriptional coactivator called NCOA2 (Nuclear Receptor Coactivator 2) controls gene expression in response to hormonal and metabolic cues. Activating SIRT6 increases PPARGC1A activity, improving mitochondrial efficiency and energy availability for wound healing. SIRT6 increases HIF1A (hypoxia-inducible factor 1-alpha) activity, helping in angiogenesis and supply oxygen to healing tissues. SIRT6 activation of SMARCC2 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin subfamily C member 2) helps control gene expression required for cell proliferation and repair during the wound healing. PKM (Pyruvate Kinase M) is involved in glycolysis. It helps create ATP from glucose. SIRT6 activation of PKM supports energy production.

3.3 Pathway Analysis output with Enrichment analysis of SIRT 1, 3, and 6

3.3.1 Enrichment Analysis of SIRT 1

We analyzed sirtuin 1 in silico and created a heat map to depict the pathways regulated by its interaction with individual genes, including NR1H3, TAF1B, MYC, FOXO3, PPARGC1A, MEF20, NNT, RELA, MYOD1, and KAT5. This fold enrichment with a significant p-value (<0.05, after false discovery rate (FDR) correction) are shown in Figure 3.4 below. The pathways that are involved in many biological processes, including the, Insulin resistance, Adipocytokine signaling pathway, AMPK signaling pathway, Insulin Signaling pathway, Alcohol liver disease, Non-alcoholic fatty liver disease. The heat map clearly shows how SIRT1 controls numerous pathways through its interactions with these genes, emphasizing SIRT1's broad involvement in cellular function. This

investigation sheds light on the probable processes by which SIRT1 contributes to diabetic wound healing treatment responses.

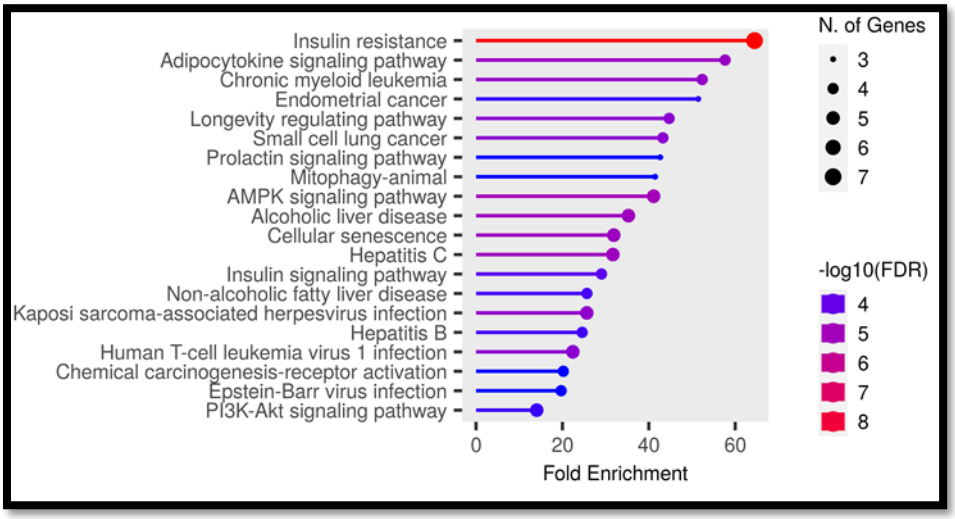

Figure 3.4: Fold Enrichment Analysis of SIRT1-associated genes.

The Fold Enrichment chart represents the SIRT1-related genes in the molecular and cellular mechanisms. The relationship between the genes associated with SIRT1 and the many processes in which they function following FDR correction is depicted in the graphic above. The number of genes involved in the pathway is called the "Bar size." A color scale is used to define the FDR value. As a result, the association increases if it is closer to red and vice versa for blue. The percentage of the chosen genes engaged in a pathway divided by the rate of reference genes is known as fold enrichment. A more significant number indicates a more substantial enrichment because it evaluates the enrichment magnitude—source: ShinyGO 0.80.

3.3.2 PPI Network Analysis of SIRT1

The 10 proteins (NR1H3, TAF1B, MYC, FOXO3, PPARGC1A, MEF20, NNT, RELA, MYOD1, and KAT5) were subjected to a STRING PPI (Protein-Protein Interaction) study, a total of 28 interactions shown in Figure 3.5 a highly integrated network in which these proteins interact together to control vital biological functions.

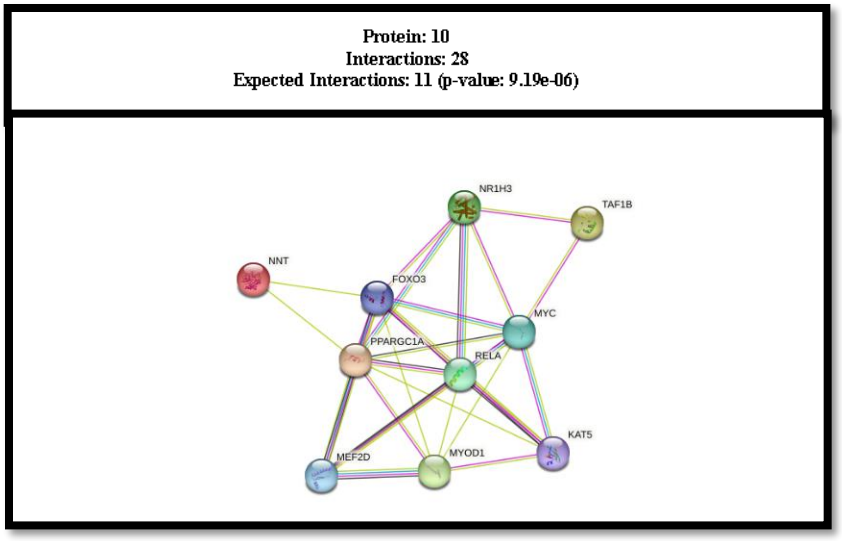

Figure 3.5: Protein-protein interaction of SIRT1 associated gene.

Source: STRING

3.3.3 Enrichment Analysis of SIRT3

We analyzed SIRT3 in-silico and created a heat map to depict the pathways regulated by its interaction with individual genes, including ATP50, ACSS1, PDHA1, SOD2, SDHA, FOXO3, CERS2, CERS6, CERS1. This fold enrichment with a significant p-value (<0.05, after false discovery rate (FDR) correction) is shown in Figure 3.6 below. The involved pathways are essential in many biological processes, including, Metabolic pathways, Carbon metabolism, TCA cycle, Glycolysis/gluconeogenesis, FoxO signaling pathway, Oxidative phosphorylation. The heat map shows that SIRT3 controls numerous pathways by interacting with these genes. Sirtuin 3 has broad involvement in cellular function. This investigation shows SIRT3's role in processes contributing to diabetic wound healing treatment responses.

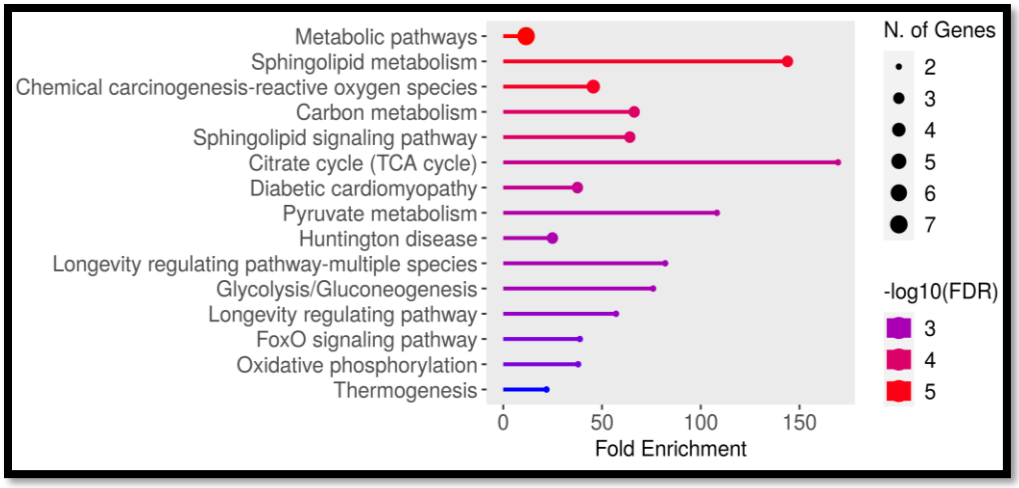

Figure 3.6: Fold Enrichment analysis of SIRT3-associated genes.

The Fold Enrichment chart represents the SIRT3-related genes in the molecular and cellular mechanisms. The relationship between the genes associated with SIRT3 and the many processes in which they function following FDR correction is depicted in the graphic above. The number of genes involved in the pathway is called the "Bar size." A colour scale is used to define the FDR value. As a result, the association increases if it is closer to red and vice versa for blue. The percentage of our chosen genes engaged in a pathway divided by the rate of reference genes is known as fold enrichment. A more significant number indicates a stronger enrichment because it evaluates the enrichment magnitude—source: ShinyGO 0.80.

3.3.4 PPI Network Analysis of SIRT3

The 9 proteins ATP50, ACSS1, PDHA1, SOD2, SDHA, FOXO3, CERS2, CERS6, CERS1 were subjected to a STRING PPI (Protein-Protein Interaction) study, a total of 17 interactions shown in Figure 3.7 a highly integrated network in which these proteins interact together to control vital biological functions.

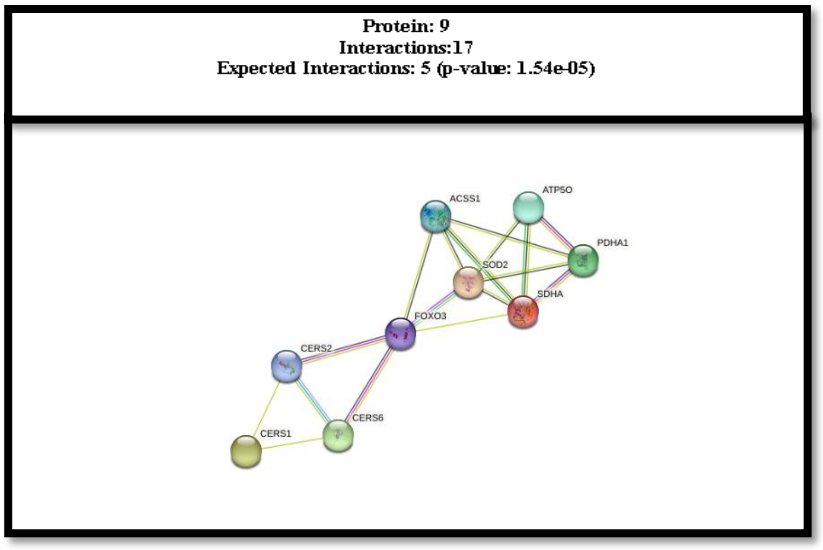

Figure 3.7: The PPI network analysis of SIRT3-associated genes.

Source: STRING

3.3.5 Enrichment Analysis of SIRT6

We analyzed SIRT6 in-silico and created a heat map to depict the pathways regulated by its interaction with individual genes, including NNT, FOXO1, FOXO3, KAT2A, NCOA2, PPARGC1A, PKM, SMARCC2, HIF1A, and TRIM28. This fold enrichment with a significant p-value (<0.05, after false discovery rate (FDR) correction) are shown in Figure 3.8 below. The involved pathways are essential in many biological processes, Non-homologus end joining, Glucagon signaling pathway, Apipocytokines Signaling pathway, AMPK Signaling pathway, FoxO signaling pathway, Insulin signaling pathway. SIRT6 regulates vital processes, including lipid metabolism, energy synthesis, and DNA repair, it is essential for wound healing.

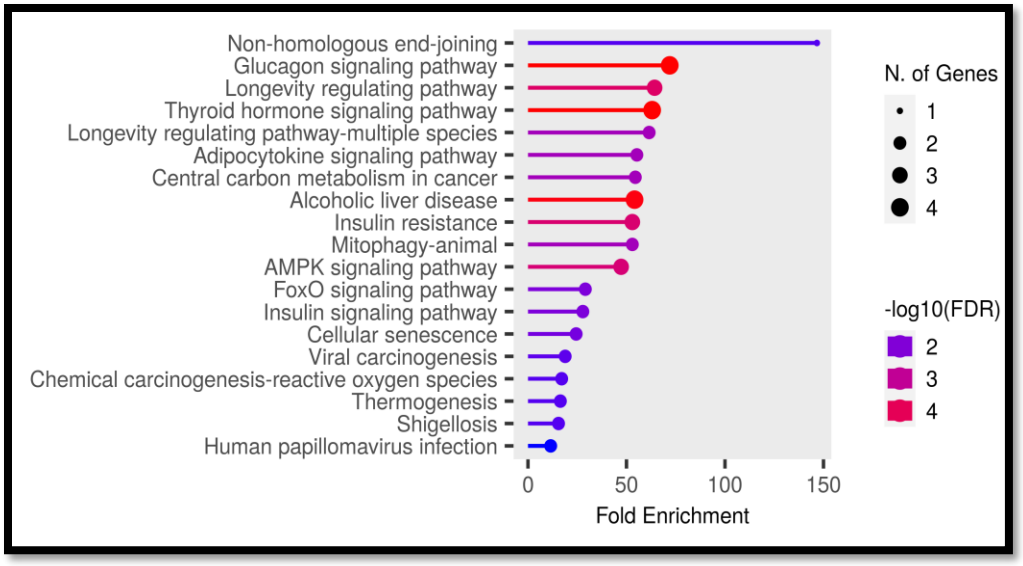

Figure 3.9: Fold Enrichment Analysis of SIRT6-associated genes.

The Fold Enrichment chart represents the SIRT6-related genes in the molecular and cellular mechanisms. The relationship between the genes associated with SIRT3 and the many processes in which they function following FDR correction is depicted in the graphic above. The number of genes involved in the pathway is called the "Bar size." A color scale is used to define the FDR value. As a result, the association increases if it is closer to red and vice versa for blue. The percentage of our chosen genes engaged in a pathway divided by the rate of reference genes is known as fold enrichment. A more significant number indicates a stronger enrichment because it evaluates the enrichment magnitude—source: ShinyGO 0.80.

3.3.6 Network Analysis of SIRT6

The 10 proteins NNT, FOXO1, FOXO3, KAT2A, NCOA2, PPARGC1A, PKM, SMARCC2, HIF1A, and TRIM28. were subjected to a STRING PPI (Protein-Protein Interaction) study, a total of 31 interactions shown in Figure 3.9 a highly integrated network in which these proteins interact together to control vital biological functions.

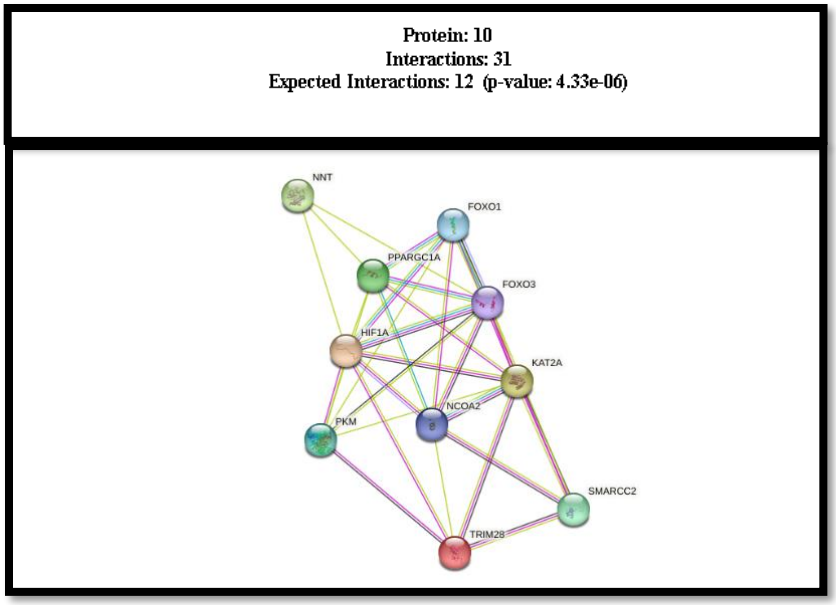

Figure 3.10: The PPI network analysis of SIRT6-associated genes.

Source: STRING

3.5 Cross- Analysis of Potential Gene Targets of SIRT 1, 3 and 6

A cross-analysis of these gene targets in Figure 3.16 of sirtuin 1, 3, and 6 helped us find the common genes involved with SIRT 1, 3, and 6 activity.

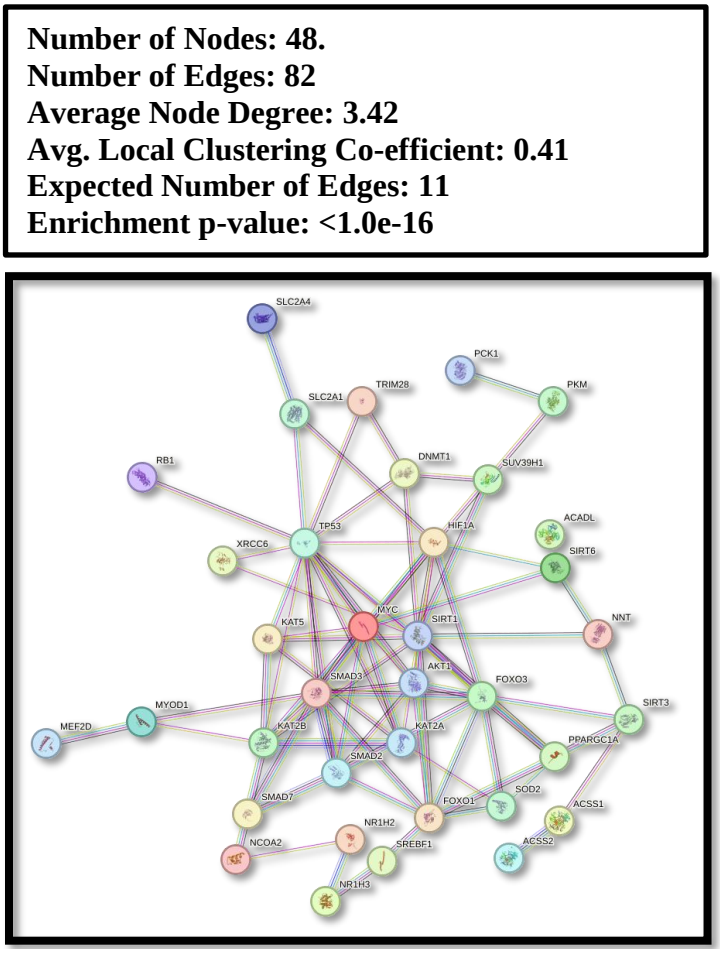

Figure 3.12: Protein-protein interaction of SIRT1, 3, and 6 gene targets.

This data helped us find the common gene targets of sirtuin 1, and 6 involved in diabetic wound closure. Source: STRING

3.5.1 Key Targets of Sirtuin1,3, and 6

Cytoscape analysis was performed using the CytoHubba plugin to identify key hub genes of SIRT1, SIRT3, and SIRT6 that are important in the healing of diabetic wounds. Analysis showed that key hub genes include XRCC6, HIF1A, P53, PPARGC1A, PPARγ, PPARα, FOXO3, GLUT1 and NNT. These hub genes are important in regulating important cellular processes such as DNA repair.

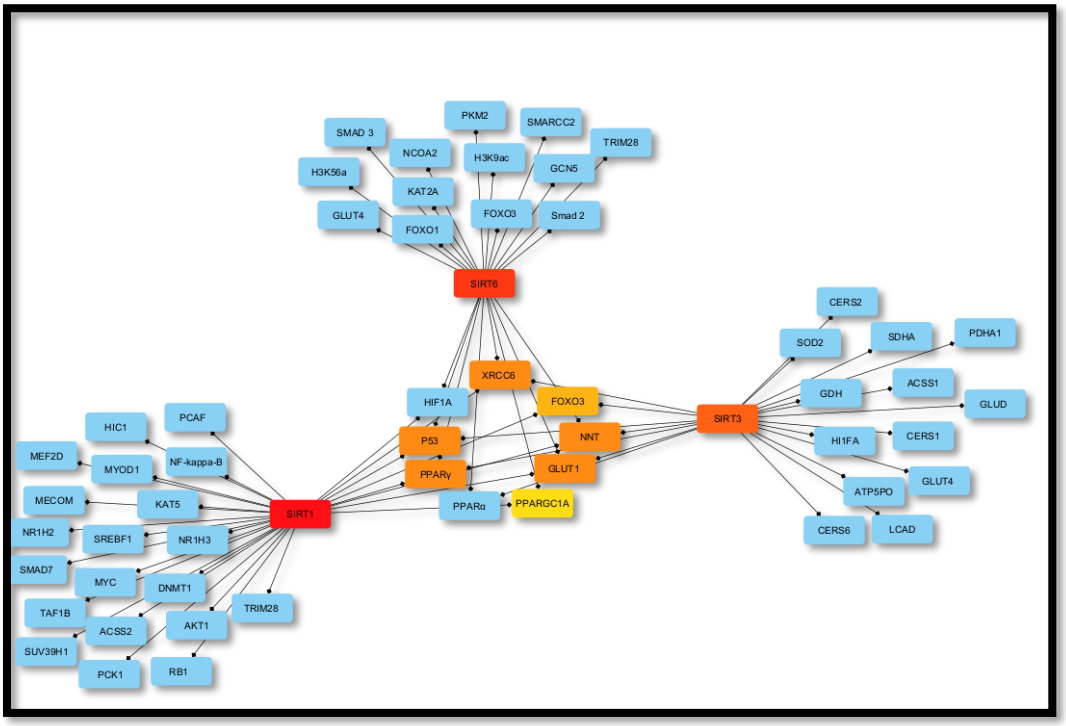

Figure 3.14: Essential Hub Genes Identified in the Network of Common Targets for SIRT1, SIRT3, SIRT6.

3.5.2 Fold Enrichment Analysis of Common Genes

We analysed sirtuin 1,3, and 6 in silico and created a heat map to depict the pathways regulated by its interaction by these genes, including XRCC6, HIF1A, P53, PPARGC1A, PPARγ, PPARα, FOXO3, GLUT1 and NNT. This fold enrichment with a significant p-

value (<0.05, after false discovery rate (FDR) correction) are shown in Figure 3.12 below. The pathways that are involved in many biological processes, including, AMPK signalling pathway, Non-homologus end joining, Nicotinate nicotinamide metabolism, Adipocytokines signalling pathway, and Foxo signalling pathway, these pathways are known to involved in diabetic wound healing.

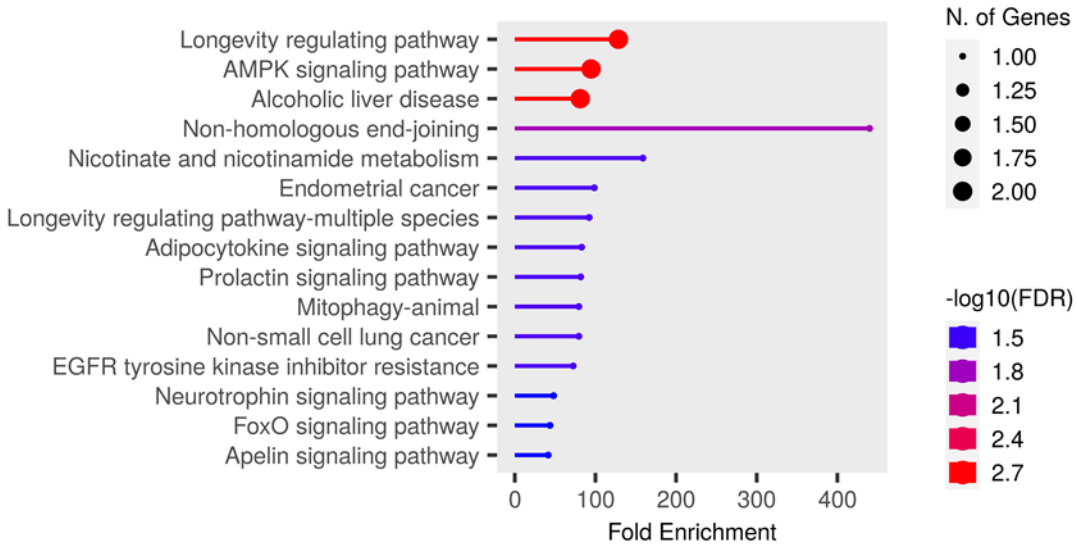

Figure 3.16 Fold Enrichment Analysis of SIRT1, 3, and 6 associated genes.

CHAPTER 4: DISCUSSION

Diabetes mellitus, a range of illnesses characterized by hyperglycemia due to a relative or absolute deficit in insulin synthesis or function, is a condition that can be managed to prevent severe complications. (Alam et al., 2014). Diabetes patients usually develop ischemic vascular disease or a wound healing problem. Type 2 diabetes is associated with increased atherosclerosis, endothelial cellular dysfunction, glycosylation of ECM proteins, and vascular degeneration. These problems ultimately hinder neovascularisation and diabetic wound healing (Kolluru et al., 2012). Impaired vasculature soon after the precipitating injury prevents oxygen from reaching the wound, resulting in a hypoxic surrounding environment. Chronic wounds that do not receive oxygen for an extended period are less likely to heal because it inhibit the cell formation of ECM, blood vessel development, and epithelial regeneration (Guan et al., 2021).

Mammalian sirtuins (SIRTs) comprises seven members of the class III histone deacetylases (HDACs) family of historically conserved proteins. These sirtuins have an enzymatic activity, they bind to NAD+ and can function effectively on various substrates depending on their physiological functions (Carafa et al., 2016). According to Thandavarayan et al. (2015), proteins of the sirtuin family have a crucial role in regulating several pathological processes, including inflammation, glycolysis, DNA repair, and the promotion of lifespan. The three primary mechanisms involved in wound healing are proliferation, migration of cells, and inflammation. Sirtuins are critical in promoting cell migration and proliferation due to their anti-inflammatory characteristics, potentially enhancing wound healing (Bibi et al., 2021).

Studies have indicated that when it comes to promoting the healing of diabetic wounds, SIRT 1, 3, and 6 are important targets (Beegum et al., 2022). SIRT1 shows promise in improving diabetic wound healing. Research has shown that SIRT1 expression is reduced in diabetes individuals with delayed wound healing. SIRT1 activators have great promise for improving diabetic wound healing because of their remarkable properties in stimulating angiogenesis via proliferation, migration, and developing tubes in vitro in human umbilical vein endothelial (HUVEC) cells. SIRT1 knockout mice showed changes in the growth and recruitment of fibroblasts, mast cells, neutrophils, and macrophages to the wound site and in the production of connective tissue (Beegum et al., 2022). SIRT3, a deacetylase found in mitochondria, is crucial for oxidative stress management and energy metabolism. In individuals with diabetes, elevated blood sugar levels combined with a deficiency in SIRT3 eventually resulted in oxidative stress, which limited angiogenic reactions, including the synthesis of VEGF. The presence of SIRT3 can significantly reduce oxidative stress, increase angiogenic responses, and accelerate wound healing in diabetics (Yang et al., 2020). In contrast, SIRT6 expression is low in numerous human tissues in diabetic patients, which may indicate that SIRT6 protects against diabetes-related illnesses (Li et al., 2023). Targeting the SIRT6 might be a viable treatment for poor wound healing (Thandavarayan et al., 2015).

Lapachol, a naphthoquinone, has a wide variety of medicinal properties, including anti-inflammatory, anticancer, anti-ulcer, antiseptic, and bactericidal activities (Hussain et al., 2007). Recent research indicate that lapachol enhances wound healing by increasing the production of Rac1/Cdc42/α-Pak proteins through sirtuin 1 and 3. As a result, it might be regarded a promising therapeutic option for wound healing (Bibi et al., 2021). To support

the current findings, this study looked at the molecular targets of active sirtuins 1, 3, and 6 in diabetic wound healing. When lapachol was administered topically to diabetic mice with poor wound healing, the results were promising. The fact that the wounds healed satisfactorily in the treated mice suggests that lapachol's increased SIRT1 and SIRT3 levels considerably aided the healing process.

In this work, an in vivo experiment was carried out on a diabetic mouse model to assess the efficiency of lapachol in wound healing. Three groups were used: a control diabetic group, a vehicle-treated group, and a 0.1% lapachol-treated group. Digital pictures of the wounds were collected from day 0 to day 10 after wound creation in order to monitor the process of wound closure, as shown in Figure 3.1. The results were promising, as the wounds in the lapachol-treated group showed the most significant healing, with nearly complete closure observed by day 10, as illustrated in the corresponding figures.

Figure 3.2 shows that histological analyses were performed to substantiate these findings further. Hematoxylin and eosin (H&E) staining revealed enhanced epidermal formation in the 0.1% lapachol-treated group, depicting an increase in wound repair than the control and vehicle-treated groups. Masson's trichrome staining was also used to assess collagen production, a critical factor in wound healing. The lapachol-treated group demonstrated the most substantial collagen production in Figure 3.3, suggesting that lapachol accelerates wound closure and enhances tissue repair quality by promoting collagen synthesis. These results underscore the therapeutic potential of lapachol in improving wound healing outcomes in diabetic conditions.

The computational analysis was performed to find gene targets of the activated sirtuins involved in diabetic wound healing, and several functional studies were performed using tools (ShinyGO) and databases such as the QIAGEN Ingenuity Pathway Analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) and Cytoscape to identify key genes in SIRT1,3 and 6 network.

An in silico analysis was conducted to find these gene targets of the activated sirtuins involved in diabetic wound healing. These genes were used to perform several in silico functional analyses (considering corrected p-values < 0.05 as statistically significant) using tools (ShinyGO) and databases such as the Qiagen and Kyoto Encyclopedia of Genes and Genomes (KEGG). The STRING and was used to find Protein-protein interaction ShinyGO 8.0 data was used for in-fold enrichment analysis. This helped us better understand the molecular mechanisms through which sirtuins help in wound healing. The analysis determined the Insulin resistance pathway as the most significantly enriched pathway in association with gene targets of Sirtuin 1, and subsequent Adipocytokines signaling pathways, AMPK signalling pathway, cellular senescence, and Insulin signaling pathway are known to be involved in diabetic wound closure shown in figure 3.4. These pathways are integral to understanding how SIRT1 contributes to wound healing in diabetic conditions. The FoxO signalling pathway, in particular, is crucial for regulating stress resistance and cellular repair mechanisms, which are essential for effective wound healing in diabetes. These pathways are crucial to understanding Sirtuin 1's role in diabetic wound healing. FOXOs (forkhead box O) play a crucial role in sustaining homeostasis by reacting to and mitigating environmental disruptions. Their primary biological function is responding to stress, not to serve as an essential mediator of in under normal physiological

conditions (Eijkelenboom & Burgering, 2013). The 10 proteins (NR1H3, TAF1B, MYC, FOXO3, PPARGC1A, MEF20, NNT, RELA, MYOD1, and KAT5) were subjected to a STRING PPI (Protein-Protein Interaction) study and total of 28 interactions were showed in Figure 3.5 a highly integrated network in which these proteins interact together to control vital biological functions.

As seen in Figure 3.6, the fold enrichment analysis of SIRT3 genes demonstrated the involvement of these gene targets in several essential pathways. Notably, the Metabolic pathway, TCA cycle, glycolysis/gluconeogenesis and FOXO signalling pathway are known to be involved in diabetic wound healing. These pathways imply that SIRT3 is involved in more general regulation of survival, stress response, and cellular metabolism, all of which are necessary for effective wound healing. The 9 proteins ATP50, ACSS1, PDHA1, SOD2, SDHA, FOXO3, CERS2, CERS6, CERS1 were subjected to a STRING PPI (Protein-Protein Interaction) study, a total of 17 interactions showed in Figure 3.7 a highly integrated network in which these proteins interact together to control vital biological functions.

The fold enrichment analysis in Figure 3.8 found that SIRT6 gene targets are linked to various pathways, with the Non-homologous end joining pathway, Glucagon signalling pathway, Adipocytokines signalling pathway, Insulin resistance, AMPK signalling pathway, FOXO signalling pathway and Insulin signalling pathway plays the most important role in diabetic wound healing. These pathways are critical in metabolic regulation, energy production, and stress response, all required for diabetic wound healing. The 10 proteins NNT, FOXO1, FOXO3, KAT2A, NCOA2, PPARGC1A, PKM, SMARCC2, HIF1A, and TRIM28. were subjected to a STRING PPI (Protein-Protein

Interaction) study, a total of 31 interactions shown in Figure 3.9 a highly integrated network in which these proteins interact together to control vital biological functions.

Cytoscape analysis was performed using the CytoHubba plugin to identify key hub genes of SIRT1, SIRT3, and SIRT6 including XRCC6, HIF1A, P53, PPARGC1A, PPARγ, PPARα, FOXO3, GLUT1, and NNT are involved in diabetic wound healing. XRCC6 (X-Ray Repair Cross Complementing 6), aka Ku70, is an important factor in the repair of DNA double-strand breaks by non-homologous end joining (NHEJ), which is essential for the stabilization of DNA double-strand breaks during cellular damage and stress. SIRT1 may improve DNA repair ability and repair protein Ku70 deacetylation. However, this repair activity decreased when SIRT1 siRNA was used to inhibit endogenous SIRT1 expression, suggesting that SIRT1 can control a cell's ability to repair DNA strand breaks (Jeong et al., 2007). Sirtuin 3 deacetylates Ku70 and plays a role in stabilizing the Ku70-BAX interaction. Because Ku70 can assist block BAX transporting into mitochondria and reduce cell death, the Sirt3 protein may have a function in cell survival (Luo et al., 2018). SIRT6 activation deacetylase Ku70 and dramatically increased cell proliferation while suppressing apoptosis. (Feng et al., 2017). SIRT6 is involved directly in the DNA repair mechanism and is involved in the deacetylation of XRCC6 which enables more effective DNA repair.

SIRT1 directly interacts with HIF1α and can activate glycolysis-related genes and control ROS and ATP production (Yu et al., 2018). SIRT1 deacetylation inhibits the activity of HIF-1α expression which may contribute to cell death (Pan et al., 2020). SIRT3 activation downregulates the expression of HIF1α (Jo et al., 2018). SIRT3 regulates the metabolic shift toward oxidative phosphorylation, ensuring that cells under toxic stress produce

energy effectively. It promotes cell survival by mediating HIF1A-mediated activity. Overexpression of SIRT6 increased HUVEC (human umbilical vein endothelial cells) invasion, migration, proliferation, and tube formation by modulating levels of HIF-1α. (Yang et al., 2021). Overexpression of SIRT6 inhibits HIF1α and VEGF expression, enhancing the expression of PHD2 (Prolyl hydroxylase domain 2). PHD2 inhibits angiogenesis, potentially lowering HIF1α and VEGF levels (Wang et al., 2018).

SIRT1 can stop the p53-dependent cell cycle and prevent apoptosis, hence facilitating the apoptosis process. At the same time, it helps to protect the genome's integrity. Promote cell survival and improve DNA repair processes. Regulation of the SIRT1-p53 axis may influence the fate of somatic and progenitor cells during tissue regeneration and repair (Ong & Ramasamy, 2018). Increased Nampt activity causes cells to create more NAD+ from nicotinamide, activating SIRT1 deacetylase activity. Increased SIRT1 activity inhibits p53 activity, preventing p53-dependent cellular senescence (Yi et al., 2010). In the absence of external stress, silencing JNK2 causes p53-dependent and JNK1-dependent cell death in the human epithelial cell line HCT116. Co-silenced JNK2 with SIRT3 protects cells from JNK2-mediated cell death. These findings imply that SIRT3 is required for JNK2-induced p53-independent cell death (Chen et al., 2017). The suppression of glucose levels caused by p53 was reduced in mice with liver-specific Sirt6-KO. SIRT6 regulates glucose metabolism by connecting p53 transcription activity and gluconeogenesis (Zhang et al., 2014).

Oxidative damage is directly linked to the SIRT1/PGC-1α pathway. By deacetylating PGC-1α, SIRT1 may help regulate cellular activity. It also has a role in energy production, mitochondrial biogenesis, and several physiological processes, such as ageing and cellular

stress response (Liang et al., 2020). SIRT1 suppression subsequently lowers the levels of PGC-1α, SOD2 and UCP2 (uncoupling protein 2) (S.-J. Wang et al., 2015). Knockdown of mitochondrial sirtuin 3 enhanced basal ROS levels and stopped PGC-1α's suppressive effect on the cellular formation of ROS (Kong et al., 2010). SIRT3 is essential for PGC-1α-induced mitochondrial gene expression and biogenesis. SIRT3 downregulation reduces PGC-1α-mediated mitochondrial biogenesis, due to lower expression of NRF-1 (Nuclear respiratory factor 1) and mtTFA (mitochondrial transcription factor A). MtTFA is involved in mitochondrial DNA replication and transcription (Brenmoehl & Hoeflich, 2013). SIRT6 stimulates the PGC-1α/NRF1, NRF2 pathway by AMPK phosphorylation and direct deacetylation of PGC-1α. PGC-1α which in return increases mitochondrial DNA stability and decreases oxidative stress (Li et al., 2020).

The Ppargc1a gene encodes the PPARγ coactivator 1-α (PGC1α). The transcriptional coactivator of PPARα and PPARγ modulates cardiac FAO, mitochondrial biogenesis, and respiration (Kalliora et al., 2019). Sirt1 protein suppresses genes regulated by PPAR-γ, including those involved in fat storage. Sirt1 inhibits PPAR-γ via interacting with its cofactors NCoR (nuclear receptor co-repressor) (Picard et al., 2004). The SIRT1 expression is reduced by PPAR-γ in response to ligands, while SIRT1 activity can be modulated by NAD and small molecular organic substances (Han et al., 2010). SIRT6 regulates fatty acid transporter genes via the PPARγ transcription factor. Sirtuin 6 binds to PPARγ's DNA-binding domain to control activity (Khan et al., 2021). SIRT6 suppresses glycolysis and promotes fatty acid oxidation, and supports PPARγ's role in maintaining lipid reserves and reducing inflammation, both crucial for wound healing.

PPARα activation reduces inflammatory marker CD40 expression through SIRT1-mediated deacetylation of NF-κB in VECs and adipocytes (W.-r. Wang et al., 2015). PGC-1α and Sirt3 activate enzymes involved in mitochondrial fuel catabolism, promoting metabolic reprogramming. Sirt3 controls the production of ATP from oxidative phosphorylation to maintain mitochondrial energy balance (Rato et al., 2014). SIRT6 acts as a co-activator of PPARα in the liver, activating fatty acid β-oxidation genes. SIRT6 may influence lipid metabolism by regulating the PPAR family of transcription factors at the tissue and isoform levels (Khan et al., 2021).

The TF FoxO3a is de-acetylated by sirtuin 1 and regulates the activity of FoxO3a to generate resistance to OS. In the endothelial cells, FoxO3a controls a group of key antioxidant genes with the interaction with transcriptional co-activator PGC-1α (Olmos et al., 2013). The hydrogen peroxide induces sirtuin 3 to de-acetylate FOXO3 at K271 and K290. The expression of many FOXO3-dependent genes, which are essential for mitotic equilibrium, is stimulated by this deacetylation. Mt homeostasis contributes to maintaining the integrity and functionality of mitochondria by the production of new mitochondria (Tseng et al., 2013). Sirtuin 6 interacts with FOXO3, which promotes its ubiquitination while decreasing its stability (Hu et al., 2018). FoxO3 and Sirt6, two longevity genes, can lower LDL cholesterol levels by regulating the Pcsk9 gene (Tao et al., 2013).

SIRT1 activation reduced the expression of HIF-1α, HO-1, and GLUT1 mRNA, indicating that it regulates the PI3K/MTOR signalling pathway and reduces hypoxia-induced oxidative stress in cardiomyocytes and inflammation(Ma et al., 2022). SIRT3 destabilized the metabolic reprogramming of HIF1α which directly affects the GLUT1 activity decreasing its ability to uptake glucose (Srivastava et al., 2018). The Sirt6 inhibitor

enhanced the expression of the glucose transporters GLUT1 and -4 in muscle, hence increasing the activity of the glycolytic pathway (Sociali et al., 2017).

Enzymes like nicotinamide and nicotinamide nucleotide transhydrogenase (NNT). The enzyme mononucleotide Adenyltransferase 3 (NMNAT3) controls the amount of NAD+ in the mitochondria. As cells age, their levels of mitochondrial NAD+ drop along with a reduction in SIRT3 activity. These modifications seriously impair the transition of cell destiny (Son et al., 2016). Through its regulation of pathways that improve mitochondrial function and lower oxidative stress, SIRT1 indirectly affects NNT. During wound healing, NNT aids in preserving mitochondrial function and detoxifying ROS, particularly in diabetes circumstances when oxidative stress is increased. Through its indirect involvement in glucose metabolism regulation and DNA stability maintenance, SIRT6 supports NNT's role in oxidative stress regulation. SIRT6 guards against excessive glycolysis and promotes oxidative metabolism to maintain the effectiveness of mitochondrial activity. Hence, during tissue regeneration in diabetic wounds,

SUMMARY OF RESEARCH WORK

This research investigated the healing effect of lapachol in diabetic wound healing through in vivo experiments and computational studies. In vivo experiments were conducted using diabetic mouse models with three groups: a diabetes control group, a vehicle-treated group, and a 0.1% lapachol-treated group. Digital images demonstrated that the Lapachol-treated group had the most significant wound closure, with almost complete healing by day 10. Staining confirmed this finding. The group treated with 0.1% Lapachol demonstrated higher epidermal development and collagen synthesis, indicating more wound healing than the other groups.

Computational analysis suggested that XRCC6, HIF1A, P53, PPARGC1A, PPARγ, PPARα, FOXO3, GLUT1, and NNT are the targets of sirtuins 1, 3, and 6. To support this finding, from the literature, we found that out of these nine genes, only XRCC6, HIF1A, P53, PPARGC1A, PPARγ, PPARα, FOXO3, GLUT1, and NNT directly interact with SIRT1, 3, and 6, making them critical genes. Since activating these sirtuins regulates healing, these genes are probably essential for diabetic wound regulation.

Based on our in vivo findings, in silico analyses were conducted to identify target genes involved in wound healing, particularly on sirtuins known to be activated by lapachol. Statistical analysis showed that potential target genes for SIRT1 included NR1H3, TAF1B, MYC, FOXO3, PPARGC1A, MEF20, NNT, RELA, MYOD1, and KAT5. Targets identified for SIRT3 included ATP50, ACSS1, PDHA1, SOD2, SDHA, FOXO3, CERS2, CERS6, and CERS1. In addition, SIRT6 was found to target genes such as NNT, FOXO1, FOXO3, KAT2A, NCOA2, PPARGC1A, PKM, SMARCC2, HIF1A, and TRIM28. Out

of these genes, XRCC6, HIF1A, P53, PPARGC1A, PPARγ, PPARα, FOXO3, GLUT1, and NNT are shown to be the common gene target of activated SIRT1, 3, and 6. These genes are involved in critical metabolic and signaling pathways that impact energy metabolism, DNA restoration mechanisms, and cellular responses to stress. These processes are all necessary for effective wound healing.

Fold enrichment analysis indicated that major pathways such as the AMPK signaling pathway, the insulin signaling pathway, the glucagon signalling route, and the FoxO signalling pathway are the most significant ones involved with Sirtuins 1, 3, and 6. These pathways are crucial in metabolism and stress response, which are required for effective wound healing. PPI interaction analysis found complicated interactions between these target genes, suggesting that SIRT1, SIRT3, and SIRT6 have various roles in cellular responses required for diabetic wound repair.

CONCLUSION AND FUTURE RECOMMENDATIONS

The primary findings of this research revealed that XRCC6, HIF1A, P53, PPARGC1A, PPARγ, PPARα, FOXO3, GLUT1, and NNT are key gene targets of activated sirtuin 1, 3, and 6, and these genes are probably involved in diabetic wound regulation. Lapachol is a compound involved in wound healing for diabetic conditions. It activates Sirt1, 3, and probably 6. In vivo experimentation suggested that the mice treated with 0.1% lapachol showed the most accelerated wound closure compared to the solvent-treated (vehicle-treated) and no-treated diabetic group, signifying lapachol's healing capability. The computational analyses were performed to identify the gene targets involved in the diabetic wound repair mechanism. Sirtuins 1 and 3, followed by SIRT6, were included in the analysis due to their known role in diabetic wound healing. The in-silico analysis also supports our in-vivo experimental results.

These findings highlight the complicated interplay between sirtuins and their gene targets in wound healing, particularly diabetes. To the best of our knowledge, the XRCC6, HIF1A, P53, PPARGC1A, PPARγ, PPARα, FOXO3, GLUT1, and NNT are genes, which are the key targets of sirtuins 1, 3, and 6, have not been identified before. Identifying these gene targets gives valuable insights into the molecular mechanisms by which lapachol exerts its beneficial effects and potential ability pathways that could be targeted in future healing treatments for improving wound repair in diabetics.

4.3 Future Recommendations

It could be helpful to look into the possible synergistic effects of mixing Lapachol with other well-known wound healing agents or therapies. Examining how Lapachol interacts with other drugs may result in combination treatments for diabetic wounds will be more significant. While SIRT1, SIRT3, and SIRT6 were the main focus of this investigation, investigating the function of other sirtuins in wound healing may offer a more thorough knowledge of the sirtuin family involved in this process, perhaps leading to the discovery of novel therapeutic targets and strategies for the repair of diabetic wounds. Future research endeavors should integrate preclinical trials with animal models that more closely resemble human diabetes diseases to aid in translating these results into clinical practice. Ultimately, the necessity of it as a therapeutic alternative for individuals with diabetes shows that lapachol is both safe and effective. Future studies are provided by discovering essential gene targets controlled by SIRT1, SIRT3, and SIRT6 in diabetic wound healing. Modulating these genes and the pathways linked to them can offer the drug targeting these genes to manage wound healing in diabetic patients.

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