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Molecular basis of burn wound healing in diabetics: The effect of Vitamin B17, metformin and autologous fat stem cells

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With Much Love ..

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DEDICATION

I dedicate My Doctorate thesis to:

- **My family**: especially to my mother for her priceless presence in my life, my helpful sisters and unique brother who are the light of my days and the candle of my nights. You are the reason behind my positive energy and endless happiness.

- **My father** who is living inside my soul and in every single corner of my memory.

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Hanine,

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Glossary and abbreviations:

ABA: American Burn Association

Acute: having a rapid onset, severe symptoms and short course; not chronic

ADA: American Diabetic Association

Allograft (homograft): graft of tissue between individuals of the same species

Apoptosis: fragmentation or disintegration of a cell into membrane-bound particles that are then eliminated by phagocytosis

Autograft: graft transferred from one area of a patient's body to another area of the same patient.

BSA: body surface area

Chronic: persisting for a long period of time with little change; opposite of acute

Clysis: administration or injection of fluid into the body by other than the oral or intravenous routeS

CMS: Center for Medicare Services, Department of Health and Human Services of the United States

Conversion: process of progressive necrosis, in depth and width of a burn following initial injury; process in which burn depth increases due to progressive apoptosis, e.g., from partial thickness to full thickness, hours or days after the initial injury.

Cutaneous: referring to the skin

Demarcation: the boundary between the burn wound and the peripheral unburned skin. Also, the observation of a distinct boundary between a partial-thickness wound and a wound which is full-thickness (that may not have been clinically detectable earlier after the burn injury)

Desquamate: a normal process in which the cornified layer of the epidermis is sloughed off in fine scales. In the context of burns, an accelerated process of peeling of the epidermis and loss of deeper layers of the skin.

DM: Diabetes mellitus

Epithelial: pertaining to or composed of epithelium, the covering of the external and internal organs and lining of the internal passageways of the body.

Erythematous: pertaining to redness or inflammation of the skin or mucous membranes caused by congestion of the capillaries

Eschar: a slough (area of necrotic tissue), crust or dry scab resulting from a burn.

Escharectomy: the surgical removal of dead soft tissue (eschar) from a burn wound.

Heterogeneous: consisting of dissimilar elements or parts; derived from different individuals or species

Hypermetabolism: abnormally increased rate of metabolic activity

Hypertrophic scar: abnormal area of scarred tissue that replaces normal tissue at the site of an injury due to an increase in the size of the area's constituent cells

Mechanical shearing: in relation to burns, a cut or tear in tissue or a graft due to a physical force or strain placed on the area.

Meshing: the process of creating small openings across the length and breadth of a harvested piece of skin to be used for grafting so that the graft can be expanded or stretched to cover a burn wound recipient site.

Pathogen: microorganism capable of producing disease.

PBD: post burn day

Peripheral vascular disease: an abnormal condition that affects the blood vessels outside the heart and brain and often involves a narrowing of vessels that carry blood to the legs, arms, stomach or kidneys. Causes can include obesity, smoking, and numerous metabolic disorders (e.g., diabetes) and involve structural changes in the blood vessels such as inflammation and resulting tissue damage, ulceration, etc.

Sebaceous gland: one of many small oil-secreting glands located in the dermis, often lying adjacent to hair follicles.

Skin appendages: referring to the hair follicles, sweat glands, and sebaceous glands in the skin

Skin replacement: tissue or graft that permanently replaces lost skin with healthy skin.

Skin substitute: a biomaterial, engineered tissue or combination of materials and cells or tissues that can be substituted for skin autograft or allograft in a clinical procedure.

TNF-α: tumor necrosis factor-α

Vasoconstrictive: causing narrowing of blood vessels

WHO: world health organizations

Vasodilation: dilation or widening of the lumen of blood vessels

Xenograft (heterograft): a graft between two different species (e.g., animal to human)

Abstract

Introduction: Chronic wounds and persistent cutaneous burns complications represent a worldwide challenge and a critical health problem particularly for diabetic persons since diabetics have a higher propensity for infection. Nowadays, new advances and advanced new technologies are being developed to optimize and improve the burn healing process. However in case of diabetic wounds in general or skin burns in particular, this process is complicated since diabetic persons have the capacity to tolerate chronic inflammations.

Aims: In order to enhance the process of burn wound healing in time and quality, this study linked the science of nutrition, endocrinology and the skin medical and surgical pathologies to the therapeutical effect of a combination based on Vitamin B17 and/or metformin in the presence or absence of a potential source of autologous mesenchymal stem cells found in the subcutaneous adipose tissue.

Methodology: A total of 190 Sprague-Dawley male rats were divided into 2 main groups: 70 animals were non-diabetic and 120 rats developed diabetes after streptozotocin injection. All animals were subjected to burns, and then they were grouped into 10 conditions or subgroups based on the presence or absence of relocated autologous adipose tissue under the burnt skin and the treatment administered: single or combined injection of vitamin B17 and/or metformin. Vitamin B17 was injected in the subcutaneous area whereas metformin was injected intraperitonialy. Assessment was performed by clinical observation, histological analysis, TEWL record, mast cells and ROS activities determination, as well as, by molecular analysis of cytokines (IL-1 alpha, IL-6 and IL12) and heat shock protein HSP70 at 6 different time points (D1, D3, D7, D14, D21, and D28).

Results: Results showed that the injection of vitamin B17 alone, or in combination with relocated fat tissue formed two optimal conditions in diabetic burnt rats and promoted the better healing processes compared to other conditions. There were remarkable anatomical results represented by a faster skin detachment (at day 14) and by the smaller scar area left on day 28. Besides, Vitamin B17 improved the burn wound healing for diabetics since it promoted remarkable immuno-modulatory effect of interleukins (IL-1α, IL-6, and IL-12) represented by a delayed and limited inflammation which is correlated with several similar studies which used Vitamin B17 for cancer treatment. Moreover ROS modulation and HSP70 expression were positively regulated during the different steps of wound healing.

Conclusion: This work has great implications in dealing with wound healing complications such as chronic skin inflammation and diabetic skin closure which can save the life of thousands of injured persons

Keywords: Diabetes, wound healing, amygdaline (vitamin B17), inflammation, Metformin, adipose tissue stem cells, IL-2, IL-6, IL-10, and HSP-70.

I. Introduction:

The skin forms one of the largest organs in the body, in surface area and weight, and plays many roles starting with protection and ending with the immune regulation and sensation (Mclafferty, Hendry, & Farley, 2012). It is the first barrier which faces numerous external factors that are surrounding our body. In some cases, an accident or a factor can cause harmful damage to our skin tissue which has the inherent power to repair. However, skin burn wound healing remains the most important and challenging skin repair process, since its accomplishment requires the cooperation of many cellular, physiological and molecular mechanisms (Gonzalez, Costa, Andrade, & Medrado, 2016). Moreover, an additional factor is needed namely, the presence of a functional immune system in order to have a successful healing and skin repair (MacLeod & Mansbridge, 2016). These facts prove that the burn wound healing process forms a complex between cells, signalling pathways, and extracellular matrix. The healing mechanism passes through 4 major steps and each step has its own complications; they all form a critical scenario (Mendonça & Coutinho-Netto, 2009). In diabetic persons the wound healing process is interrupted and in most cases the wound takes long time to heal due to many pathophysiological and pathological changes compared to a normal person (Falanga, 2005). Burns constitute a major cause of injury worldwide and can lead to open wounds, disability, death, and psychological complications. Therefore, burn patients require subsequent rehabilitation, reconstruction and long-term anti-scar therapy (Kavitha et al., 2014). In this context, new medications are being developed and tested in order to improve the healing process, understand its related mechanisms as well as the potential therapeutic molecules that enhance the process, thus leading to a better outcome with less complications and side effects. On this basis the following research project has been undertaken with diabetes being the main target and the complexity of diabetic ulcer in mind.

A. Background:

According to the world health organization (WHO), more than 300,000 people die annually from burn worldwide, from which 180,000 are fire-related burns. The prevalence of burns is significantly high in low and mid income countries (WHO, 2018). Furthermore, according to the Center for Disease Control (CDC), every year, 1.1 million people suffer burn injuries that require medical attention. In parallel, in the United States, approximately 17 million (6.2%) of the U.S. population are diabetic; this disease is commonly spread among the American

population and stands behind the seventh leading cause of death in the U.S. Moreover, every year 10,000 people in the United States die as a result of burn-related infections (review, 2019).

Several new studies reported on improving wound healing by using administered chemicals, by developing new surgery for skin graft (Madiedo, Gaviria-Castellanos, & Zapata-Ospina, 2018), or by applying new technologies. In this context, laser use is a newly developed approach in the management of burn scar (Sobanko & Alster, 2009). Furthermore, new exciting technologies for burn treatment are under development to improve on how burns are treated and expedite the healing process, in particular, the use of stem cells, which became one of the most prominent medical approaches in medicine (fadi ghieh et al 2015).

Adipose-derived stem cells (ADSCs) are adult mesenchymal stem cells in adipose tissue with self-renewal and multi-directional differentiation potential. New studies showed the importance of the application of ADSCs in the treatment of wounds based on the achieved good results and outcome (Kim & Heo, 2014), (Miana & González, 2018). Moreover, during the last 3 decades, research on adipose tissues has spread in parallel with the increase of obesity. Several observations converged on the idea that adipose tissues are organized as a large organ with endocrine and plastic properties . In addition, multiple recent studies have proved and confirmed the great potential of autologous ADSC's in achieving a faster and better wound healing in animal experiments (Chang et al., 2018).

Metformin is known for decades to play an important role in diabetes and was used as a hypoglycaemic medication which can lower the glucose level for diabetic persons (Cheng & Yang, 1983). A published study in 2016 tagged metformin with an additional role in diabetes. In this study, metformin proved to enhance the angiogenic functions of endothelial progenitor cells via activating the AMPK/ eNOS pathway in diabetic mice (Yu et al., 2016). Moreover, metformin accelerated wound closure and angiogenesis in diabetic mice. Additional new studies highlight an unexpected adverse effect of metformin-induced MSC (Mesenchemical stem cells) apoptosis through AMPK-mediated mTOR suppression, which is attenuated by an AMPK inhibitor (He et al., 2018). These findings were interesting and pushed us to test the hypothesis that metformin can play a role in improving burn wound healing alone in combination to Vitamin B17, which is the main focus of our study.

Vitamin B17 or Amygdalin is known to delay and inhibit cell proliferation and angiogenesis in some cancer cases(Makarević, Rutz, Juengel, Kaulfuss, Reiter, et al., 2014). It was also reported to be an immunomodulator with some anti-inflammatory effects and consequently might play some role in burn wound healing. On this basis, our goal was to characterize the mechanism of burn wound healing, and the possibility to tag a role for each of Vitamin B17, Metformin and autologous fat tissue as a potential source of stem cells, in burn wound healing regulation.

In this study, we were investigating the regulation of burn wound healing in diabetic rats, in the presence of a potential source for autologous mesenchymal stem cells found in subcutaneous fat. Moreover, the Vitamin B17 and metformin were being tested for their implication in the healing process in the presence or absence of autologous adipocyte tissue as a potential promoter of growth factors regulation and stem cells manipulation. The accomplishment of this work has great implication in wound healing complications such as chronic skin inflammation and diabetic skin closure which can save the life of thousands of injured persons.

B. Diabetes mellitus overview:

Diabetes mellitus (DM) or so called the "silent killer" became one of the most spread and mortal diseases worldwide, it complicates the life of diabetic persons and leads directly or indirectly to death. DM is a chronic lifelong condition that prevents our body from efficiently using the energy from the food we eat. To better understand this disease, it is essential to know how the body uses and metabolises food for energy production. The blood vessels and blood are considered as the main highways that transport sugar from stomach and liver to the cells in order to use it or to store it. Sugar cannot go into the cells by itself. The pancreas releases an essential helper which is the insulin into the blood letting the sugar enters to different cell types of our entire body.

1. Definition and epidemiology

Scientifically the WHO defines diabetes as a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas or the ineffectiveness of the insulin produced. Such a deficiency results in increased concentrations of glucose in the

blood, which in turn affect the body at a multi-organ level. The blood vessels and nerves are particularly susceptible to this damage(Kolluru, Bir, & Kevil, 2012).

Worldwide in 2017, 424.9 million people aged 20–79 years or 451 million people aged 18–99 years lived with diabetes. It was also predicted that the number of people with diabetes aged 20–79 years will rise to 629 million or to 693 million among 18–99 years by 2045 (Cho et al., 2018). The prevalence of diabetes in adults aged 18–99 years was estimated to be 8.4% in 2017 and predicted to rise to 9.9% in 2045. It was also estimated that, in 2017, approximately 5.0 million deaths were attributable to diabetes among people aged 20–99 years. Hence, diabetes accounted for 9.9% of the global all-cause mortality among people within this age range (Shaw, Sicree, & Zimmet, 2010).

2. Diabetes types and causes

Diabetes has different types that can be simplified into 3 **main ones**:

Type 1 diabetes, formerly known as insulin-dependent diabetes, is due to a pancreatic failure at the level of producing the insulin which is essential for survival. This form develops most frequently in children and adolescents, but is being increasingly noted later in life (Kerner & Brückel, 2014).

Type 2 diabetes, or the non-insulin-dependent diabetes, results from the body's inability to respond properly to the action of insulin produced by the pancreas. Type 2 diabetes is much more common and accounts for around 90% of all diabetic cases worldwide. It occurs most frequently in adults, but is being noted increasingly in adolescents as well (Kerner & Brückel, 2014).

Gestational Diabetes, it is a woman specific diabetes type that usually develops during pregnancy (gestation**).** Gestational diabetes is considered as temporary diabetes, since the blood sugar usually returns to normal soon after delivery. However, in some cases, women can be at risk of developing type 2 diabetes. Where the necessity to work with a health care team to control and manage the blood sugar (Seshiah et al., 2009).

Other diabetes types can be recorded and are related directly to different stress types, nutrition and life style; most of these complications are temporary and are reversible.

3. Diabetes and genetics:

Referring to the American Diabetes Association, Type 1 and type 2 diabetes have different causes. Some are related to genetic predisposition and others are related to our lifestyle.

The inherited predisposition to the disease is not enough; an additional environmental factor is needed in order to trigger the diabetes. Unlike some traits, diabetes does not seem to be inherited in a simple pattern. Yet clearly, some people are born more likely to develop diabetes than others (Zimmet, Alberti, & Shaw, 2001).

Genes alone are not enough. One proof of this is identical twins. Identical twins have identical genes. Yet when one twin has type 1 diabetes the other gets the disease, at most, only half the time. Moreover, certain defined genetic markers have been shown to increase the risk of developing Type 1 diabetes. Type 2 diabetes is strongly familial, but it is only recently reported that some genes have been consistently associated with increased risk for Type 2 diabetes in certain populations. Both types of diabetes are complex diseases caused by mutations in more than one gene, as well as by environmental factors (Ley, Schulze, Hivert, Meigs, & Hu, 2015).

4. Diabetes discovery and evolution:

Diabetes is not a new disease; it was well documented in ancient civilizations since more than 3500 years ago. It was known by the Egyptian, Greek, Indian, Latin, Persian, and other civilizations through history(Lakhtakia, 2013). It was well noticed by Egyptians 1500 years BC and it was named as diabetes in 250 BC, referring to the excess fluid excretion by kidneys (White, 2014). Then, it gained a second name (mellitus) or honey in Latin around the 11^{th} century. Diabetes was also separated by an ancient Indian physician into 2 types. The deep knowledge of this disease became wider with time evolution, also the diagnosis related to the urine smell and colour goes back to the 11^{th} century.

Between 1700 -1800, the reason behind the sweet taste in the urine of diabetic persons was linked to an excess sugar that is present in their blood and urine (White, 2014).

Table1 simplifies the main collected information which helped in the discovery and achievement steps towards understanding this disease over the last five centuries.

Table1: evolution of diabetes mellitus management

Time line	Main discovery or achievement	The part	Ref
17^{th} century (1675-1898)	Syrop of poppines was prescribed as a treatment of diabetes mellitus over 200 years.	Different physicians	(Lakhtakia, 2013)
(1770-1800)	Identification the reason behind the sweet taste of the urine which back to an excess of sugar in the urine and blood	Mathew Dobson	(Diagnostics, 2017)

2nd half of 18th century	High fat and protein diet first use for treating diabetes	John Rollo	(White, 2014)
Early 19th century	First chemical tests to detect sugar in urine	Bouchardat	(Lakhtakia, 2013)
Early 19th century	The discovery of glycogen and the first link between diabetes, glycogen and metabolism	Claude Bernard	(White, 2014)
1869	The discovery of Islets of Langerhans	Paul Langerhans	(Akash, Rehman, & Chen, 2013)
1889 (20 years later)	Discovery of a pancreatic extract which help to reduce hyperglycaemia and glycosuria and so called after " insulin " or islands in Latin	Joseph von Mering and Oskar Minkowski	(Akash et al., 2013)
Early 20th century	Invention of a new method to measure glucose in urine (Benedict's solution)	Stanly Rossiter Benedict	(Goldman, Zajac, Shrestha, Patel, & Poretsky, 2016)
1916	First book about " the treatment of Diabetes Mellitus"	Elliot Joslin	(Goldman et al., 2016)
1919	Book publication titled : "The Dietary restrictions in treatment of diabetes" which invented a starvation diet that causes some death cases among the patients	Frederick Allen	(Goldman et al., 2016)
July 1922	The first bottle of Lilly's Iletin (Insulin)	Eli Lily	(Goldman et al., 2016)
1923	The first commercial insulin production	Eli Lilly and company	(White, 2014)
1949	Insuline was suggested to play an important role as a key transporting glucose into cells	Rachmiel Levine	(White, 2014)
1949 (same year)	Production of insulin of a standardized insulin syringe designed and approved by the American Diabetic association (ADA)	Becton Dickinson and company	(Goldman et al., 2016)
1953	The first hospital blood glucose monitoring	Elliot proctor and staff	(Goldman et al., 2016)
1959	Development of a radioimmunoassay technique to measure insulin in blood	Solomon Berson and Rosalyn yalo	(Lakhtakia, 2013)
1970	Introduction of first glucose meter	The Ames company	(Lakhtakia, 2013)
1972	U100 insulin syringes with low dosage error became available	Different companies	(Lakhtakia, 2013)
1976	First insulin pump were invented	Dean Kamen and Rosalyn yalow	(Goldman et al., 2016)
1979	New diabetes classification into 4 conditions: - Insulin-dependent or type 1 Diabetes - Non-insulin-dependent or type2 Diabetes - Gestational diabetes - Diabetes linked to other syndromes or conditions	National diabetes Data group	(White, 2014)
1982	The first biosynthetic human and non-animal insulin (humulin) were produced and distributed in different countries	FDA approved	(Lakhtakia, 2013)

1984	The first insulin pen NOVO pen	Novo nordisk	(Goldman et al., 2016)
1989	Release of the first standards of care to guide physicians in the treatment of diabetes	The American Diabetes Association	(Lakhtakia, 2013)
1991	November 14 was launched as the world diabetes day (the birthday of Frederick banting)	The world health organization WHO	(Goldman et al., 2016)
1997	The fasting glucose level for diagnosing diabetes was lowered from 140mg/dl to 126mg/dl		(Goldman et al., 2016)
Mid 1900	Metformin discovery and evolution in markets		
1918	Metformin was first discovered		(Goldman et al., 2016)
1940	Metformin was rediscovered		(White, 2014)
1957	Metformin was reported for the first time to treat diabetes		(Goldman et al., 2016)

5. Diabetes mellitus nowadays:

Nowadays, diabetes mellitus is largely understood as a disease, since the cooperation of cumulative efforts and approaches through centuries helped the management of diabetes and led to a better understanding of this disease.

Physicians of today can easily diagnose diabetes with different blood and urine testing techniques. Moreover, it became easy to differentiate between Type 1 and Type2 diabetic patients, as well as the linked treatment approaches.

Insulin injection remains the standard treatment for people having Diabetes type 1. Since it can compensate for the absence or the lack of secretion of the main pancreatic hormone.
Metformin or Biguanide is usually used for lowering glucose levels or glycaemia in both diabetes type 1 and 2. Up till now, metformin is the most used drug either alone or in combination with other molecules. However, a panoply of molecules targeting specific receptors or enzymes have been developed recently and are present in the market, they include: canagliflozin, dapagliflozin, and empagliflozin.
Despite the presence of this large series of specific therapeutic molecules, metformin remains the drug of choice in most circumstances.

C. Metformin:

Metformin or N, N-dimethylbiguanide is the first line medication for treating type 2 diabetes. It was first discovered in 1917 and described in 1922 by Emil Werner and James Bell as a product in the synthesis of N, N-dimethylguanidine(Scheers et al., 2018).

Considerable efforts have been made since the 1950s to better understand the cellular and molecular mechanisms of action of metformin, a potent antihyperglycemic agent now recommended as the first line oral therapy for type 2 diabetes (T2D).
The origin of metformin trace back to a folk remedy derived from a toxic plant Galega officinalis or French lilac. Galega officinalis contains the phytochemicals galegine and guanidine both of which decrease blood sugar levels (Scheers et al., 2018). The plant was used in medieval times to treat the symptoms of diabetes. Clinical trials have proven that galegine and guanidine were too toxic for humans. However, when two guanidine molecules were joined together with slight modification, the result gave the biguanides: phenformin, buformin and metformin. Of this family of drugs only metformin remains, others were discontinued for their high risk of lactic acidosis and mortality (Hakeem-Habeeb, 2011)

Metformin main functions are illustrated in (figure.1) which simplifies the hormonal and glycaemia regulation of metformin. Briefly, the most important is that Metformin has remarkable effect on activating the AMPK pathway (Hakeem-Habeeb, 2011). In addition Metformin proved to improve the angiogenic functions of endothelial progenitor cells via activating the AMPK/ eNOS pathway in diabetic mice (Yu et al., 2016), the same study proved that metformins accelerate wound closure and angiogenesis in diabetic mice, however, the mechanism was not determined (Yu et al., 2016).

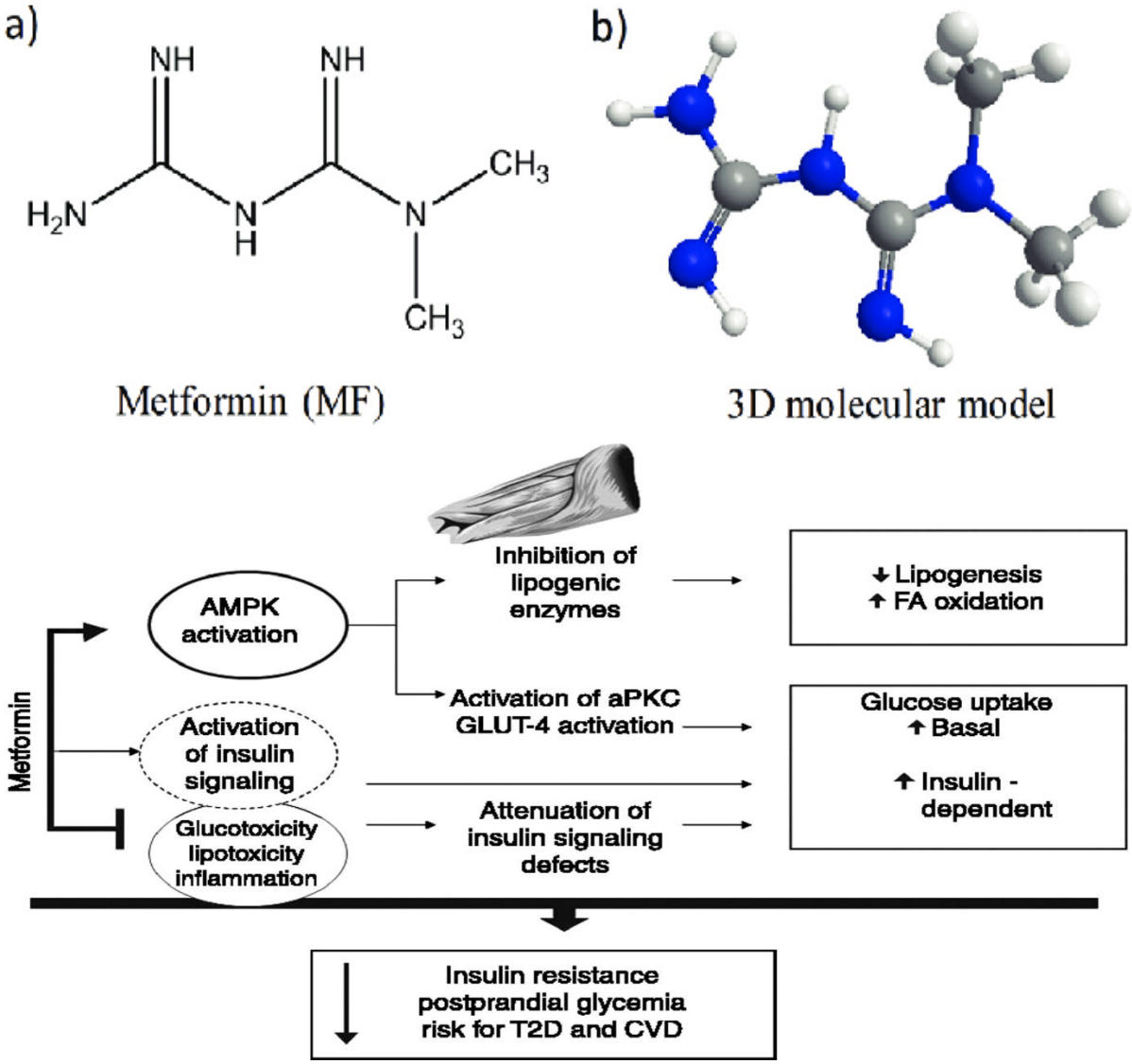


Figure1: molecular aspects and Main functions of metformin (Zhou et al., 2001)

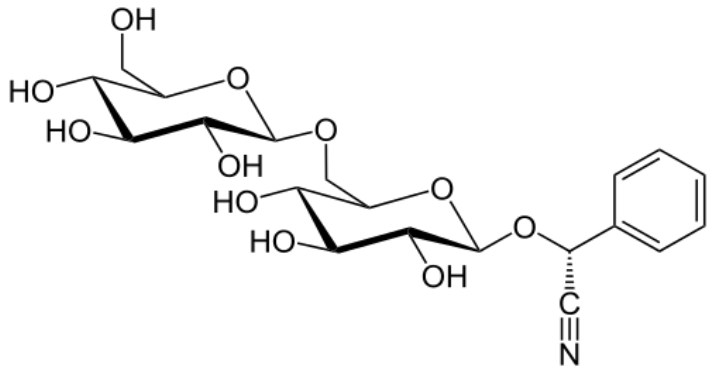
D. Vitamin B17 (Amygdalin):

Amygdalin (from Ancient,Greek: *amygdálē* "almond") is a naturally occurring chemical compound, famous for falsely being promoted as a cancer cure.

Vitamin B17, also called amygdalin, and more commonly laetrile, is one of the most controversial vitamins of the last few decades. Moreover, some sources believe that neither amygdalin nor laetrile is a vitamin.

1) Vitamin B17 Chemical formula and hydrolysis:

Amygdalin is classified as a cyanogenic glycoside, a large group of secondary metabolites that are widely distributed in the plant kingdom, including many plants that are commonly consumed by humans. This classification is due to a nitrile group (figure. 2), which can be released as the toxic cyanide anion by the action of a beta-glucosidase: eating amygdalin will cause it to release cyanide in the human body, and may lead to cyanide poisoning (Newton, Schmidt, Lewis, Lawrence, & Conn, 1981).



Chemical formula	$C_{20}H_{27}NO_{11}$
Molar mass	457.429

Figure2: Molecular aspects of Amygdalin and its chemical formula (wikepedia)

2) Amygdalin main sources:

Amygdalin is mainly found, in apples, apricots and almonds. It is widely distributed in the plant kingdom, and being present in more than 2500 plant species (Bolarinwa, Orfila, & Morgan, 2014). Amygdalin content of seeds from rosacea species, of non-Rosacea seeds amygdalin, and of processed products, are well documented in tables (2, 3, and 4).

Table 2: amygdalin content of seeds from rosacea species (Bolarinwa et al., 2014)

Amygdalin content of seeds from Rosaceae species

Fruit Seeds	Amygdalin Content (mg/g)
Apricot	14.37 ± 0.28
Cherry (Black)	2.68 ± 0.02
Cherry (Red)	3.89 ± 0.31
Nectarine (Summer Fire)	0.12 ± 0.01
Peach	6.81 ± 0.02
Plum (Green)	17.49 ± 0.26
Plum (Black; Friar Black)	10.00 ± 0.14
Plum (Purple; Larry Anne)	2.16 ± 0.02
Plum (Yellow; Son Gold)	1.54 ± 0.02
Plum (Red; Laetitia)	0.44 ± 0.04
Apple (Royal Gala)	2.96 ± 0.02
Pear (Conference)	1.29 ± 0.04

Each value is expressed as mean ± standard deviation (n = 3 extractions)

Table 3: amygdalin content of non-Rosacea seeds (Bolarinwa et al., 2014)

Amygdalin content of non-Rosaceae seeds

Fruit Seeds	Amygdalin Content (mg/g)
Courgette	0.21 ± 0.13
Cucumber	0.07 ± 0.02
Marrow	0.06 ± 0.01
Melon (Honey Dew)	0.12 ± 0.07
Squash (Crown Prince)	0.11 ± 0.22
Squash (Acorn)	0.07 ± 0.03
Squash (Red Kabocha)	0.07 ± 0.11
Squash (Butternut)	0.01 ± 0.04

Each value is expressed as mean ± standard deviation (n = 3 extractions)

Table 4: amygdalin content of processed products (Bolarinwa et al., 2014)

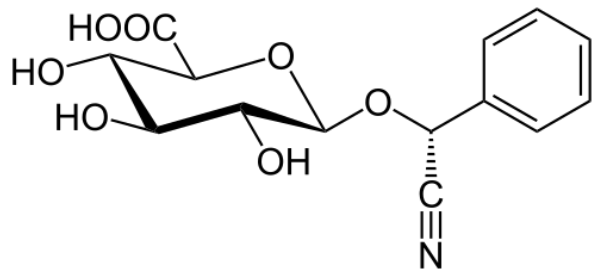
Amygdalin content of processed products	
Processed Products	Amygdalin content (mg/g)
Almond (Toasted)	0.12 ± 0.06
Almond Milk	0.05 ± 0.01
Almond Cocoa Dessert	0.04 ± 0.02
Almond Flour	0.03 ± 0.01
Apple Juice (100% pressed Bramley)	0.09 ± 0.03
Apple & Beetroot Juice (pressed apple)	0.02 ± 0.02
Apple Juice UHT (3 brands)	0.004 ± 0.01
Apple Puree	0.02 ± 0.01
Apricot & Honey Cereal Bar	nd
Apricot Slices tinned in Juice	0.05 ± 0.07
Cider (2 brands)	nd
Fruit Smoothie (pasteurized)	0.01 ± 0.02
Peach Drink	0.04 ± 0.05
Peach Slices tinned in Juice	0.06 ± 0.01
Prune Slices tinned in Juice	0.03 ± 0.03
Pumpkin (Toasted)	nd
Marzipan	0.02 ± 0.01

Each value is expressed as mean ± standard deviation (n = 3 extractions). nd - not detected.

Since the early 1950s, both amygdalin and a modified form named **laetrile** (Figure. 3) have been promoted as alternative cancer treatments, often using the misnomer **vitamin B_{17}**. However, studies have found them to be clinically ineffective in the treatment of cancer, as well as potentially toxic or lethal when taken by mouth, due to cyanide poisoning (Dang, Nguyen, & Tran, 2017).

Laetrile was originally developed in 1952 by the biochemist Ernst Krebs (NutritonReview.org, 2012) . Krebs originally isolated laetrile from apricot pits, and with his son, began to promote it as a cancer preventative and a wonder cure. Extensive testing of laetrile by the National Cancer Institute found laetrile to be of no value as a cancer treatment, and it was further rejected by the Food and Drug Administration on the grounds it might be

poisonous due to its cyanide content. Taking excessive amounts of laetrile are dangerous, and also used improperly, can be lethal.



Chemical formula	$C_{20}H_{27}NO_{11}$
Molar mass	457.429

Figure3: Molecular aspects of Laetrile and its chemical formula (wikepedia)

History of laetrile

Historically, Amygdalin was first isolated in 1830 from bitter almond seeds (Prunus dulcis) by Pierre-Jean Robiquet and Antoine Boutron-Charlard. In addition Liebig and Wöhler found three hydrolysis products of amygdalin: sugar, benzaldehyde. and prussic acid (hydrogen cyanide, HCN). Later research showed that sulfuric acid hydrolyzes it into D-glucose, benzaldehyde, and prussic acid; while hydrochloric acid gives mandelic acid, D-glucose, and ammonia (Gonzales & Sabatini, 1989).

In 1845 amygdalin was used as a cancer treatment in Russia, and in the 1920s in the United States, but it was considered too poisonous. In the 1950s, a purportedly non-toxic, synthetic form was patented for use as a meat preservative, and later marketed as laetrile for cancer treatment(Markle, Petersen, & Wagenfeld, 1978).

The U.S. Food and Drug Administration prohibited the interstate shipment of amygdalin and laetrile in 1977. Thereafter, 27 U.S. states legalized the use of amygdalin within those states (Dorr & Paxinos, 1978).

While there is ongoing research, to date, there has been no effective proof that laetrile helps prevent or cure cancer. The main medical criticism commonly directed at laetrile is that people with potentially curable cancer may choose to take laetrile while avoiding conventional treatments, waiting until it is too late to gain benefit from effective therapy.

In our research we will be investigating the implication of amygdalin on the wound healing process. Previous studies showed that Amygdalin analogues Inhibit IFN-γ signalling and reduce the inflammatory response in human epidermal keratinocytes (Paoletti et al., 2013). Moreover, an additional study proved that Amygdalin inhibits angiogenesis in the cultured endothelial cells of diabetic rats (Mirmiranpour et al., 2012). In parallel amygdalin exerts inhibitory effects on angiogenesis in aortic rings of diabetic rats and may pave a new way for treatment of unfavourable angiogenic conditions. Besides, Amygdalin inhibits angiogenesis in the cultured endothelial cells of diabetic rats (Mirmiranpour et al., 2012).
All mentioned informations pushed us to think that amygdalin may have a role in modulating and regulating the wound healing process which involves: inflammation, cell proliferation and angiogenesis.

Amygdalin, the controversial literature and main research findings:

After a wide and deep search in the literature, a non-countable number of papers and reviews were encountered about Vitamin B17 (Amygdalin). Some articles reported some controversial results already, where Amygdalin was highlighted to play an important role in the modulation of cancer cell growth and at the same time was blamed to have a poisoning effect. Thus, the main published works about Vitamin B17 were covering mainly four essential topics: its antitumor action on different human cancer types (Song & Xu, 2014), its potential modulatory effect of cell growth, its important role in apoptosis and potential apoptotic capacity, and its toxicity. In addition, other covered topics were linked to the science of physiology, toxicology, pharmacology, metabolic genetics, oncology and many others.

It is important to mention, that there was no single or no remarkable study that linked Amygdalin to burn wound healing. However, there were only 6 publications which covered diabetes and the use of vitamin B17

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E. Skin anatomy, function and complications:

For the purpose of this work it was found pertinent to have an overview about the skin anatomy and physiology, in order to evaluate better the burn wound healing process and the effect of the different variables, mainly vitamin B17 and metformin injection, in addition to the contribution of autologous fat tissue, in wound healing and skin repair for diabetic and non-diabetic persons.

The skin (cutis, integument) and its appendages (hair, mammary glands, sweat glands, sebaceous glands and nails) constitute the integumentary system, which is already known as the external cover of our body and forms the largest organ(Mclafferty et al., 2012). The skin represents alone (15% to 20%) of our body weight. It is a complex organ composed of different cell types that differ anatomically and physiologically. Moreover each skin cell type has an ability to work with other cells and cooperate in order to provide many functions that are necessary to face the external environment and to keep a healthy internal homeostasis(Mclafferty et al., 2012).

1. Main skin function:

The major functions of the skin include the following (Mclafferty et al., 2012):

- It represents the first barrier against the external environment and protects us from different types of physical, chemical and biological agents such as bacteria and viruses. Additionally it can resist to all kinds of pressures including mechanical pressure, chemical pressure and heat pressure by forming a balanced permeability barrier.
- It holds a primary responsibility in immunological responses and promotes initial information and reaction, in order to call for the appropriate effector cells in the lymphatic tissue.
- It regulates the body temperature, skin hydration and water loss for better modulation of homeostasis.
- It connects us with the external environment by providing sensory information through the nervous system.
- It serves as site for vitamin D synthesis and has an endocrine function by secreting hormones, growth factors and cytokines.
- It performs a main role in secretion and has many excretory products from different exocrine glands such as sebaceous, sweat and apocrine glands.

2. Skin histology:

Two main layers compose the skin: the epidermis (ectodermal part) and dermis (mesodermal part). Both layers have different compositions and extensions (figure.4), in addition to the hypodermis which is formed mainly of variable amounts of adipose tissue. Each of the 3 layers has its own composition and characteristics:

The **epidermis** consists of four main layers, so called strata (stratum); they increase to five in the case of thick skins (figure.5-6). These layers have a high turnover rate and grow continuously. Beginning from the deepest layer they are as follow:

Stratum basale (SB) or so called stratum germinativum since it serves as reservoir of mitotically active epidermal stem cells. This layer is in touch with the basal lamina which comes directly above the connective tissue of the dermis and provides renewal of the epidermis which is composed mainly of keratinocytes (figure.5). Anatomically the SB is represented by a single layer of stem cells with are small and have less cytoplasm than the cells in the layer above (figure.5). They are closely spaced packed and include various amounts of melanin. They are connected to each other and to Keratinocytes by desmosomes. New keratinocytes arise from this layer by mitotic divisions and ensure the continuity of epidermis regeneration.

Stratum spinosum (SS), or spinous layer, comes directly above the SB (figure) with larger keratinocytes which become increased in size and flattened. Moreover the nucleis of these cells become elongated, instead of being oval, in the upper layer of SS, where the keratinocytes exhibit numerous cytoplasmic processes or spines, which give this layer its name, and the cells change shape to acquire squamous shape.

Stratum Granulosum (SG), whereby the keratinocytes at this level contain numerous keratohyalin granules, hense the name of the layer. These granules are composed of : the cysteine- rich and histidine-rich proteins as main precursors of filaggrin, which play an important role in aggregating the keratin filaments in the stratum corneun that comes directly above the SG.

Stratum Lucidum (SL) is a layer that is limited only to thick skin and can be considered as a substratum of the stratum corneum (figure 6). It contains eosinophilic cells where the process of keratinization is advanced.

Stratum Corneum (SC) is a layer is composed of the most differentiated keratinized cells in the skin. At this superficial layer, keratinocytes have no nucleis, they lose their cytoplasmic organelles and become filled with keratin filaments (figure 5-6). In the deeper portion of this layer, the plasma membrane of these cornified, keratinozied cells is covered with an extra layer of lipids and forms the water barrier in the epidermis of the skin.

Under the epidermis comes directly the **dermis** which is mainly formed of connective tissue and offers the mechanical support and strenght to the skin and defines its thickness. It is formed mainly of 2 layers: the papillary layer and the reticular layer.

The papillary layer is the superficial layer of the dermis and is directly connected to the stratum basale of the epidermis. It is formed of loose connective tissue and collagen fibers that are thinner than those of the reticular layer. They form a network of collagen mainly collagen1 and collagen 3. The papillary layer is thin and includes:

- The dermal papillae
- The dermal ridges
- The blood vessels which serve the different epidermal layers without entering the epidermis
- The nerve processes that reach the dermis and penetrate the basal lamina in order to enter the epithelial compartment.

The reticular layer: is the deepest part of dermis and differs in thickness depending on the position of the part of the body. Compared to the papillary layer it is thicker and less cellular. It is mainly composed of type 1 collagen and coarser elastic fibers.

An additional layer comes directly deep to the reticular layer and serves as energy storage and also provides insolation. It is a fat rich layer so called the **layer of adipose tissue**. This layer constitutes the hypodermis or subcutaneous fascia.

The hypodermis:

The hypodermis is mainly composed of connective tissue septa separating the adipose tissue which is arranged into lobules. The adipose lobules have different amounts of adipose tissue.

The hypodermis lies deep to the dermis and subcutaneous fascia and can be thick for individuals living in cold climates and for those who are well nourished.

In addition, skin thickness varies depending on the location in the body from 1 to 5 mm . The thickest skin is found usually in the upper part of the back.

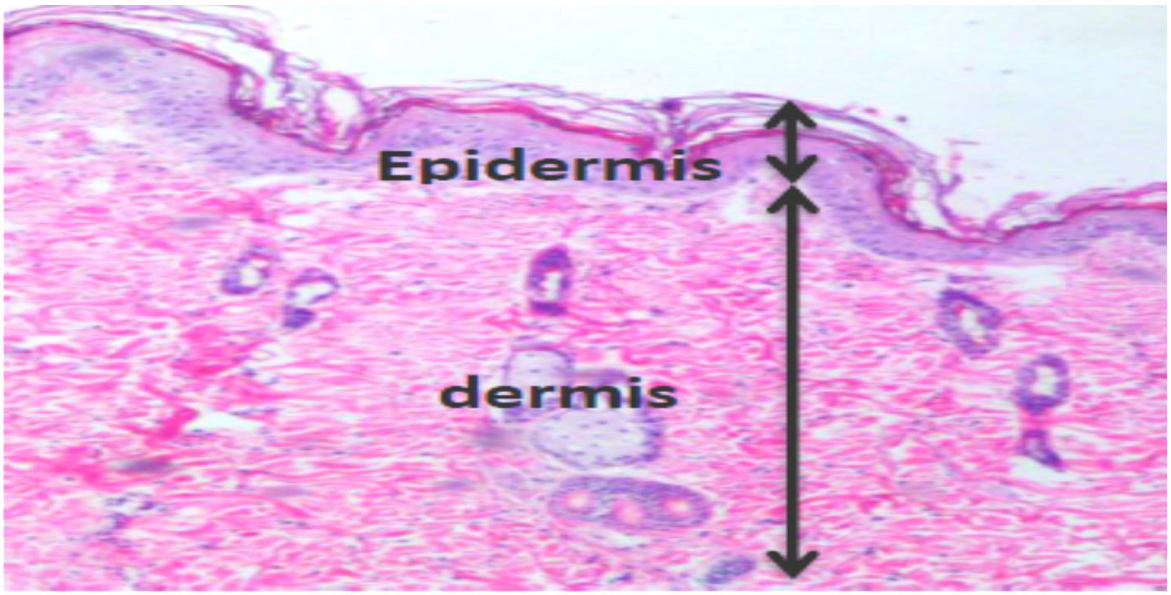


Figure 4: haematoxylin and eosin (H&E)-stained slide (2 x 10) from rat skin. It shows the two main layers of the skin: the **dermis** and **epidermis**. The epidermis appears as the surface layer and it is formed of stratified squamous epithelium that is keratinized. The dermis directly under the epidermis and includes two layers the papillary which comes after the epidermis (mainly more cellular) and the reticular layer which meets the hypodermis.

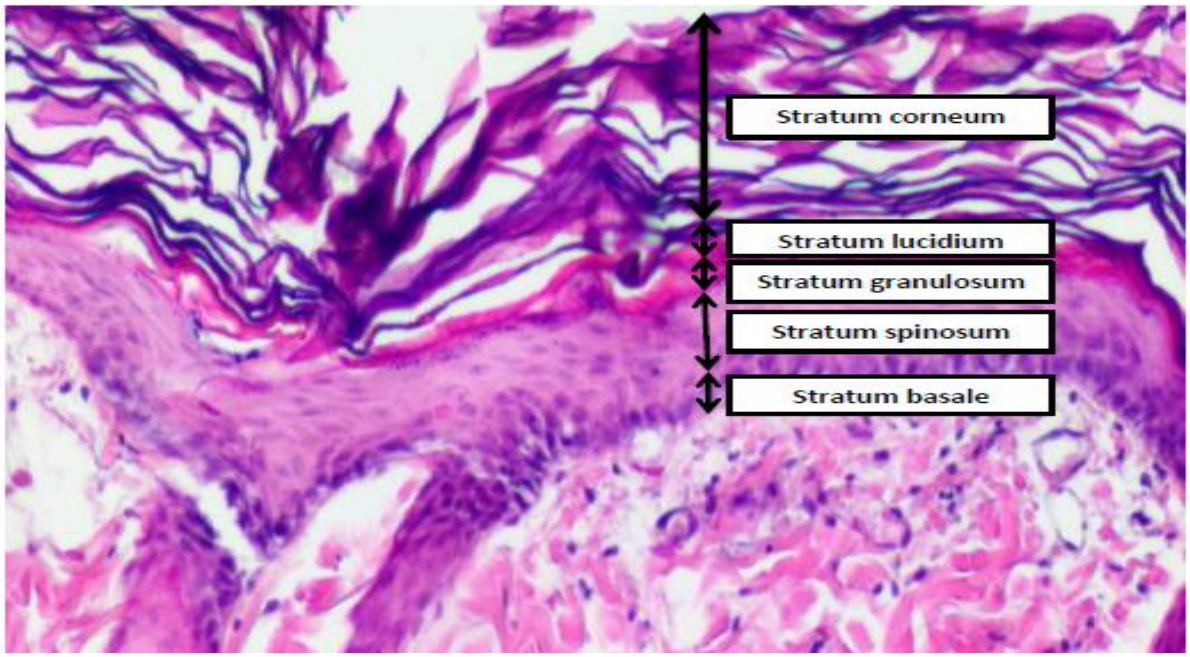


Figure 5: ***a photomicrograph showing the different layers of rat skin.*** **:** a haematoxylin and eosin (H&E)-stained slide (4x10) from Rat skin shows the different layers of epidermis containing an extremely thick stratum corneum and the remaining four superposable strata (lucidiumm granulosum, spinosum and basale)

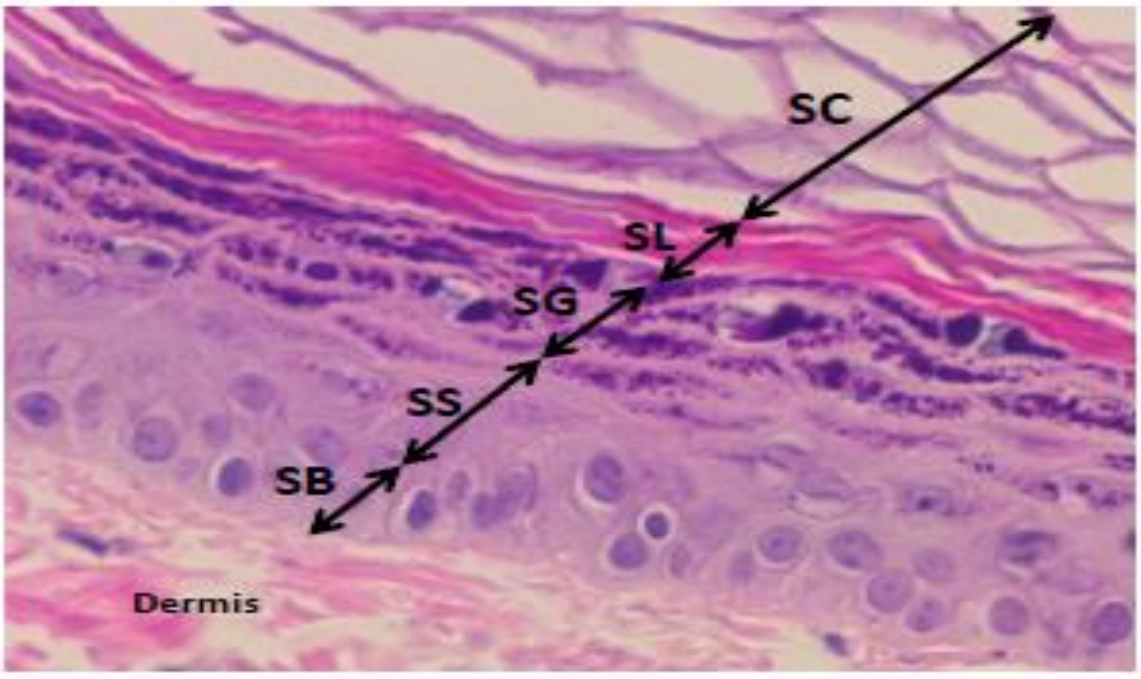


Figure 6: a haematoxylin and eosin (H&E)-stained slide (10x10) from rat skin. It shows the different layers of epidermis. The **SC (**stratum corneum) with anucleate and desquamed cells, the **SL** (stratum lucidium) as a sublayer of stratum corneum, the **SG** (stratum granulosum) where cells exhibit typical apoptotic nuclear morphology including fragmentation of their DNA, the **SS** (stratum spinosum) where the keratinocytes exhibit numerous cytoplasmic processes or spines, which give this layer its name, and the cell shape changes acquires the squamous shape, and the **SB** (stratum basale) which is involved mainly in cell division.

3. Main skin cells:

Four main cell types can be found in the epidermis (Roberts, 2009):

3-1-Cells of the Epidermis:

a) **Keratinocytes:** they are highly specialized epithelial cells, and have the capacity to separate the organism from its external environment. They predominate the epidermis and originate in the stratum basale. They handle two main functions, producing the keratins and formation of the epidermal water barrier which is responsible of maintaining body homeostasis.

b) **Melanocytes:** they produce the pigment of the epidermis. They are loctated in the stratum basale and have elongated nuclei and a clear cytoplasm in the stratum basale. They produce the pigment melanin and secrete it; moreover, they have the capacity to distribute it into keratinocytes. The secreted melanin serves as a protector against the damaging effect of non-ionizing ultraviolet irradiation.

c) **Langerhans cells:** they are the main cells that are responsible of signalling and are involved in the immune system. They have clear cytoplasm and can be clearly seen in the stratum spinosium. Like melanocytes They are dendritic-appearing cells with a tenis shape. Moreover, these cells are considered as antigen-presenting cells in the epidermis. They are first located in bone marrow and originating from the common lymphoid progenitor cells (CLP). The Langerhans cells have the capacity to migrate via the bloodstream in order to enter the epidermis and be differentiated into immunocompetent cells that are involved in mononuclear phagocytosis. After performing antigen phagocytosis they migrate to the nearest lymph nodes where they meet T lymphocytes. Finally they have common features with phagocytes where both express MHC1 and MHC2 in addition to Fc receptors for immunoglobulin G (IgG), for this reason they are involved in delayed-type hypersensitivity reactions.

d) **Merkel's cells:** they are linked to cutaneous nerve response and associated to sensory nerve endings. Their origin is not well known but they are found in the stratum basale and are dendritic in shape with a lobed nucleus. The merkel cells express both

epidermal and neural antigenic markers. They are connected to keratinocytes and contain intermediate keratin filaments in their cytoplasm with some melanosomes. They are mainly characterized by the presence of dense-cored neurosecretory granules. They combine with neuron in the epidermis and with sensitive mechanoreceptors called Merkel's corpuscle.

3-2 Nerve supply

The skin is characterized by the presence of various types of sensory receptors which form the peripheral terminals of sensory nerves. These nerves supply the sweat glands, with motor nerve endings to the blood vessels, and arrector pili muscles.

The free nerve endings in the epidermis lack a connective tissue or shwan cell investment. They terminate in the stratum granulosum and are the most numerous neuronal receptors in the epidermis. They serve different sensory functions including heat, cold, and fine touch. Moreover, they form networks surrounding the hair follicles and are sensitive to hair movement and react as mechanoreceptors.

Other nerve endings can be found encapsulated in a connective tissue capsule. These encapsulations vary in function and include: **Pacinian corpuscles** which are involved in the pressure changes and vibration detection, **Meissner's corpuscles** that are responsible mainly for sensivity to light touch, and **Ruffini's corpuscles** which detect with sensitivity the skin stretch and torque .

3-3 Epidermal skin appendages:

There are four main types of epidermal skin appendages, including: hair follicles, sebaceous glands, eccrine sweat glands, and the apocrine sweat glands (122-125). They form a main store of epithelial stem cells that can play an efficient role in skin wound repair. Each appendage has its products and functions to perform as seen in table 2. However the hair follicle is composed of three segments that differ in their histologic and extention appearance (table 5).

Table 5: The main products and functions of the different skin appendages (123)

Appendage	Product	Localization	Function
• Hair follicle	Hair	Almost present over the entire body except the palmar surfaces of the hands, sides (humans), tails (rats), and the plantar surface of the feet, the lips, and the region around the urogenital orifices.	- Regulation of body temperature - The hair follicle is responsible for the production and growth of a hair.
• Sebaceous Glands	Sebum		- Secretion of an oily protective substance.
• Eccrine sweat glands	Sweat		- Regulation of body temperature.
• Apocrine sweat glands	Sweat with high concentration of lipids, proteins, and carbohydrates.		- Produce a serous secretion containing pheromones -

4. Main differences between human skin and Rat skin:

A study done in 2004 proved that skin histology is almost the same between humans and rats, however, the rat skin is more permeable to all tested substances than human skin (Van Ravenzwaay & Leibold, 2004) Moreover, an additional study showed that the permeation rates of three administrated model drugs through the rat skin were about twice those through human skin(Takeuchi et al., 2012).

In our research work we were using **Clysis methods,** and mainly **subcutaneous and interaperitonial** injections**.** These methods are non-affected by the skin permeability and may not score remarkable differences between human and rat skins.

5. Skin complications in diabetes:Diabetic persons face potentially serious skin problems like dehydration and itching among others, but the most important and challenging skin complications are wounds caused by burn or what we call burn wounds.

F. Burns and burn wound healing:

1. Burn definitions and epidemiology:

Chronic wounds and persistent cutaneous burns complications represent a worldwide challenge and a critical health problem, particularly for diabetic persons since diabetics have a higher propensity for infection(Nicks, Ayello, Woo, Nitzki-George, & Sibbald, 2010). A wound may be described in many ways; referring to its severity, anatomical location, the method of closure and many other factors related to the healing time and scar formation or shape. The American Burn Association defines the burn as an injury to the skin or other organic tissue primarily caused by thermal or other acute trauma(Association, 2009). Burns are also a global public health problem, accounting for an estimated 180 000 deaths annually, according to the World health organization (WHO). Moreover, in 2004, nearly 11 million people worldwide were burned severely enough to require medical attention. In 2008, over 410 000 burn injuries occurred in the United States of America, with approximately 40 000 requiring hospitalization(Günter & Machens, 2012) .

2. Burn classifications:

Burns can alter only the superficial layer or causes damage to deeper layers and sometimes arrive to bones. Burns can destroy one or many types of cells in the skin or other tissues. This damage can be caused by different factors such as hot liquids (scalds), hot solids (contact burns), or flames (flame burns) (Association, 2009), (Gaieski & Mikkelsen, 2016). In some cases, tissue injuries that are caused by radiation, radioactivity, electricity, friction or contact with chemicals are also identified as burns(Gaieski & Mikkelsen, 2016) .

The classification of burn wounds varies depending on many factors such as the depth of the burn, the heat temperature, the duration of contact of injuring agent, the thickness of the skin and finally the blood supply to the injured area (Roberts, 2009). Another classification can be also based on the duration of existence such as acute wounds, which usually heal within 3 weeks, to chronic wounds which may last for more than 3 months.

The adopted classification will classify wounds into four types depending on the severity of burns: The superficial wounds (first burn degree), superficial partial-thickness wounds

(second partial burn degree), deep partial-thickness wounds (second deep burn degree), and full-thickness wounds (figures7 and 8).

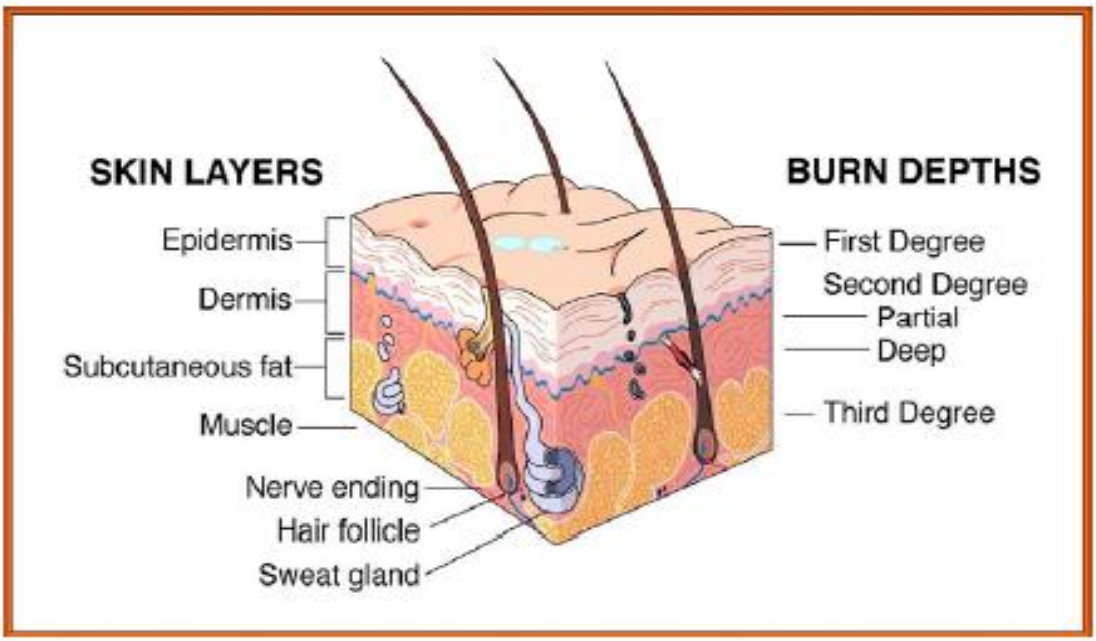


Figure 7: burn classification depending on burn depth and the different altered skin layers: 4 major burn degrees :1- Superficial wounds (first degree wounds), 2- second degree partial-thickness wounds 3- Deep partial-thickness wounds, and 4- Full-thickness wounds (third degree burn)(Günter & Machens, 2012).

Superficial wounds: where damage only affects and extends over the epidermis area of skin with an intact dermis. These wounds heal completely within 8 days without leaving any scars behind (review, 2019).

Superficial partial-thickness wounds: in this case, the wounds extend over epidermis and superficial parts of the dermis, mainly the papillary layer of the dermis. This type of burns forms blisters which can be painful if exposed to air and takes between 2 to 3 weeks to heal .

Deep partial-thickness wounds: they extend from epidermis to deep dermis layers. The papillary and the reticular layers of the dermis, 3 weeks are needed as a minimum for a total healing. Moreover, most of times a surgery is needed to remove the necrotic layer of the skin.

Full-thickness wounds: in this case the subcutaneous tissue layer is damaged and no spontaneous healing, they end with scar formation. There is a big chance of infection and

trauma since the protection barriers are being lost, the need of wound closure with high sterility in such forms is the best treatment approach (Association, 2009).

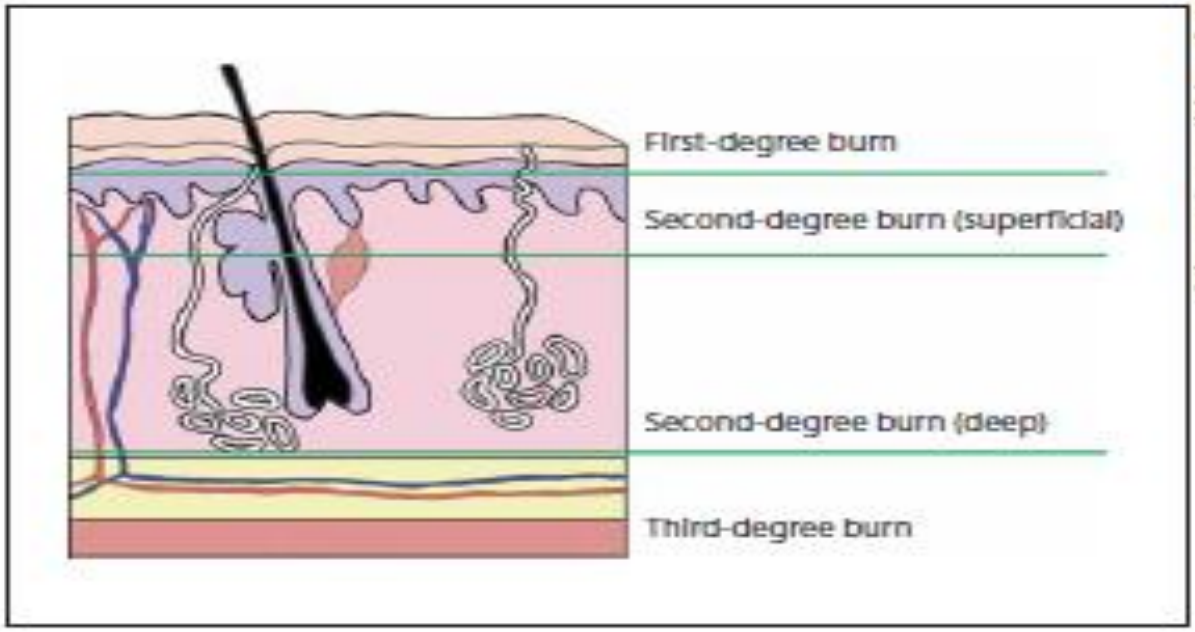


Figure 8: the four categories of thermal injury to the skin and their specific healing time: ***First-degree burn*** (spontaneous healing in 5–10 days, no scar formation); ***Second degree burn, superficial*** (spontaneous healing in 10–21 days, no scar formation); ***Second-degree burn, deep*** (spontaneous healing in 21 days, scar formation); ***Third-degree burn*** (no spontaneous healing, scar formation).(Günter & Machens, 2012)

Note: burns that heal spontaneously are not always straightforward, and many burn wounds have a mixture of superficial and deep burns, making precise classification of the entire wound difficult. Moreover, a burn appearing shallow on day 1 may appear considerably deeper by day 3.

In this study the main focus was on cutaneous burns, deep second degree burns, and wound healing process.

Regardless the cause of the burn, all healing processes should pass by four consecutive steps which are considered common for all wound degrees and can be simplified into 3 main overlapping phases.

1. Burn wound healing process:

The process of restoring skin to its original structure after any injury is called wound healing. Skin burn wound healing remains the most important and challenging skin healing process, since its accomplishment requires the cooperation of many cellular, physiological and molecular mechanisms (Gonzalez et al., 2016). Moreover, an additional factor is needed, namely the presence of a functional immune system in order to have a successful healing and skin repair. These facts prove that the burn wound healing process forms a complex between cells, signalling pathways, and extracellular matrix. The healing mechanism passes through 4 major steps that can be sometimes grouped into 3. Each step has its complications and forms a critical scenario. Following skin injury or burn wound, the damaged tissue is repaired by a normal cutaneous healing response which takes days even years in some cases. Wound healing is recognized as an important process for the survival of all living organisms (Günter & Machens, 2012). In brief, wound healing is a complicated process formed of overlapping stages: Inflammation or Homeostasis, Proliferation, Remodelling or Maturation (Guo & DiPietro, 2010).

Physiology of adult skin wound healing

Skin wound healing begins directly after wounding and might last for variable periodes. It is a dynamic process that is highly regulated by cellular, humoral and molecular mechanisms. Skin regeneration is a specific substitution of a tissue, where the need of a renewal of the superficial epidermis, mucosa and other skin layer is a must. However, skin repair is not homogenous and can differ from case to another depending on many factors leading sometimes to an unspecific form of healing fibrosis and scar formation (Shai & Maibach, 2005). A deficiency of a cell type or the absence of a mediator could be compensated for by others that are involved in wound healing; hence, the repair can still occur. The acute wound healing process can be divided into 3 overlapping stages defined below and described in table 5 and (figure.7 and 8).

1- Stage one: Hemostasis

This stage takes place immediately after injury and lasts for some hours. In this phase, the inflammatory process is initiated. The inflammatory phase of the wound healing cascade gets activated during the coagulation phase and can roughly be divided into an early phase with

neutrophil recruitment and a late phase with the appearance and transformation of monocytes(Reinke & Sorg, 2012) and (DiPietro & Polverini, 1993).

During the hemostasis phase, many cells and factors for the healing the process are involved with no mechanical strength in the wound yet(Robson, Steed, & Franz, 2001). The different clotting cascades are then initiated by extrinsic clotting factors from the injured skin, in addition to the intrinsic thrombocytes that get activated by exposed collagen for aggregation. In brief, this stage involves: vasoconstriction, mast cells and neutrophils recruitment and activation of macrophages(DiPietro & Polverini, 1993).

Vasoconstriction:

During homeostasis, the injured vessels go through a 5 to 10-min vasoconstriction caused by the platelets in order to reduce blood loss and fill the tissue gap by a blood clot containing cytokines and growth factor(DiPietro & Polverini, 1993). The blood clot contains also fibrin molecules, fibronectin, vitronectin and thrombospondins, the basis for fibrosis and scar formation later on. The sequence of events taking place in skin wound healing resembles an orchestra or acts of a drama. A deficiency of a cell type or the absence of a mediator could be compensated for by others that are involved in wound healing; hence, the repair can still occur. Consequently, a provisional matrix is formed as a scaffold for many cells to migrate including leukocytes, keratinocytes, fibroblasts and endothelial cells(Eming, Krieg, & Davidson, 2007). This matrix also acts as a reservoir of growth factors.

The vasoconstriction is life-saving whereby clot formation causes a local perfusion failure. This lack of perfusion will result in a consecutive lack of oxygen, increased glycolysis and changes in pH. A vasodilation, in which thrombocytes invade the provisional wound matrix, will then follow the vasoconstriction (Robson et al., 2001) . In addition, the infiltration of leukocytes is influenced by chemotactic factors secreted by platelets, mast cells, and the leukocytes themselves would release cytokines and growth factors to:

1) initiate the inflammatory process (IL-1α, IL-1β, IL-6 and TNF-α)
2) stimulate the collagen synthesis (FGF-2, IGF-1, TGF- β)
3) activate the transformation of fibroblasts to myofibroblasts (TGF-β)
4) start the capillary sprouting (FGF-2, VEGF-A, HIF-1 α, TGF-β)

Vasodilation can be recognized by an edema (hyperemia) and by a local redness of the wound (Bauer, Bauer, & Velazquez, 2005).

Mast cells and Neutrophils recruitment:

In response to degranulated platelets, mast cells and by-products of bacterial degradation, neutrophils are recruited to the site of injury for 2–5 days(Reinke & Sorg, 2012). Neutrophils are crucial within the first days to injury due to their ability of phagocytosis and protease secretion that may kill local bacteria and assist in degrading necrotic tissue, and to act as chemoattractants for other cells that are involved in the inflammatory phase, and release mediators (TNF-α , IL-1β and IL-6) (Eming et al., 2007). These mediators will amplify the inflammatory response and will stimulate VEGF and IL-8 for an adequate wound repair(Reinke & Sorg, 2012).

Macrophages main role comes at day3 post burn:

About three days after injury, macrophages move to the injury zone to support the ongoing process. They phagocytose pathogens and cell debris, in addition to the secretion of growth factors, chemokines and cytokines(Sindrilaru & Scharffetter-Kochanek, 2013). Furthermore, these secretions keep the healing process going and are able to initiate the next phase of wound healing: the proliferative phase. The inflammatory response is very essential as it supplies growth factors and cytokine signals that regulate cellular and tissue movements, which are very crucial for the wound repair mechanisms in adult mammalians. Evidence suggests that the severity of inflammation is very important in the extent of scar formation(DiPietro & Polverini, 1993).

Macrophages have many functions in wound healing. Some of these are host defense, promotion and resolution of inflammation. In addition, they remove apoptotic cells, support cell proliferation, and restore tissue after an injury(Sindrilaru & Scharffetter-Kochanek, 2013). They also function as antigen-presenting cells. Moreover, phagocytes during wound repair play an integral role in a successful healing response via the synthesis and secretion of numerous potent growth factors such as TGF- β, TGF- α, basic FGF, PDGF and VEGF. These growth factors promote cell proliferation and synthesis of extracellular matrix (ECM) molecules by cells present in the skin(DiPietro & Polverini, 1993).

2- Stage two:

Stage 2 includes proliferation, reepithelialisation, binding of growth factors to their receptors and activation as well as formation of an acute granulation tissue(Mendonça & Coutinho-Netto, 2009).

Proliferation: The second stage is the phase of proliferation. It lasts for 3–10 days after wound infliction. In this stage, the main aim of the healing process is to cover the wound surface, to form a granulation tissue, and to restore the vascularization(Endrich & Menger, 2000). Therefore, besides migration "of local fibroblasts along the fibrin network and the beginning of reepithelialization from the wound edges", neovascularization and angiogenesis are triggered by capillary sprouting. Granulated tissue formation stops via apoptosis, thus forming a mature wound being avascular and acellular(Endrich & Menger, 2000). Besides, the basis of a new matrix of connective is laid down. It is regulated by cytokines like IFN-γ and TGF-β, whereby, collagen synthesis, fibronectin, and other basic substances, needed for wound healing by fibroblasts, are used for the closure of tissue gaps and the restoration of the mechanical strength of the wound. "Subsequently, the synthesis of collagen increases throughout the wound, while the proliferation of fibroblasts declines successively, adjusting a balance between synthesis and degradation of the ECM" (Madden & Peacock Jr, 1971).

Reepithelialization

Reepithelialization is ensured by local keratinocytes at the wound edges in addition to epithelial stem cells located at hair follicles or sweat glands (Li et al., 2013).This process of covering the wound by an epithelial lining is activated by signaling pathways of epithelial and nonepithelial cells from nearby intact skin, thus releasing a myriad of different cytokines and growth factors: EGF, KGF, IGF-1 and NGF among others (Li et al., 2013). "Furthermore, the abolition of the contact inhibition and physical tension at desmosomes and hemidesmosomes produces lipid mediators and activates membrane-associated kinases (SRC kinases) resulting in an increased permeability of the membranes for ions, e.g. calcium"(Reinke & Sorg, 2012). This signaling to the cells at wound edges causes retraction and reorganization of their intracellular tonofilaments to adjust direction of the cellular migration. The loosening of Intercellular desmosomes via collagenase and elastase enzymatic activity causes keratinocytes to migrate along the maintained fibrin blood clot in the higher layers of the granulation tissue (Jacinto, Martinez-Arias, & Martin, 2001). This process is called the 'shuffling' of keratinocytes and is characterized by the ability of such cells to migrate along a chemotactic gradient formed by mediators e.g. IL-1, and over a fibronectin-rich matrix to the wound's center (Jacinto et al., 2001).

The migration itself is accomplished via lamellipodial crawling and is directed into the defective site due to the polymerization of cytoskeletal actin fibers. The formation of a new

focal adhesion at the ECM, which is regulated by integrins (Korting, Schöllmann, & White, 2011), proceeds until the migrating cells touch each other which the n leads to a reorganization of the cytoskeleton (Jacinto et al., 2001).

Binding of the growth factors to their receptors

Binding of the growth factors to their receptors on the endothelial cells of existing vessels is the first step in neovascularization, thereby activating intracellular signaling cascades.. Endothelial cells that are activated will secrete proteolytic enzymes to dissolve the basal lamina and thus be able to proliferate and migrate into the wound area. This process is called 'sprouting'. The endothelial cells orient themselves at superficial adhesion molecules, e.g. integrins (αvβ3, αvβ5, α5β1). Then, they will exocytose matrix metalloproteinases at the front of proliferation to lyse surrounding tissue, and hence support endothelial proliferation. Small tubular canals form the newly built sprouts. In turn, the sprouts will interconnect to others forming a vessel loop. The latter new vessels will differentiate into arterioles and venules. Stabilization of these mature vessels is established via pericytes and the recruitment of smooth muscle cells (Reinke & Sorg, 2012).

Formation of an acute granulation tissue

The last step in the proliferation phase is the formation of an acute granulation tissue, a transitional tissue which replaces the fibrin/fibronectin-based provisional wound matrix. Hence, it might produce a scar by maturation (Krafts, 2010) (Enoch & Leaper, 2005). Furthermore, it is composed of highly dense fibroblasts, granulocytes, macrophages, capillaries and loosely organized collagen bundles. This high amount of cellular compounds gives the granulation tissue its name. As angiogenesis is not yet completed, the tissue is highly vascular (Reinke & Sorg, 2012) making it appear with a classic redness that might be traumatized easily (Korting et al., 2011). However, fibroblasts dominate in this phase, fulfilling different functions such as collagen bundles formation and ECM substances production (i.e. fibronectin, glycosaminoglycans, proteoglycans and hyaluronic acid). ECM, at this stage, presents a scaffold for cellular adhesion in addition to regulation and organization of growth, movement, and cellular differentiation within it(Barker, Rosson, & Dellon, 2006). Fibroblasts are precursors of the provisional wound matrix on and in which cell migration and organization occur (Jacinto et al., 2001). By the end of this phase, the numbers of mature fibroblasts will be reduced by myofibroblast differentiation and consecutively terminated by apoptosis (Hinz, 2007).

3- Stage three: Remodeling:

Remodeling is the last phase of wound healing and occurs from day 21 to up to 1 year after injury. The formation of granulation tissue stops through apoptosis of the cells (Korting et al., 2011).

A mature wound is, therefore, characterized as being avascular as well as acellular. During the maturation of the wound, the components of the ECM undergo changes. Collagen III, which was produced in the proliferative phase, is now replaced by the stronger collagen I (Reinke & Sorg, 2012). This type of collagen is oriented in small parallel bundles and is, therefore, different from the basket-woven collagen in healthy dermis. Later on, the myofibroblasts cause wound contraction by their multiple attachments to collagen and help decrease the surface of the developing scar . Furthermore, the angiogenic process diminishes, the wound blood flow declines, and the acute wound metabolic activity slows down and finally stops (Arnold & West, 1991).

4- Scarring:

The physiological endpoint of mammalian wound repair displays the formation of a scar, which is directly linked to the extent of the inflammatory process throughout wound healing. There are different situations which provide evidence in support of this statement (Reinke & Sorg, 2012). First, there is the fact that fetal wound healing, which shows a lack of the typical inflammatory response, is scarless up to a certain age.

Table 7: Summary of Stages of wound healing and the main referred processes.

	Stages of healing		
Main processes	**Hemostasis and inflammation**	**Proliferation**	**Remodelling and maturation**
	Hemostasis	Dermis Release of growth factors by macrophages and fibroblasts	Reorganization and remodeling of the ECM
	Hemostasis Vasoconstriction	Fibroblast migration and proliferation Synthesis of matrix proteins (fibronectin and collagen)	Myofibroblast formation
	Formation of fibrin clot Inflammation	Angiogenesis	Contraction of the wound
	Release of cytokines and growth factors by platelets and immune cells, and from the disrupted matrix	Epidermis: Keratinocyte migration, proliferation and differentiation Contributions from hair follicle stem cells	Cell apoptosis
	Invasion of inflammatory cells (neutrophils, monocytes macrophages)	Possible contribution from interfollicular epidermal stem cells	Maturation

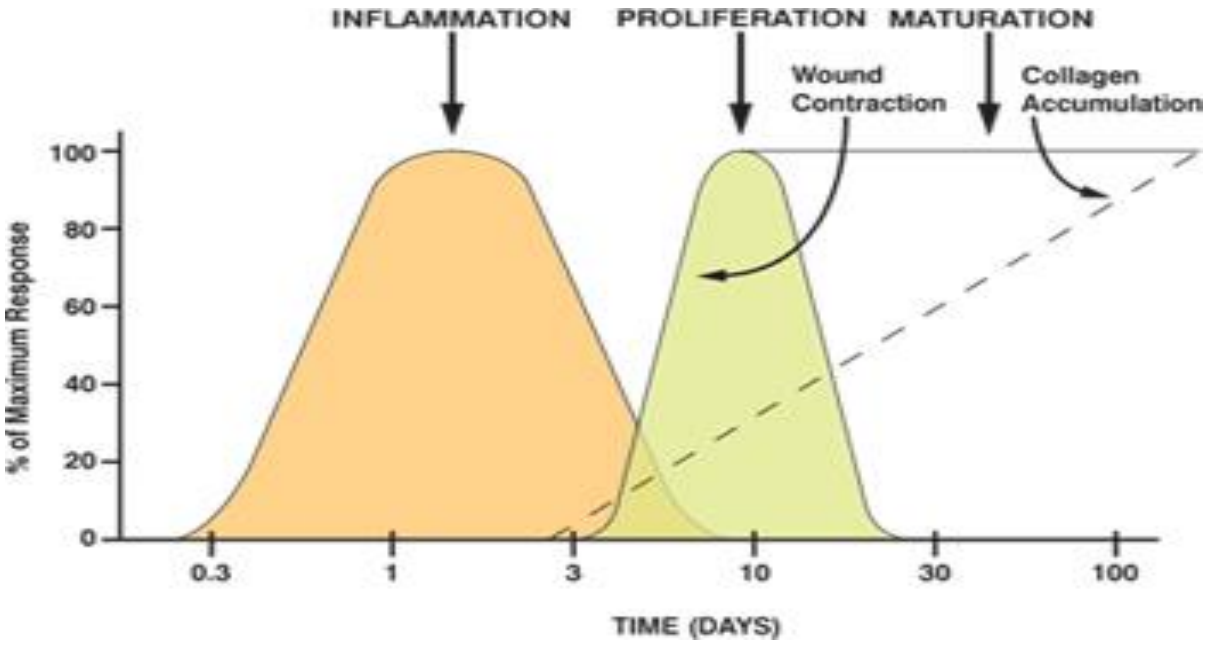


Figure 9: wound healing process and time related mechanisms

Phase	Cellular and Bio-physiologic Events
Hemostasis	1. vascular constriction 2. platelet aggregation, degranulation, and fibrin formation (thrombus)
Inflammation	1. neutrophil infiltration 2. monocyte infiltration and differentiation to macrophage 3. lymphocyte infiltration
Proliferation	1. re-epithelialization 2. angiogenesis 3. collagen synthesis 4. ECM formation
Remodeling	1. collagen remodeling 2. vascular maturation and regression

Figure 10: Normal wound healing process: cellular and biophysiologic events

2. growth factors, interleukins and cytokines in the wound healing process:

The wound healing process involves the coordinated efforts of several cell types including keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. The migration, infiltration, proliferation, and differentiation of these cells will culminate in an inflammatory response, the formation of new tissue and ultimately wound closure. This complex process is executed and regulated by an equally complex signalling network involving numerous growth factors, cytokines and chemokines. Of particular importance is the epidermal growth factor (EGF) family, transforming growth factor beta (TGF-b) family, fibroblast growth factor (FGF) family, vascular endothelial growth factor (VEGF), granulocyte macrophage colony stimulating factor (GM-CSF), platelet derived growth factor (PDGF), connective tissue growth factor (CTGF), interleukin (IL) family, and tumour necrosis factor-α family (TNF- α) .

Cytokines in wound healing:

Cytokines are peptides and glycoproteins that are primarily produced by inflammatory cells with a molecular weight varying from 5 to 30 KDa. Cytokines play important roles in the regulation of inflammatory and immune responses during wound healing by activating various cells. The chemokines, lymphokines, monokines, interleukins, colony-stimulating factors, and interferons are all cytokines, they are different by the type of cells they influence.

Interleukins-1β in wound healing:

In acute wound, IL-1beta is produced by numerous cell types such as monocytes, macrophages, fibroblasts, and keratinocytes. IL-1beta acts as a paracrine factor and autocrine signal that induces the migration and proliferation of keratinocytes. Once exogenously administrated, Il-1β has been shown to promote healing of partial-thickness wounds in swine.

Growth factors in wound healing:

Growth factors form a large family of regulatory peptides that are synthesized and secreted by many of cell types involved in wound healing, including inflammatory cells, platelets fibroblasts, epithelial cells, and endothelial cells. Growth factors are classified into several families based on their characteristics . The most relevant growth factor families for wound healing are the epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming

growth factor β (TGF-β), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF).

G. Factors affecting wound healing:

Multiple factors can lead to impaired wound healing, some are defined as local factors because they influence the characteristics of wound, while other factors are known to be systematic factors related to an overall health and disease state figure 9. **The main local factors are:** Oxygenation and Infections, while **The main systematic factors are:** Age, stress, sex hormones, diabetes, medications, obesity, alcohol consumption, smoking, and nutrition (Guo & DiPietro, 2010).

Local Factors	Systemic Factors
Oxygenation	Age and gender
Infection	Sex hormones
Foreign body	Stress
Venous sufficiency	Ischemia
	Diseases: diabetes, keloids, fibrosis, hereditary healing disorders, jaundice, uremia
	Obesity
	Medications: glucocorticoid steroids, non-steroidal anti-inflammatory drugs, chemotherapy
	Alcoholism and smoking
	Immunocompromised conditions: cancer, radiation therapy, AIDS
	Nutrition

Figure 11: Factors affecting wound healing(Guo & DiPietro, 2010).

In our study of the burn wound healing process, we were interested in evaluating two main factors the oxygenation as a local factor and Diabetes as a systematic factor and their impact on burn wound healing.

Oxygenation:

Oxygen is a vital element in life, it is important for cell metabolism and prevents wounds from infections. Moreover, It Induces angiogenesis, increases keratinocyte differentiation, migration, and reepithelialisation. It also enhances fibroblast proliferation and collagen synthesis and in parallel, it promotes wound contraction.

During the first stages of burn wound healing, the microenvironment of the early wound faces a drop in oxygen levels and is remarkably hypoxic. This is due to vascular disruption and the overconsumption of oxygen by metabolically active cells, for this reason, chronic wounds are notably hypoxic.

In wounds where oxygenation is not restored, healing is impaired. Temporary hypoxia after injury triggers wound healing, but prolonged or chronic hypoxia delays wound healing. In acute wounds, hypoxia can induce cytokines and growth factors production from macrophages, keratinocytes, and fibroblasts. Cytokines that are produced in response to hypoxia include PDGF, TGF-β, VEGF, (TNF-α), and endothelin-1, they are crucial promoters of cell proliferation, migration and chemotaxis, and angiogenesis in wound healing.

In normally healing wounds, the reactive oxygen species (ROS), hydrogen peroxide (H2O2) and superoxide (O2) are thought to act as cellular messengers to stimulate key processes associated with wound healing, including cell motility, cytokine action (including PDGF signal transduction), and angiogenesis. Both hypoxia and hyperoxia increase ROS production, but an increased level of ROS transcends the beneficial effect and causes additional tissue damage .

Briefly, the proper oxygen level is crucial for optimum wound healing. Hypoxia stimulates wound healing such as the release of growth factors and angiogenesis, while oxygen is needed to sustain the healing process

.

H. **Burn wound healing complications in diabetes:**

Diabetic patients are known to experience more infections in clean wounds than non-diabetic patients and to heal more slowly, especially in the extremities. (Table5). Moreover, diabetic patients face a prolonged hypoxia which may be caused by an insufficient angiogenesis.

Hypoxia can amplify the early inflammatory response, thereby prolonging injury by increasing the levels of oxygen radicals (230). Hyperglycemia can also add to the oxidative stress when the production of ROS exceeds the anti-oxidant capacity. The formation of advanced glycation end-products (AGEs) under hyperglycemia and the interaction with their receptors (RAGE). RAGEs are associated with impaired wound healing in diabetic mice as well. High levels of metalloproteases (MMP) are a feature of diabetic foot ulcers, and the MMP levels in chronic wound fluid are almost 60 times higher than those in acute wounds. This increased protease activity supports tissue destruction and inhibits normal repair processes.

As a sum up, the healing complications for persons with diabetes are due to many impaired influencing functions related to this disease involving:

- hypoxia,
- dysfunction in fibroblasts and epidermal cells,
- impaired angiogenesis and neovascularization,
- high levels of metalloproteases
- damage from ROS and AGEs
- decreased host immune resistance, and
- neuropathy

The influence of these factors on wound healing is summarized in figure 12 and table 8.

In brief, reflecting on the state of the art in understanding the process of burn wound healing and its complications in diabetics, it was considered very pertinent to point out the great relevance of assessing the combination of:

(1)- A potential source of stem cells and cytokines, the autologous adipose tissue,

(2)- The immune modulatory, cell proliferation and anti-inflammatory effects of vitamin B17.

(3)- The multiple effects of metformin.

Using an established animal model for burn wound healing, the data emanating from this research will be able to highlight the various mechanisms and signalling pathways involved.

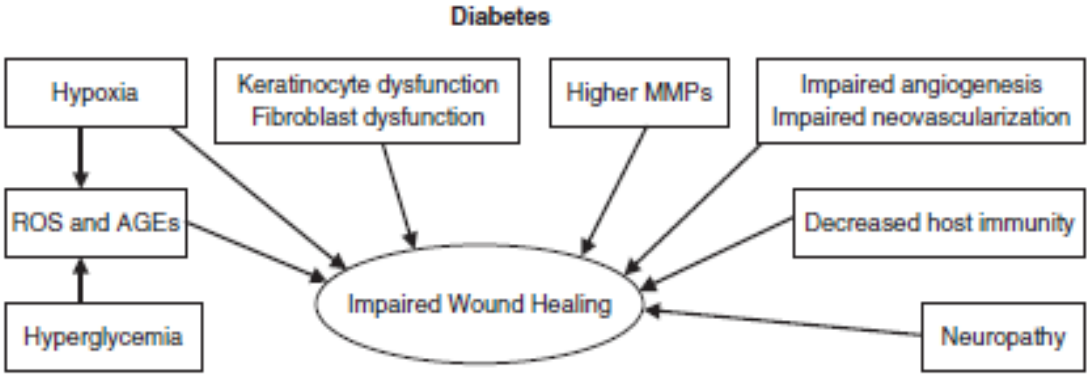


Figure12: The potential effects of diabetes on wound healing. MMPs, matrix metalloproteases; ROS, reactive oxygen species; AGEs, advanced glycation end-products (Guo & DiPietro, 2010).

Table 8: Summary of Factors Related to Wound Impairment in Obesity

Local Wound Conditions	**Associated Diseases and Conditions**	**Factors Altering Immune and Inflammatory Responses**
1. adipokines: leptin, adiponectin, resistin 2. cytokines: TNF-alpha, IL-1, IL-6, IL-8, IL-10 3. chemokines: IL-8, MCP-1, IP-10 MCP, monocyte chemoattractant protein-1; IP-10 interferon-gamma-inducible protein	1. decreased vascularity in adipose tissue 2. skin folds harbor micro-organisms 3. friction caused by skin on skin 4. increased wound tension 5. increased tissue pressure 6. hematoma and seroma formation 7. venous hypertension 1. hard to reposition 2. coronary heart disease 3. atherosclerosis 4. type 2 diabetes 5. cancer 6. hypertension 7. dyslipidemia 8. stroke 9. respiratory problems	1. adipokines: leptin, adiponectin, resistin 2. cytokines: TNF-alpha, IL-1, IL-6, IL-8, IL-10 3. chemokines: IL-8, MCP-1, IP-10 MCP, monocyte chemoattractant protein

(*S. Guo and L.A. DiPietro:* Affecting Wound Healing, *Dent Res* 89(3):219-229, 2010Factors)**

II. Hypothesis and aims:

Diabetes complicates the life of millions of people every year, it is also called the silent killer and one of humanity's greatest health challenges. Moreover, every year the mortality caused by burn is increasing, especially for diabetic persons. Therefore, the elucidation of the signalling mechanisms regulating burn wound healing is primordial to better understand the skin closure and restoration of complications that are related to abnormal and delayed healing. On this basis, our goal was to characterize the mechanism of burn wound healing, and the possibility to tag a role of each of Vitamin B17, Metformin and autologous fat tissue as a potential source of stem cells, in burn wound healing regulation.

In brief the Hypothesis is:

To study our hypothesis, 4 main aims were under investigation:
Amygdalin or vitamin B17, in presence of autologous adipose tissue and metformin improves burn wound healing in diabetic patients. Such an effect can be modulated by vitamin B17 and/or Metformin by regulating the inflammatory response and cell proliferation.

Main Aims:

Aim 1: to study the main differences between burn wound healing in diabetic and non-diabetic rats and the modulation of relevant parameters like scar characteristics, trans epidermal water loss (**TEWL),** healing time, glycemia , HSP70 , IL1, IL 2, IL6 and IL12 as well as Reactive Oxygen Species (ROS).

Aim 2: to study the modulatory effect of vitamin B17 on burn wound healing in diabetic and non-diabetic rats through the regulation of the above parameters.

Aim 3: to evaluate the possible additive effects of metformin and vitamin B17 on burn wound healing in normal and diabetic rats.

Aim 4: to assess the additive effect of added autologous adipose tissue and vitamin B17 on burn wound healing in normal and diabetic rats.

III. Research design and methods:

Animal part:

Animal housing:

Adult male Sprague-Dawley rats (320-380g) were used in this study. All rats were kept in humidity and temperature controlled rooms (21°C±2°C), on a 12/12 dark/light cycle and had free food and water access. All protocols were approved by the Institutional Animal Care and Use Committee of the American University of Beirut.

Research design:

The **190** animals were divided into 2 main groups (diabetic and non-diabetic) as outlined in the research design in (**figure13)**. The rats were received in batches of 48 rats every time. each batch was divided into 2 subsets A and B. the timeline of animal manipulation is described in (Table 9).

Animal groups and numbers

The study covered 10 conditions or subgroups (figure 14) and each condition included 6 time points (D1, D3, D7, D14, D21, and D28). Four animals were used in each time point in order to satisfy the statistical and technical replicates, thus ending by 24 animals per condition. It is important to mention that all sets covered the time points D1, D3, D14 and D28 while, D7 and D21 were excluded in sets 3 and 4 in order to minimize the number of used animals to **190 rats**.

Processes:

All rats were subjected to burning, the burn was done 1 week or 10 days after the induction of diabetes **(figure 14 and 15).** The metformin and Vitamin B17 treatments started either directly after burn or the next morning (at 7am) for the operated animals.

Animal caging:

Once received, the animals were organized in 12 cages, thus animals of the same DOB (date of birth) and same weight were grouped together (up to 4 rats in the same cage).

After operation, each animal was transferred into a separate medium sized cage with daily cleaning and observation to avoid any complication.

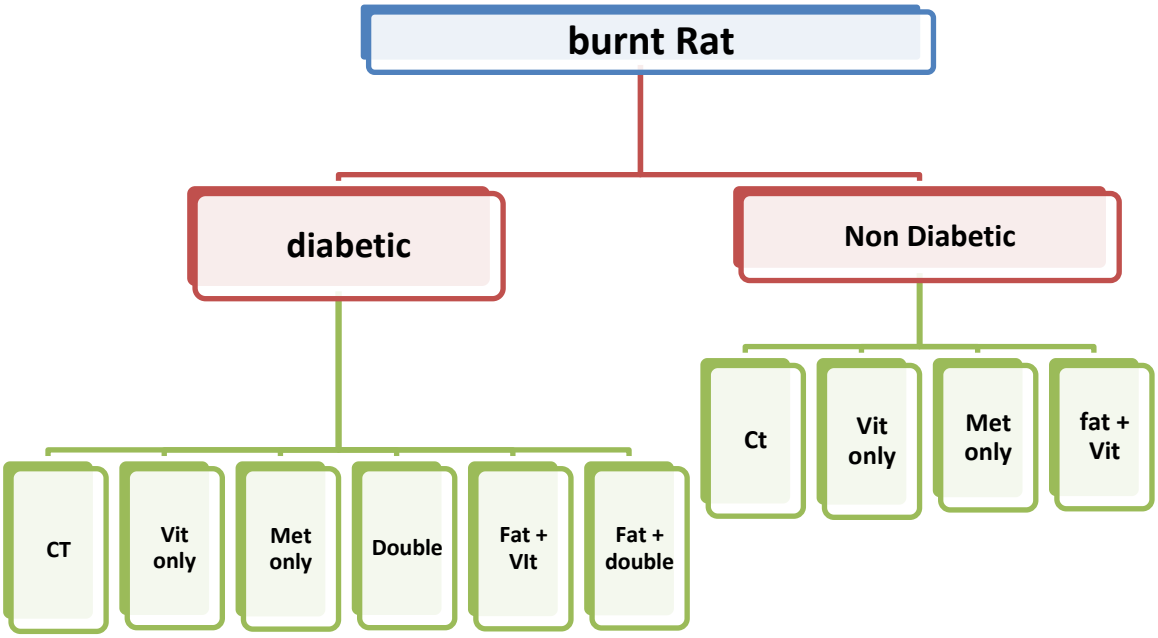


Figure 13: Research design

Table 9: Animal sets and timeline

Set number	Manipulation timeline 2018	Condition 1	Condition 2
1	January to February	D+V	D+V+M
2	April to may	D+V+F	ND +V+F
3	May to June	ND + V	ND+M
4	June to July	D+V+F+M	D+F+M
5	July to august	Ct diabetic	Ct non diabetic

NB: D=diabetic rats; ND= Non Diabetic rats; M= metformin injection; V=Vitamin B17 injection and F = autologous fat tissue relocation.

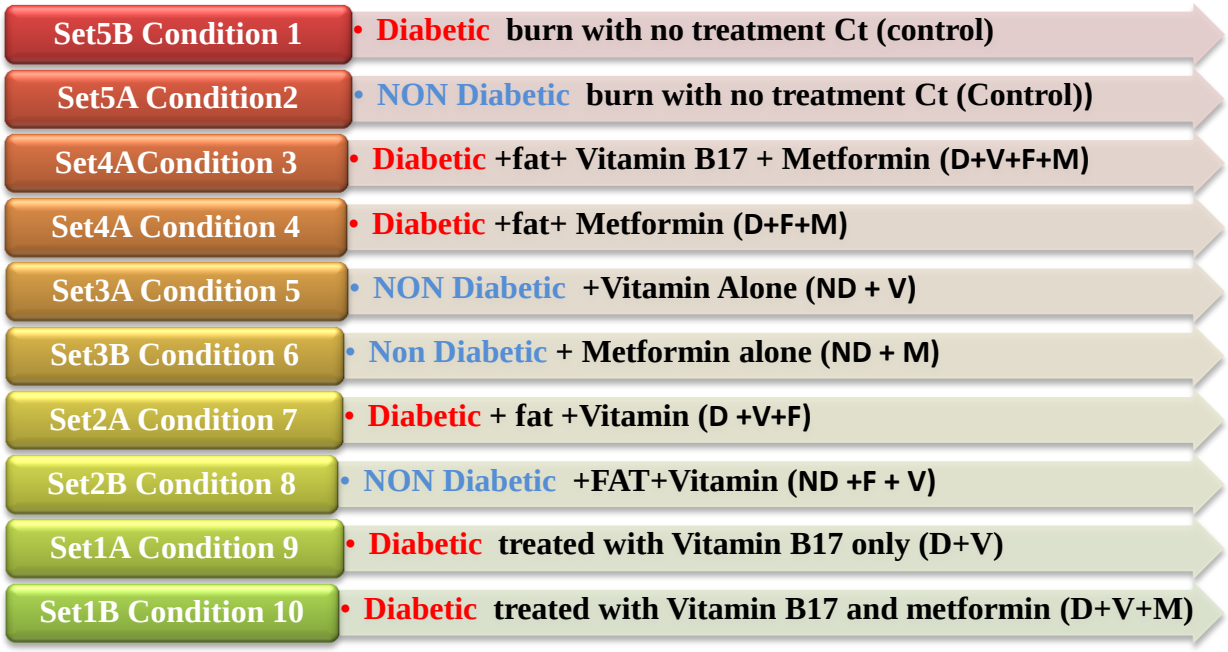


Figure 14: male rats were divided into 2 main groups, diabetic and non-diabetic and then grouped according to the different administered treatments into a total of 10 conditions.

NB: D=diabetic rats; ND= Non Diabetic rats; M= metformin injection; V=Vitamin B17 injection and F = autologous fat tissue relocation.

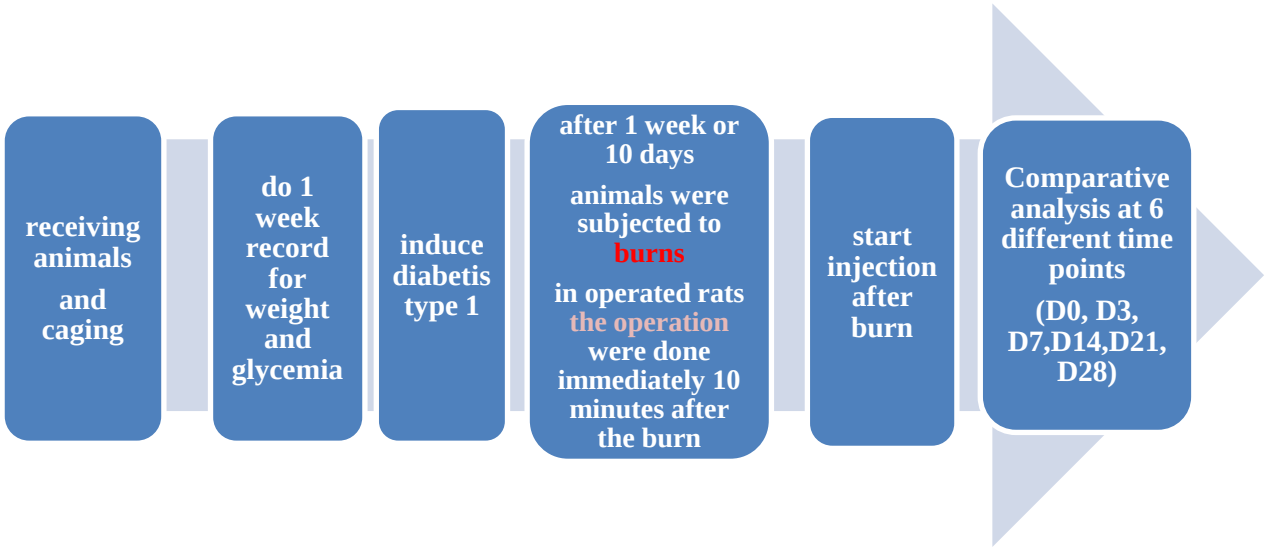


Fig 15: different processes to which the rats were subjected

NOTE: After sacrificing all sets, the animals were numbered (from 1 to 190) then simplified to representative replicates from (1 to 155) as seen in tables (2, 3, 4, 5, and 6)

This labelling method simplified the molecular, clinical and histological experiment.

Table10: list of numbers of non-diabetic and diabetic rats with no injections or operation (Controls)

Samples (1-30): Condition 1 and2 (controls, set 5)

sample ID	set	Day	sacrifice date	D/ND	Condition
1	5	D3	20/07/2018	D	CTR diabetic D3-1
2	5	D3	20/07/2018	D	CTR diabetic D3-2
3	5	D3	20/07/2018	D	CTR diabetic D3-3
4	5	D3	20/07/2018	ND	CTR ND D3-1
5	5	D3	20/07/2018	ND	CTR ND D3-2
6	5	D3	20/07/2018	ND	CTR ND D3-3
7	5	D7	24/07/2018	D	CTR diabetic D7-1
8	5	D7	24/07/2018	D	CTR diabetic D7-2
9	5	D7	24/07/2018	D	CTR diabetic D7-3
10	5	D7	24/07/2018	ND	CTR ND D7-1
11	5	D7	24/07/2018	ND	CTR ND D7-2
12	5	D7	24/07/2018	ND	CTR ND D7-3
13	5	D14	31/07/2018	D	CTR diabetic D14-1
14	5	D14	31/07/2018	D	CTR diabetic D14-2
15	5	D14	31/07/2018	D	CTR diabetic D14-3
16	5	D14	31/07/2018	ND	CTR ND D14-1
17	5	D14	31/07/2018	ND	CTR ND D14-2
18	5	D14	31/07/2018	ND	CTR ND D14-3
19	5	D14	31/07/2018	ND	CTR ND D14-4
20	5	D21	7/08/2018	D	CTR diabetic D21-1
21	5	D21	7/08/2018	D	CTR diabetic D21-2
22	5	D21	7/08/2018	ND	CTR ND D21-1
23	5	D21	7/08/2018	ND	CTR ND D21-2
24	5	D28	14/08/2018	D	CTR diabetic D28-1
25	5	D28	14/08/2018	D	CTR diabetic D28-2
26	5	D28	14/08/2018	D	CTR diabetic D28-3
27	5	D28	14/08/2018	ND	CTR ND D28-1
28	5	D28	14/08/2018	ND	CTR ND D28-2
29	5	D28	14/08/2018	ND	CTR ND D28-3
30	5	D28	14/08/2018	ND	CTR ND D28-4

Table 11: list of numbers of operated diabetic rats that are treated with metformin alone or with metformin and vitamin B17 together

Samples (31-52) Condition 3 and 4 (set 4)

sample ID	set	Day	sacrifice date	D/ND	Condition
31	4	D3	8/06/2018	D	Diabetic double injection +FAT D3-1
32	4	D3	8/06/2018	D	Diabetic double injection + FAT D3-2
33	4	D3	8/06/2018	D	Diabetic double injection +FAT D3-3
34	4	D14	18/06/2018	D	Diabetic double injection +FAT D14-1
35	4	D14	18/06/2018	D	Diabetic double injection + FAT D14-2
36	4	D14	18/06/2018	D	Diabetic double injection +FAT D14-3
37	4	D14	18/06/2018	D	Diabetic double injection + FATD14-4
38	4	D28	2/07/2018	D	Diabetic double injection +FAT D28-1
39	4	D28	2/07/2018	D	Diabetic double injection FAT D28-2
40	4	D28	2/07/2018	D	Diabetic double injection +FAT D28-3
41	4	D28	2/07/2018	D	Diabetic double injection +FAT D28-4
42	4	D3	8/06/2018	D	Diabetic only metformin +FAT D3-1
43	4	D3	8/06/2018	D	Diabetic only metformin +FAT D3-2
44	4	D3	8/06/2018	D	Diabetic only metformin +FAT D3-3
45	4	D14	18/06/2018	D	Diabetic only metformin +FAT D14-1
46	4	D14	18/06/2018	D	Diabetic only metformin +FAT D14-2
47	4	D14	18/06/2018	D	Diabetic only metformin +FAT D14-3
48	4	D14	18/06/2018	D	Diabetic only metformin FAT D14-4
49	4	D28	2/07/2018	D	Diabetic only metformin +FAT D28-1
50	4	D28	2/07/2018	D	Diabetic only metformin +FAT D28-2
51	4	D28	2/07/2018	D	Diabetic only metformin +FAT D28-3
52	4	D28	2/07/2018	D	Diabetic only metformin +FAT D28-4

Table 12: list of numbers of non-diabetic rats treated with metformin alone or vitamin B17 alone

Samples (53-75) Condition 5 and 6 (set 3)

sample ID	set	Day	sacrifice date	D/ND	Condition
53	3	D1	22/05/2018	ND	CTR ND D1 NO INJECTION-1
54	3	D1	22/05/2018	ND	CTR ND D1 NO INJECTION-2
55	3	D1	22/05/2018	ND	CTR ND D1 NO INJECTION-3
56	3	D1	22/05/2018	ND	CTR ND D1 NO INJECTION-4
57	3	D3	25-5-218	ND	ND + MET D3-1
58	3	D3	25-5-218	ND	ND + MET D3-2
59	3	D3	25-5-218	ND	ND + MET D3-3
60	3	D14	4/06/2018	ND	ND + MET D14-1
61	3	D14	4/06/2018	ND	ND + MET D14-2
62	3	D14	4/06/2018	ND	ND + MET D14-3
63	3	D14	4/06/2018	ND	ND + MET D14-4
64	3	D14	4/06/2018	ND	ND+VIT D14-1
65	3	D14	4/06/2018	ND	ND+VIT D14-2
66	3	D14	4/06/2018	ND	ND+VIT D14-3
67	3	D14	4/06/2018	ND	ND+VIT D14-4
68	3	D28	20/06/2018	ND	ND + MET D28 -1
69	3	D28	20/06/2018	ND	ND + MET D28 -2
70	3	D28	20/06/2018	ND	ND + MET D28 -3
71	3	D28	20/06/2018	ND	ND + MET D28 -4
72	3	D28	20/06/2018	ND	ND+VIT D28-1
73	3	D28	20/06/2018	ND	ND+VIT D28-2
74	3	D28	20/06/2018	ND	ND+VIT D28-3
75	3	D28	20/06/2018	ND	ND+VIT D28-4

Table 13: list of numbers of operated and Vitamin B17 treated rats, (diabetic and non-diabetic)

Sample (76-111) Condition 7 and 8 (set 2)

sample ID	Set	day	sacrifice date	D/ND	Condition
76	2	D1	21/04/2018	ND	ND+ Fat + Vit B17 D1-1
77	2	D1	21/4/2018	ND	ND+ Fat + Vit B17 D1-2
78	2	D3	23/04/2018	ND	ND+ Fat + Vit B17 D3-1
79	2	D3	23/04/2018	ND	ND+ Fat + Vit B17 D3-2
80	2	D3	23/04/2018	ND	ND+ Fat + Vit B17 D3-3
81	2	D7	30/04/2018	ND	ND+ Fat + Vit B17 D7-1
82	2	D7	30/04/2018	ND	ND+ Fat + Vit B17 D7-2
83	2	D14	3/05/2018	ND	ND+ Fat + Vit B17 D14-1
84	2	D14	3/05/2018	ND	ND+ Fat + Vit B17 D14-2
85	2	D14	3/05/2018	ND	ND+ Fat + Vit B17 D14-3
86	2	D14	3/05/2018	ND	ND+ Fat + Vit B17 D14-4
87	2	D21	3/05/2015	ND	ND+ Fat + Vit B17 D21-1
88	2	D21	3/05/2018	ND	ND+ Fat + Vit B17 D21-2
89	2	D21	3/05/2018	ND	ND+ Fat + Vit B17 D21-3
90	2	D28	17/05/2018	ND	ND+ Fat + Vit B17 D28-1
91	2	D28	17/05/2018	ND	ND+ Fat + Vit B17 D28-2
92	2	D28	17/05/2018	ND	ND+ Fat + Vit B17 D28-3
93	2	D28	17/05/2018	ND	ND+ Fat + Vit B17 D28-4
94	2	D1	21/04/2018	D	Diabetic +FAT +Vit B17 D1-1
95	2	D1	21/04/2018	D	Diabetic +FAT +Vit B17 D1-2
96	2	D1	21/04/2018	D	Diabetic +FAT +Vit B17 D1-3
97	2	D7	30/04/2018	D	Diabetic +FAT +Vit B17 D7-1
98	2	D7	30/04/2018	D	Diabetic +FAT +Vit B17 D7-2
99	2	D7	30/04/2018	D	Diabetic +FAT +Vit B17 D7-3
100	2	D7	30/04/2018	D	Diabetic +FAT +Vit B17 D7-4
101	2	D14	4/05/2018	D	Diabetic +FAT +Vit B17 D14-1
102	2	D14	4/05/2018	D	Diabetic +FAT +Vit B17 D14-2
103	2	D14	4/05/2018	D	Diabetic +FAT +Vit B17 D14-3
104	2	D14	4/05/2018	D	Diabetic +FAT +Vit B17 D14-4
105	2	D21	11/05/2018	D	Diabetic +FAT +Vit B17 D21-1
106	2	D21	11/05/2018	D	Diabetic +FAT +Vit B17 D21-2
107	2	D21	11/05/2018	D	Diabetic +FAT +Vit B17 D21-3
108	2	D28	21/05/2018	D	Diabetic +FAT +Vit B17 D28-1
109	2	D28	21/05/2018	D	Diabetic +FAT +Vit B17 D28-2
110	2	D28	21/05/2018	D	Diabetic +FAT +Vit B17 D28-3
111	2	D28	21/05/2018	D	Diabetic +FAT +Vit B17 D28-4

Table 14: list of numbers of diabetic rats treated with Vitamin B17 alone or double treated with metformin and vitamin B17

Samples (112-155) Condition 9 and 10 (set 1)

sample ID	Set	Day	sacrifice date	D/ND	Condition
112	1	D1	13/01/2018	D	diabetic + vit B17 - day 1-1
113	1	D1	13/01/2018	D	diabetic + vit B17 - day 1-2
114	1	D1	13/01/2018	D	diabetic + vit B17 - day 1-3
115	1	D1	13/01/2018	D	diabetic + vit B17 - day 1-4
116	1	D1	13/01/2018	D	diabetic + vit B17 + Met day1 -1
117	1	D1	13/01/2018	D	diabetic + vit B17 + Met day1 -2
118	1	D1	13/01/2018	D	diabetic + vit B17 + Met day1 -3
119	1	D1	13/01/2018	D	diabetic + vit B17 + Met day1 -4
120	1	D3	15/01/2018	D	diabetic + vit B17 - day 3-1
121	1	D3	15/01/2018	D	diabetic + vit B17 - day 3-2
122	1	D3	15/01/2018	D	diabetic + vit B17 - day 3-3
123	1	D3	15/01/2018	D	diabetic + vit B17 - day 3-4
124	1	D3	15/01/2018	D	diabetic + vit B17 + Met day3-1
125	1	D3	15/01/2018	D	diabetic + vit B17 + Met day3-2
126	1	D3	15/01/2018	D	diabetic + vit B17 + Met day3-3
127	1	D3	15/01/2018	D	diabetic + vit B17 + Met day3-4
128	1	D7	19/01/2018	D	diabetic + vit B17 - day 7-1
129	1	D7	19/01/2018	D	diabetic + vit B17 - day 7-2
130	1	D7	19/01/2018	D	diabetic + vit B17 - day 7-3
131	1	D7	19/01/2018	D	diabetic + vit B17 - day 7-4
132	1	D7	19/01/2018	D	diabetic + vit B17 + Met day7-1
133	1	D7	19/01/2018	D	diabetic + vit B17 + Met day7-2
134	1	D7	19/01/2018	D	diabetic + vit B17 + Met day7-3
135	1	D7	19/01/2018	D	diabetic + vit B17 + Met day7-4
136	1	D14	26/01/2018	D	diabetic + vit B17 - day 14-1
137	1	D14	26/01/2018	D	diabetic + vit B17 - day 14-2
138	1	D14	26/01/2018	D	diabetic + vit B17 - day 14-3
139	1	D14	26/01/2018	D	diabetic + vit B17 + Met day14-1
140	1	D14	26/01/2018	D	diabetic + vit B17 + Met day14-2
141	1	D14	26/01/2018	D	diabetic + vit B17 + Met day14-3
142	1	D21	2/02/2018	D	diabetic + vit B17 - day 21-1
143	1	D21	2/02/2018	D	0diabetic + vit B17 - day 21-2
144	1	D21	2/02/2018	D	diabetic + vit B17 - day 21-3
145	1	D21	2/02/2018	D	diabetic + vit B17 + Met day21-1
146	1	D21	2/02/2018	D	diabetic + vit B17 + Met day21-2
147	1	D21	2/02/2018	D	diabetic + vit B17 + Met day21-3
148	1	D28	8/02/2018	D	diabetic + vit B17 - day 28-1
149	1	D28	8/02/2018	D	diabetic + vit B17 - day 28-2
150	1	D28	8/02/2018	D	diabetic + vit B17 - day 28-3
151	1	D28	8/02/2018	D	diabetic + vit B17 - day 28-4
152	1	D28	8/02/2018	D	diabetic + vit B17 + Met day28-1
153	1	D28	8/02/2018	D	diabetic + vit B17 + Met day28-2
154	1	D28	8/02/2018	D	diabetic + vit B17 + Met day28-3
155	1	D28	8/02/2018	D	diabetic + vit B17 + Met day28-4

Induction of diabetes in rats:

The animals (Male rats) weighting 380-430 grams (75-90 days old) were injected once by streptozotocin intravenously (in the tail vein) at the dose of 55 mg/kg in a volume of 0.2 ml of the body weight. Streptozotocin (STZ) (S0130-50MG-Sigma Aldrich), induces diabetes within 3 to 10 days by destroying the beta cells. Thus, the N-nitroso-containing compound acts as a nitric oxide donor in the pancreatic islets of Langerhans and induces death of insulin-secreting cells, and thus producing an animal model of diabetes.

Burns and Surgery for Autologous adipocyte tissue transplantation:

Pre-operative preparation

Animals were operated in a sterile environment with autoclaved materials.

All animals were subjected to burns under deep anaesthesia. They were anesthetized by intra-muscular injection of ketamine and xylazine. In brief, all burn procedures and surgery steps are documented in (figure 16 and 17), respectively.

Step 1: Anaesthesia:

xylazine (21 mg/kg of body weight) and ketamine (45 mg/kg) were mixed and administered IM to 90 Sprague-Dawley rats figure 16(A). Rats regained consciousness and righting reflexes within approximately 30-40 minutes. Some were subjected to burn only (94 rats) and others (96 rats) were operated in order to perform autologous adipocyte tissue transfer. **NB:** 5 minutes were needed before starting shaving the animal, figure 16 (B-C).

Step 2: shaving:

The backs of all animals were shaved with a commercial electric shaving machine the same day of burning procedure figure 16(D and E). The ventral part was shaved only for the animals which were subjected to autologous adipose tissue transfer operation figure 16 (F).

Step 3: the burn:

Second degree burn (**Deep partial-thickness wounds**) was created by heating the skin 2 minutes at a temperature of 80 degree Celsius with an ordinary soldering iron (20W) retrofitted with 2.5 cm diameter aluminium stamp figure 16 (G-H-I). The iron was held vertically, applying its own weight with no additional pressure to ensure a reproducible experimental burn which can lead to a uniform constant surface of burn area.

Step 4: TEWL measurement

Trans epidermal water loss (TEWL) was recorded in burnt area and normal skin by using derma lab cortex machine figure 16 (J-K-L).

Operation steps (Figure 17)

Step 5 : FAAT extraction

A 0.5 cm opening in the abdominal area were used in order extract a 1 cm mass of fat autologous adipocyte tissue (FAAT) and to be relocate under the burnt area.

Step 6: FAAT insertion

A 0.5 cm opening in the burnt edge were created in order to relocate and insert the (FAAT) **under burnt area**

Step 7: stitching

Both the abdominal and dorsal openings were stitched with 4 SM-5627 Biosyn monofilament glycomere 631 (1.5 metric)

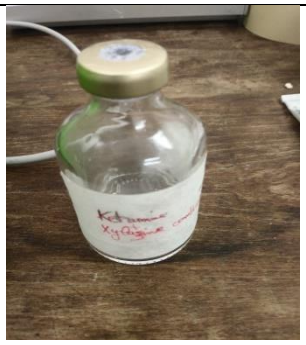
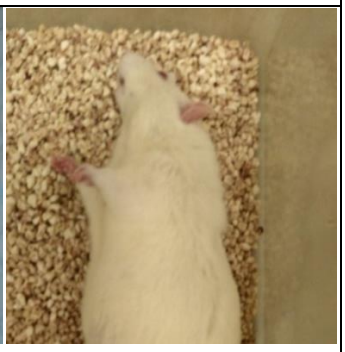
Step 1 Anaesthesia	**A**	**B**	**C**
Intramuscular injection of xylazine-ketamine Then waiting 5 to 10 minutes before shaving			
Step 2 Shaving	**D**	**E**	**F**
2 regions were shaved the dorsal side(E) for all rats and abdominal part (F) for operated rats			
Step 3 Burn	**G**	**H**	**I**
Burn created by using heat (80-82 degree) For 2 minutes			
Step 4 TEWL measurement	J	K	L
The TEWEL were measured in burnt Area (L) and normal skin(K)			

Figure 16: Burn detailed steps

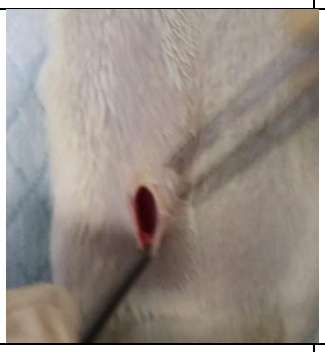
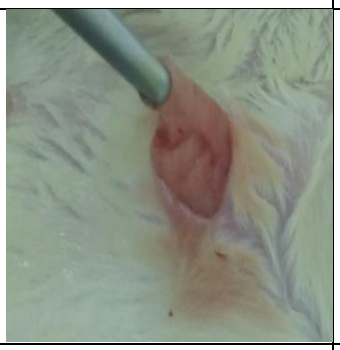
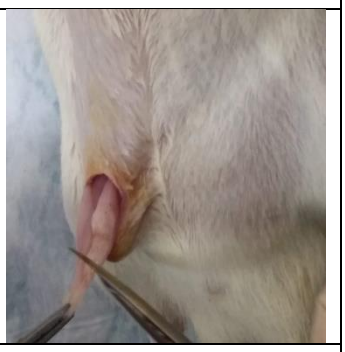
Step 5	A	B	C
Fat autologous adipose tissue extraction about 1 cm square			
Step 6 insertion under burn	**D**	**E**	**F**
insertion of fat autologous adipose tissue under burn			
Step 7 stitching	**G**	**H**	**I**
Stitching skin opening s (abdominal and dorsal)			

Figure 17: Detailed steps of the operation

Vitamin B17 injection:

Vitamin B17 (Amygdaline) can be dissolved in water and ethanol easily (Lv, Ding, & Zheng, 2005), 5mg were injected in the subcutaneous area (under the burn) in a volume of 0.2ml of physiological saline (0.9%). The injection was performed every other day.

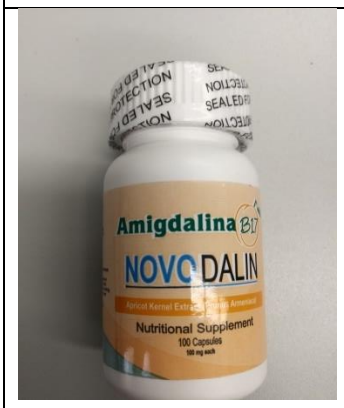
Label in frontal side	Components	Label on bottom
		

Figure 18: Vitamin B17 bottle

Metformin Injection:

150mg/kg of metformin were injected intraperitonially in a volume of 0.25 of physiological saline (0.9%). The injection was performed on daily basis. **Note: No death cases were recoded after injection, burn, anaesthesia or surgery.**

Morphologic and biochemical parameters:

Different morphologic and biochemical parameters such as wound surface area, histological alteration, and Trans epidermal water loss (TEWL) were recorded.

Macroscopic examination:

Wounds were inspected on a daily basis and findings were documented for edema, debridement, exudation, quality of the wound healing and re-epithelialization. Photos of the wounds were also taken with a mm-graded scale in frame.

Wound surface:

Wound surface was measured in square cm (area = πr2) of the burn as it progressed since the aluminium stamp was circular.

Skin biopsies:

At 6 different time points (D1, D3, D7, D14, D21, and D28), the animals were anaesthetized and 1cm biopsies were performed from a same selective area around wound and trans epidermal water loss (TEWL) was recorded from the non-biopsied excision as well from the surrounding intact skin. Biopsies were immediately placed in paraformaldehyde 4% solution. Observation of shape, texture and quality of wounds were recorded and histology slides were examined under light microscopy after routine processing and proper staining.

Light microscopy:

Fixed biopsies were embedded in paraffin and 5 μm thick sections were stained with Toluidine Blue (a metachromatic stain of mast cells), and Hematoxylin-Eosin (H&E) for routine microscopy. These slides provided a chronological histological account of the healing process, as well as, mast cell density and distribution. H&E slides were photographed by Olympus E330 camera connected to a CX41RF Olympus light microscope. Whereas the TB slides were photographed of 2048 by 1536 pixel resolution, using a VanGuard microscope fitted with MU300 camera with 3.1MP Aptina color CMOS and an AmScope capturing software version 3.7.3036.

With respect to mast cell count, it was reported as high (7-10 or more cells/field), moderate (4-6 cells/field) and low/ normal (1-3 cells/field) and very low (0-1 mast cells per field).

Real time RT-PCR:

Biopsies were snapped directly into liquid nitrogen and then stored in deep freeze at -80 degree Celsius until processing.

Gene expression in the skin was analysed by real-time RT-PCR using the ΔΔCt method. Total RNA was extracted from the skin lysate using TRIZOL reagent (Sigma-Aldrich) and converted into cDNA using the Revert First Strand cDNA Synthesis Kit (Qiagen) according to the manufacturer protocol. cDNA was measured by RT2 qPCR Biorad CFX96 using SYBR green dye and rat RT2qPCR Primers for IL1 -2, IL-12, HSP70 and GAPDH (table 7):

For **IL1a-2** forward: Fw 5'-AGGGAGTCAACTCATTGGCG-3', and reverse:

Rw 3’-GGACAGTCGAGGAGCAAACA-5’,

For **IL6-2** Forward: Fw 5’ CTGGTCTTCTGGAGTTCCGTT-3’ and reverse:

Rw 3-’ ATGAGAGATGGGGACGCACT-5’

For **IL-12** Forward: FW-5’ ATCATCAAACCGGACCCACC-3’, and reverse:

RW 3’-CAGGAGTCAGGGTACTCCCA-5’.

For **HSP70** Forward: FW 5’- TCAAGGGCAAGATCAGCGAG -3’, and reverse:

RW 3’-GCAGCCATCAAGAGTCTGTCT-5’

For **GAPDH** Forward: 5’- FW AGACAGCCGCATCTTCTTGT -3’, and reverse:

Rw 3’- CTTGCCGTGGGTAGAGTCAT-5’

GAPDH: was used as internal reference gene.

Table 15: Primers used for IL1 -2, IL-12, HSP70 and GAPDH

Gene name	**5'-3' primer sequence**	**Position (cDNA)**	**Annealing temperature**
IL1a-2	Fw AGGGAGTCAACTCATTGGCG	1577-1596	60 °C
	Rw GGACAGTCGAGGAGCAAACA	1672-1653	60 °C
IL6-2	FW CTGGTCTTCTGGAGTTCCGTT		60 °C
	RW ATGAGAGATGGGGACGCACT		60 °C
IL-12	FW ATCATCAAACCGGACCCACC	688-707	60°C
	RW CAGGAGTCAGGGTACTCCCA	776-757	60°C
HSP70	FW TCAAGGGCAAGATCAGCGAG	1850-1869	60°C
	RW GCAGCCATCAAGAGTCTGTCT	2155-2135	60°C
GAPDH	FW AGACAGCCGCATCTTCTTGT		60°C
	RW CTTGCCGTGGGTAGAGTCAT		60°C

ROS Detection:

ROS generation was assessed using oxidant-sensitive fluorogenic probe Dihydroethidium (DHE) (Invitrogen) which reacts with superoxide anions to form the red fluorescent product (2-hydroxyethidium) (Zhao H et al., 2005). Briefly, skin frozen samples were cut into 4 μm thick sections and placed on glass slides. DHE (20 μmol/l) was applied to each tissue section and the slides were incubated in a light-protected humidified chamber at 37°C for 30 min. Fluorescence was detected at excitation and emission wavelengths of 488 and 520 nm, respectively, using laser-scanning confocal microscope. Images were taken at 20x magnification lens from different fields. The average of five sections stained with DHE was taken as the value for each animal. Quantification was done using Zen light Software.

Statistical analysis:

Results are expressed as means ± SE. Statistical significance was assessed by student's unpaired t test. Significance was determined as probability (p) less than 0.05.

IV. Results:

1. Clinical profile:

The reporting of the results is divided into 2 steps. First, the overall results cover all groups and parameters with a highlight on the major findings. Second, a deeper and detailed analysis follows with a focus on the findings related to each of the aims of the study.

Clinical observation and evaluation of the skin:

The clinical observation and evaluation of all 10 animal subgroups (conditions) focused mainly on 5 main characteristics:

1. The gross wound (macroscopic) appearance at all-time points **(D1, D3, D7, D14, D21, and D28)** and the degree of redness and vascularization.
2. Skin **total detachment** percentage among rats at all-time points.
3. Area of Burn Wound (wound surface) mainly at **D28.**
4. Microscopic alterations.
5. Trans Epidermal Water Loss (TEWL) for both burnt and normal skin.

1.1 Clinical Observation:

All animals intended to become diabetic did so. The cages of diabetic rats were changed daily due to an excessive urination (urine release), while the cages of non-diabetic animals were changed every 2-3 days.

The follow-up on the progress of the wounds was based on direct observation with specific criteria as mentioned before and was documented by direct photographs of the wounds of the animals at all-time points. They are presented for each condition in replicates (figures 20 to 29) with a comparative sum up (figure 30) containing only 1 representative replicate for each time point condition.

Daily observation of the animals showed that at day 1 (D1), the wounds were well delineated with an elevated rim and a high degree of redness (+3) in the 6 diabetic burned conditions, a sign of inflammation. On the other hand, the Non-diabetic rats presented with a well delineated softer burn and little redness (+1).

On the third day, (+3) redness continued in the 6 diabetic conditions with a clear crust and some necrotic spots. However in the 4 non-diabetic conditions, no other major changes were noted among the different conditions of the same group.

After the first week, on day 8, the wounds in the diabetic animals showed more necrotic spots, redness of rims (+2) with dryness and hardening of the crusts as well as partial elevation of the rims. On the other hand, the non-diabetic animals exhibited less hardening with elevation of the rims, less necrosis and partial redness (+2).

Note: Only condition 9 (Diabetic + metformin +Vitamin B17) showed fluid retention at the burnt area over all the 4 replicates (which gave a shape of camel) at day 3 and 7 as illustrated in figure 19.

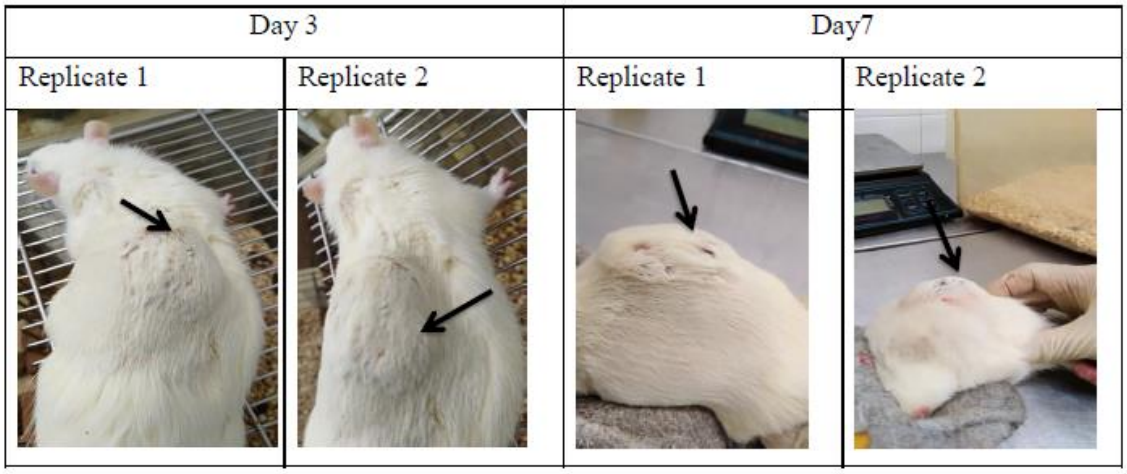


Figure 19: Camel shape seen for Condition 9 (Diabetic with metformin and vitamin B17) at day3 and day 7. **Note:** the hump shaped elevation indicated by a black arrow

Skin total detachment:

After day 7, (12 rats were left for each condition) where 4 were sacrificed at each time point D14, D21, and D28. **Table 17** shows percentage of the skin total detachment status with time for the last two weeks, from (D14 to D28).

Total skin detachment percentage was calculated by dividing the number of rats with a **completely** detached crust by the total number of remaining rats of the same condition then multiplied by 100 as described in the formula**.**

$$\%\ of\ skin\ total\ \ detachment = \frac{n}{N} \times 100$$

Where **(n)** represents the number of rats with a **completely** shed away crust and **(N)** represents the total number of remaining rats for the same condition.

Note: N decreases with time progress: N= 12, 8, and 4 for D14, D21, and D28 respectively.

For Non-diabetic rats:

At Day 14, no partial or full skin detachment were detected for non-diabetic rats in condition 2, 5 and 6 (Control non-diabetic, ND + metformin, and ND + Vitamin B17) the burn wounds showed hard elevated crusts but still in place with a rim of redness (+2), only for animals in condition 8 (ND + fat + vitamin B17) the crust was partially shed away.

For diabetic groups:

All diabetic animals showed partial skin detachment after 2 weeks. In conditions 1, 3, and 4 (control Diabetic, Diabetic + fat + metformin, and diabetic + fat + VitaminB17 + metformin), the partial detachment left a good vascularization behind (+2). While In Condition 7, 9, and 10 (Diabetic +fat + vitamin B17; Diabetic + vitamin B17; and Diabetic + vitamin B17 + metformin),the crust was completely shed away with excellent vascularization or redness (+3) for **25%** of animals of the set.

After 3 weeks (8 animals left), the crust was completely sloughed off in all animals of conditions (7, 8, 9 and 10), 50% in animals of condition 5, and 25% of animals of condition 4. While in condition 1, 2, 3 and 6 sloughing was partial for all animals. Vascularization in all groups was good; however it was best in condition 7, 8 and 9, whereby the common factor was Vitamin B17.

After 1 month, the burn wounds were checked for the last time on **day 28** before sacrifice (only 4 animals remained for each condition). The Rats for diabetic control (Condition 1) showed 100% of total skin detachment at day 28, the same for all diabetic and non-diabetic rats that were treated with B17 (Condition: 5,7,8,9 and 10) except for condition 3 where only 2 rats had a total detached skin (50%). In addition, for conditions (2, 4, and 6) 75% of rats showed a total skin detachment and only 1 rat had a partial detached skin.

Conclusion: Diabetic rats showed faster skin detachment compared to non-diabetic. In addition the healing was more advanced and healthier for non-diabetic rats undergoing the same treatment, as seen after comparing the conditions : (C1 Vs C2), (C5 Vs C9), and (C7 Vs C8). Moreover Vitamin B17 accelerated remarkably the skin sloughing time. However, in brief, the best wound healing was seen in condition 7, 8 and 9 since they all healed and got vascularized with hairs starting to grow, the all were treated with Vitamin B17 and non-treated with Metformin. In brief, Vitamin B17 treated animals presented relatively faster closure velocity especially after day 21.

Table16: Skin total detachment status rate by time

Conditions										
	C1	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**
Condition Descripuion	**Diab** CT Burnt only	**ND** Ct Burnt only	**Diab** Fat + Met +Vit B17	**Diab** Fat + Met	**ND** + vit B17	**ND** + metformin	**Diab** fat + vit B17	**ND** fat + vit B17	**Diab** + vit B17	**Diab** + vit B17 + Met
Skin Detachment percentage										
D14	0%	0%	0%	0%	0%	0%	25%	25%	25%	25%
D21	0%	0%	0%	25%	50%	0%	100%	100%	100%	100%
D28	100%	75%	50%	75%	100%	75%	100%	100%	100%	100%

Note: Diab stands for diabetic rats, and **ND stands for non-diabetic rats**

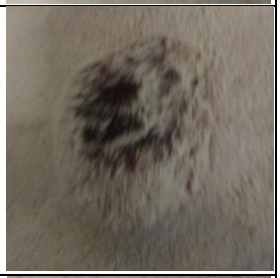
Day	Replicate 1	Replicate 2
3		
7		
14		
21		
28		

Figure 20: Gross wounds of the Condition 1 (control diabetic) at all-time point

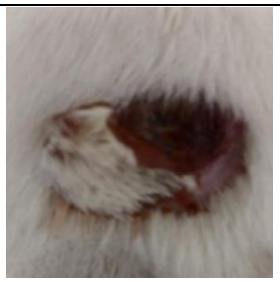
Day	Replicate 1	Replicate 2	Replicate 3
3			
7			
14			
21			
28			

Figure 21: Gross wounds of the condition 2 (control ND) at all-time points

Day	Replicate 1	Replicate 2	Replicate 3
3			
14			
28			

Figure 22: Gross wounds of the condition 3 (Diabetic + fat + met +Vit) at all-time points

Day	Replicate 1	Replicate 2	Replicate 3
3			
14			
28			

Figure 23: Gross wounds of the condition 4 (Diabetic + fat + met) at all-time points

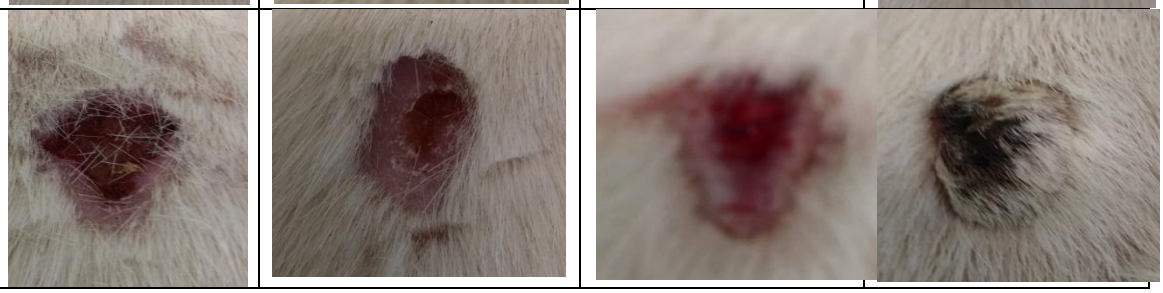
Day	0Replicate 1	Replicate 2	Replicate 3	Replicate 4
1				
14				
28				

Figure 24: Gross wounds of the condition 5 (ND +Vit) at all-time points

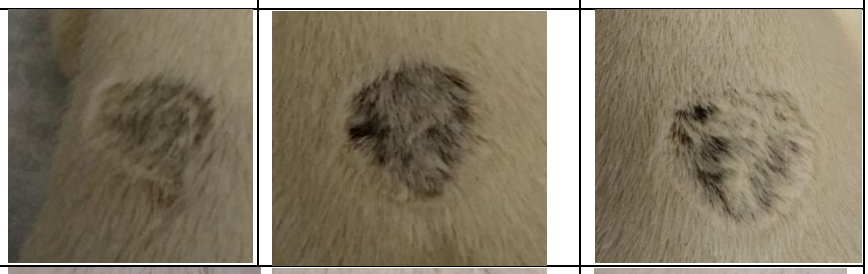
Day	Replicate 1	Replicate 2	Replicate 3
3			
14			
28			

Figure 25: Gross wounds of the condition 6 (ND + Met only) at all-time points

Day	Replicate 1	Replicate 2	Replicate 3	Replicate 4
7				
14				
21				
28				

Figure 26: Gross wounds of the condition 7 (Diabetic + fat +Vit) at all-time points

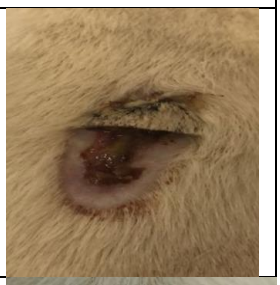
Day	Replicate 1	Replicate 2	Replicate 3
3			
7			
14			
21			
28			

Figure 27: Gross wounds of the condition 8 (ND + fat +Vit) at all-time points

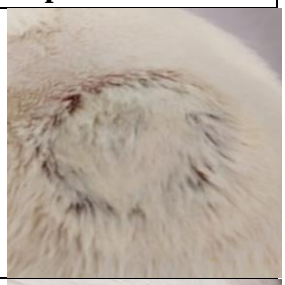
Day	Replicate 1	Replicate 2
3		
7		
14		
21		
28		

Figure 28: Gross wounds of the condition 9 (Diabetic +Vit) at all-time points

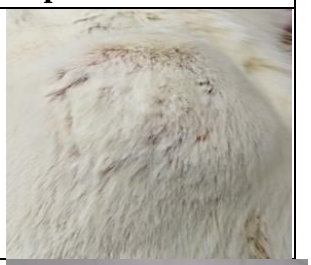
Day	Replicate 1	Replicate 2
3		
7		
14		
28		

Figure 29: Gross wounds of the condition 10 (Diabetic + met +Vit) at all-time points

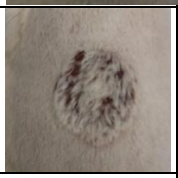
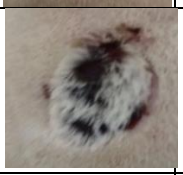
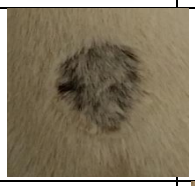
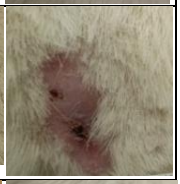
	Rat condition	D3	D7	D14	D21	D28
1	Ct diabetic Burnt only					
2	Ct ND Burnt only					
3	Diabetic fat + Met +Vit B17					
4	Diabetic fat + metformin					
5	ND + vit B17					
6	ND + metformin					
7	Diabetic fat + vit B17					
8	ND fat + vit B17					
9	Diabetic + vit B17					
10	Diabetic + vit B17 + Met					

Figure 30: panel of photos of the burn wounds of the different experimental groups at all-time points

The photos of Gross wounds of the animals at all-time points **(D3, D7, D14, D21, and D28)** are presented for each condition for the main representative replicates in figure (20 to 29).
The results were grouped later in comparative figures and tables with only one replicate for chosen conditions in order to target and follow the aims.

Skin total detachment: skin detachment doesn't occur directly it starts with wound delineated border, than partial detachment, and finally ending with a total detachment where the crust is completely shed away.

Almost all rat groups consisted of 28 rats: Four replicates were sacrificed for each time point **(D1, D3, D7, D14, D21, and D28)**. No skin detachment has been detected the first week (D1, D3, and D7). After **day 7** (12 rats remained for D14, D21, and D28). The skin total detachment status percentage by time for the last two weeks, from **(D14 to D28)** is illustrated in table 16.

1.2 Area of Burn Wound:

The area of burn at the various time points was also considered as valid indicator for the progression of the healing process. **Table 17** and **figure 31** illustrate the burn area measurements after 4 weeks (day 28) in the different experimental groups.

Measurements of the surface area of the wounds, on a regular basis, showed that during the first week (D0 to D8), there were no remarkable changes in the areas or diameters of the burns. However, they significantly differed by the end of the third week once the skin was totally detached.
According to data in Table 17 , which compared the surface area of the wounds inflected in the 10 different conditions at one time point (D28), the 7^{th} condition (diab +fat +B17) showed a significant decrease in its area (1.27± 0.05) compared to the diabetic control C1(2.93 ± 0.05), where P-value is 0.002 . The same for non-diabetic group, again comparing the 8^{th} condition non-diabetic + fat +vit B17) (1.01± 0.05) against the ND control (2.82 ± 0.05), a p-value of 0.003 indicates a significant decrease in the wound area.

Such results lead us to conclude that in both conditions 7 and 8, where animals were treated with Vitamin B17 and subjected to fat relocation, showed the smallest burn surface at day 28 compared to the controls and other conditions.

Table 17: Average wound area of different experimental groups at day 28.

Wounded skin area (Area=$A = \pi r^2$) ± 0.05										
	C1	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**
Condition Description	**Diab** CT Burnt only	**ND** CT Burnt only	**Diab** Fat + Met +Vit B17	**Diab** Fat + Met	**ND** + vit B17	**ND** + Met	**Diab** Fat + vit B17	**ND** Fat + vit B17	**Diab** + vit B17	**Diab** + vit B17 + Met
D1	3.14	3.14	3.14	3.14	3.14	3.14	3.14	3.14	3.14	3.14
D28	2.93	2.82	1.88	2.35	1.89	2.19	1.27	1.01	2.35	2.83

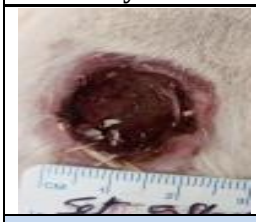
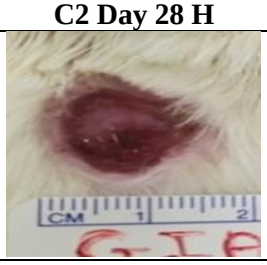
Condition 1: Control Diabetic		**Condition 2 :** Control Non Diabetic	
Day 28 H	**Day 28 V**	**C2 Day 28 H**	**C2 Day 28 V**
			
Condition 3: **Diabetic :**fat + Met +Vit B17		**Condition 4:** **Diabetic:** fat + metformin	
Day 28 H	**Day 28 V**	**C2 Day 28 H**	**C2 Day 28 V**
			
Condition 5: **ND**+ Vitamin B-17		**Condition 6:** **ND** + Metformin	
Day 28 H	**Day 28 V**	**C2 Day 28 H**	**C2 Day 28 V**
			
Condition 7: **Diabetic** +Fat+ Vitamin B-17		**Condition 8:** **ND** +fat + Vitamin B-17	
Day 28 H	**Day 28 V**	**C2 Day 28 H**	**C2 Day 28 V**
			
Condition 9: **Diabetic** + Vitamin B-17		**Condition 10:** **Diabetic** + Vitamin B-17 + Metformin	
Day 28 H	**Day 28 V**	**C2 Day 28 H**	**C2 Day 28 V**
			

Figure 31: panel of photos of burn area measurement after 4 weeks

Note: H stands for Horizontal wound diameter and **V** for vertical wound diameter

Microscopy and histological studies:

Figure (32-36) represent the histological skin sections for all diabetic and nondiabetic rats and all studied conditions.

The wound was checked daily and the wound assessment was done by evaluating the wound colour and size as described in the clinical part, in addition to the possibility of infection, inflammation, edema, re-epithelisation, and the nearby skin status.

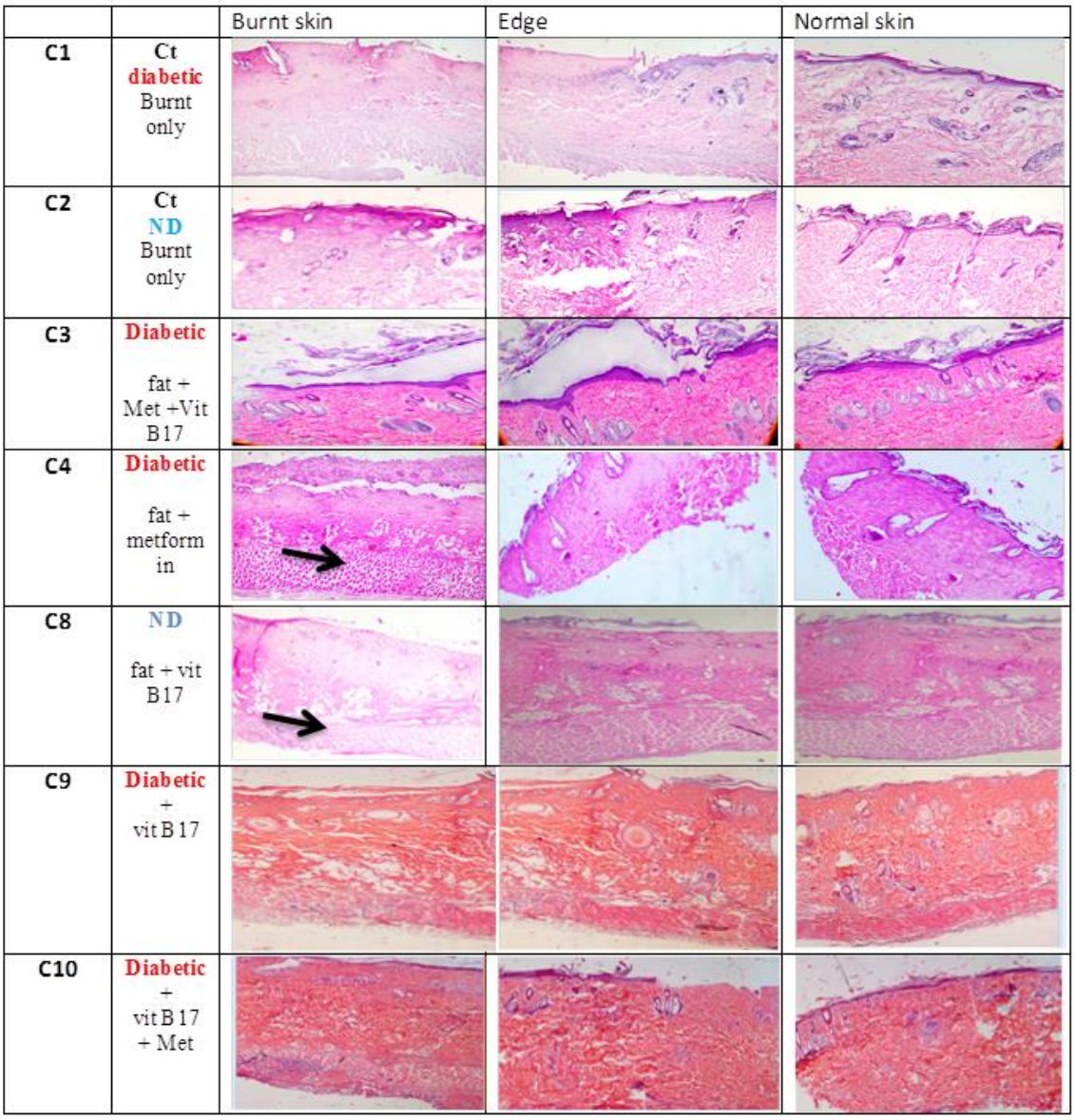


Figure 32: photomicrographs from day 3 stained with haematoxylin and Eosin (H&E), magnification (×100). Note the significant changes in the burnt skin compared to normal in the various groups C1 to C2. The arrows in C4 and C8 indicates the location of fat underlying the skin.

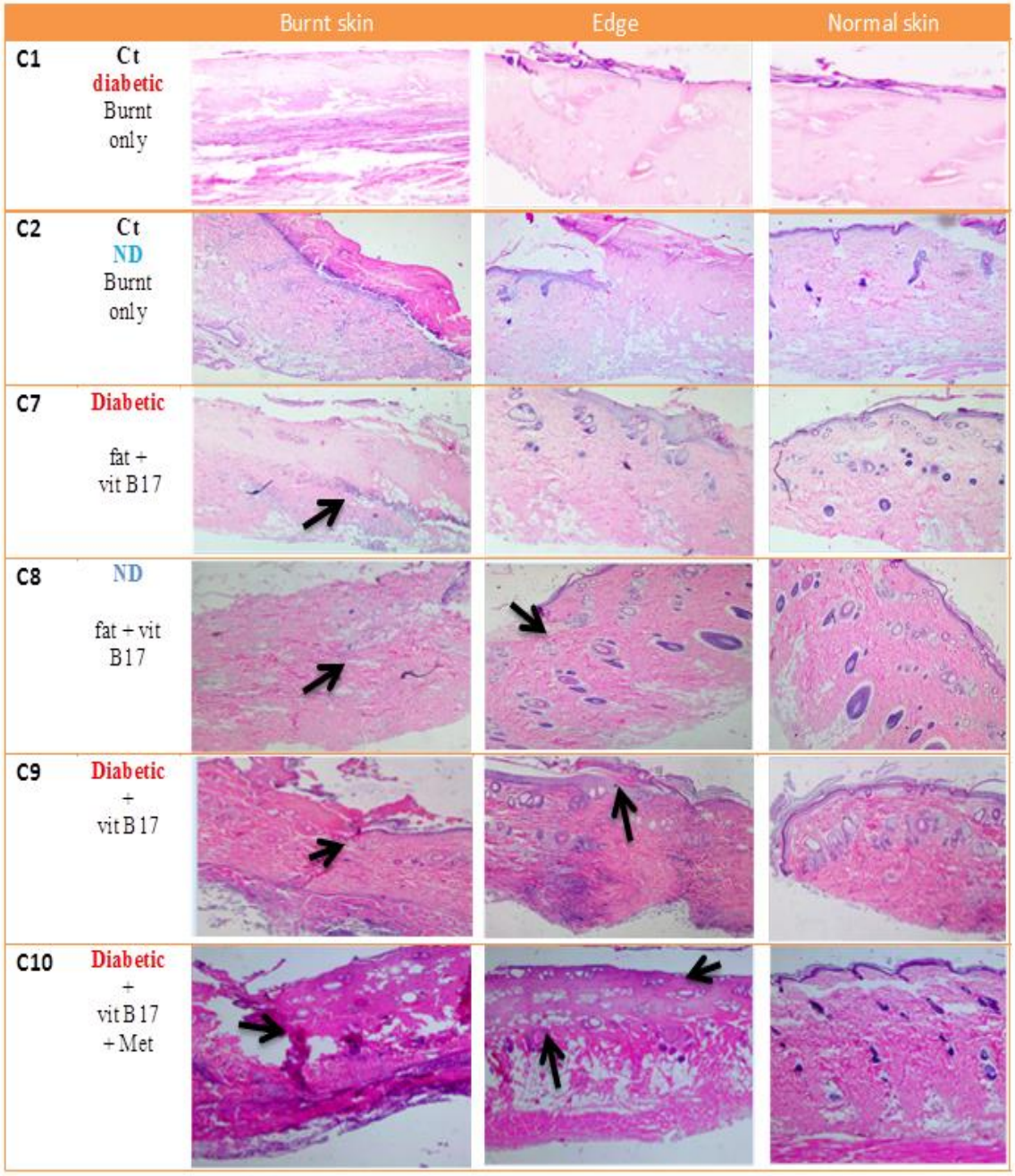


Figure 33: photomicrographs from day 7 stained with haematoxylin and Eosin (H&E), magnification (×100). The edge started to show some epithelialization, more so in the edge of C7, C8, C9, and C10. Disorganized tissues were still noted in the center of the wound and more so in C10 where the fat was absent (arrow head).

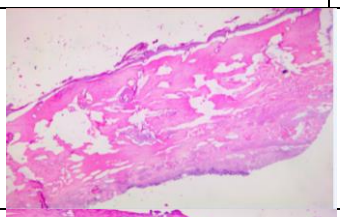
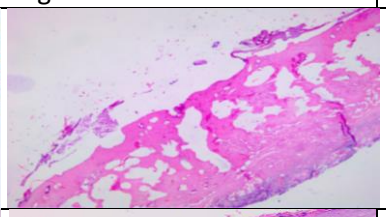
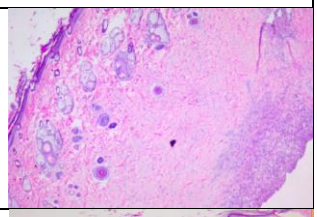
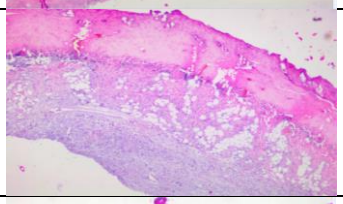
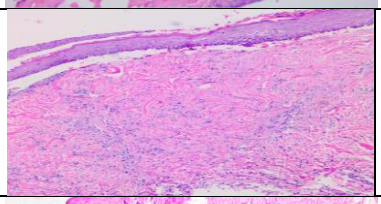
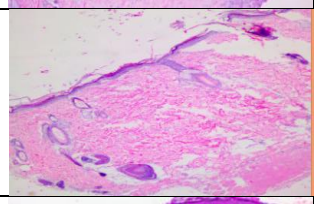
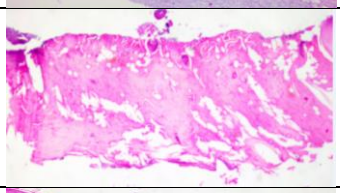
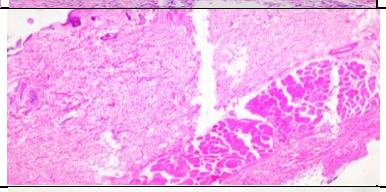
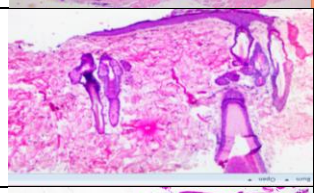
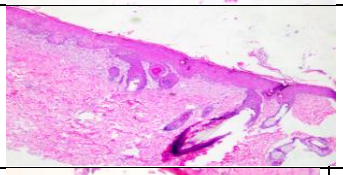
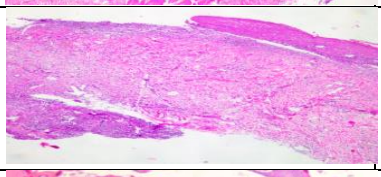
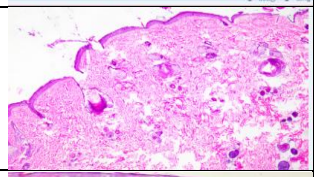
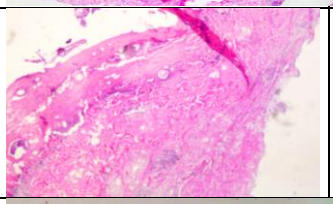
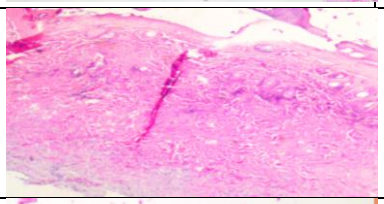
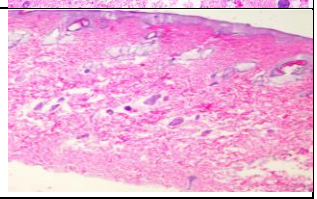
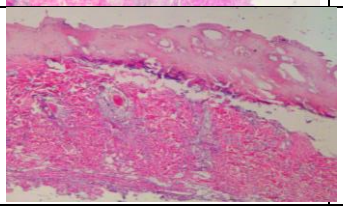
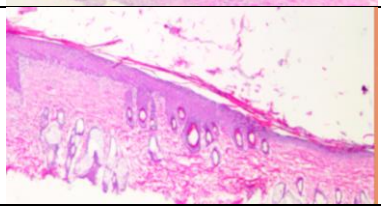
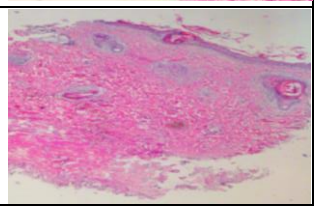
		Burnt skin	Edge	Normal skin
C1	**Ct diabetic** Burnt only			
C2	**Ct ND** Burnt only			
C3	**Diabetic** fat + Met +Vit B17			
C4	**Diabetic** fat + metformin			
C9	**Diabetic** + vit B17			
C10	**Diabetic** + vit B17 + Met			

Figure 34: photomicrographs from day 14 stained with haematoxylin and Eosin (H&E), the healing process progressed well in C4, C9 and C10 in the presence of Vitamin B17 and fat (C9) or fat and metformin (C4) but to a lesser extent in C10 in presence of metformin and vitamin B17, and much worse in C3 where fat + met + B17 were administered.

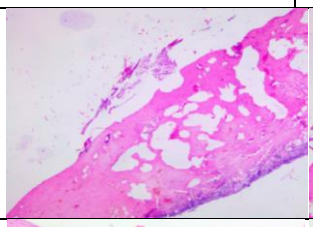
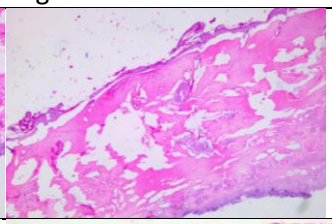
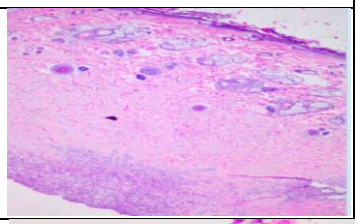
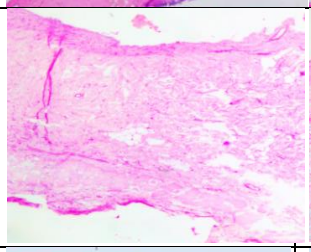
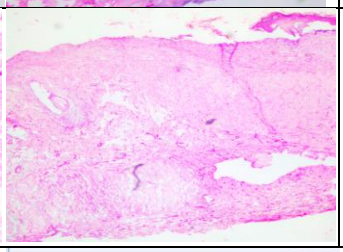
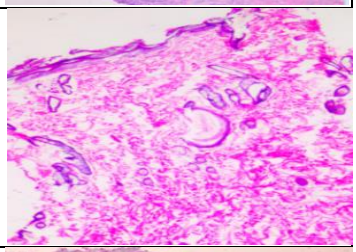
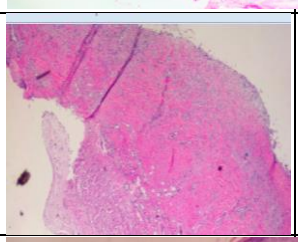
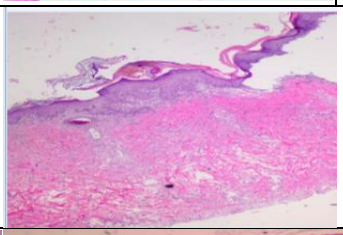
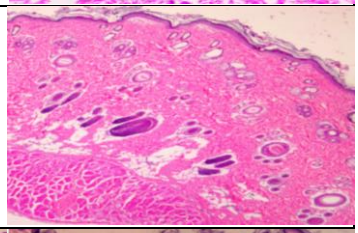
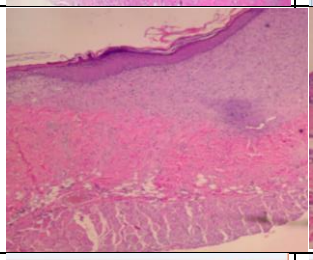
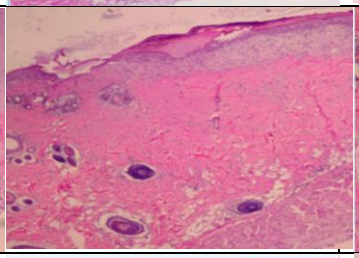
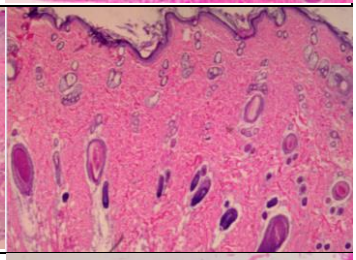
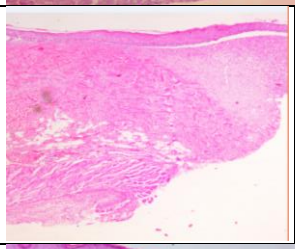
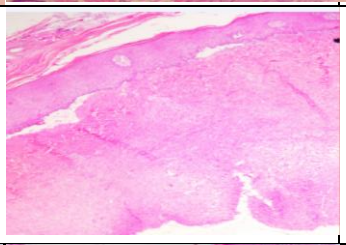
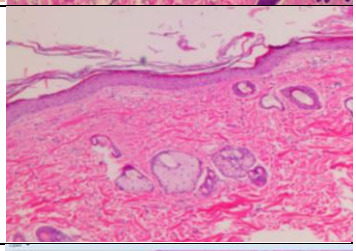
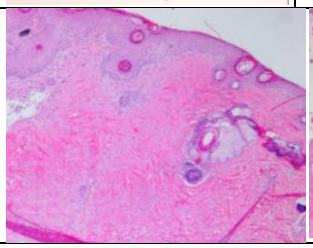
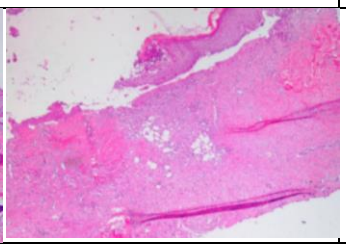
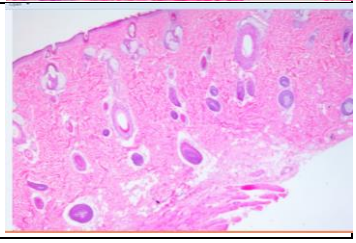
		Burnt skin	Edge	Normal skin
C1	**Ct** **diabetic** Burnt only			
C2	**Ct** **ND** Burnt only			
C7	**Diabetic** fat + vit B17			
C8	**ND** fat + vit B17			
C9	**Diabetic** + vit B17			
C10	**Diabetic** + vit B17 + Met			

Figure 35: photomicrographs from day 21 stained with haematoxylin and Eosin (H&E).
Note: the formation of new skin with variable epithelial layers. It is best in C8 (non diabetics receiving fat and Vitamin B17).

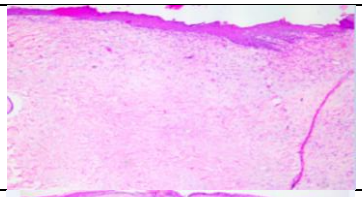
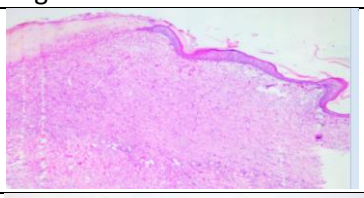
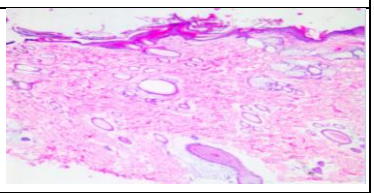
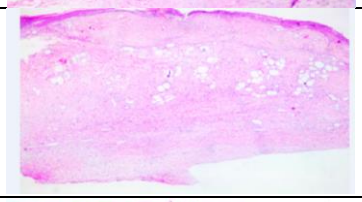
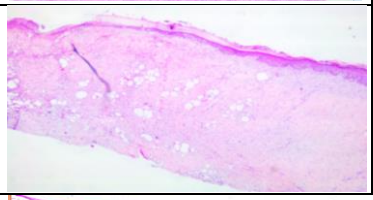
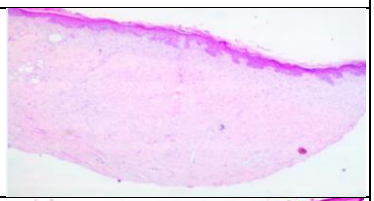
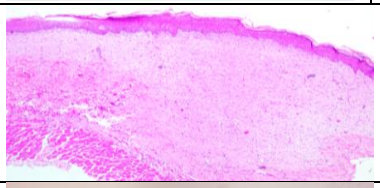
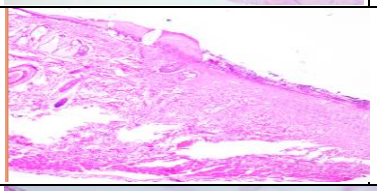
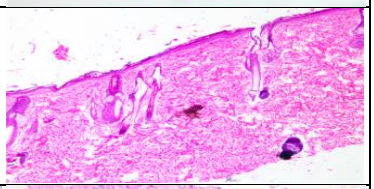
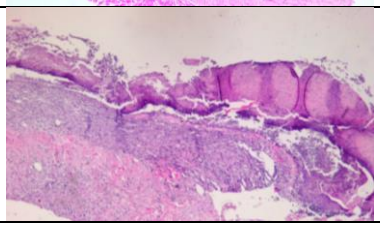
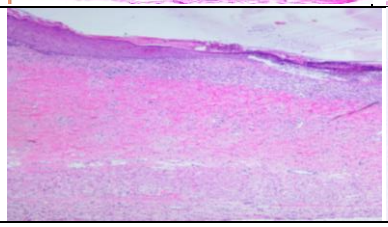
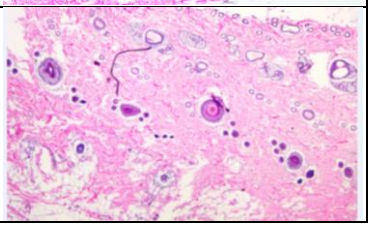
		Burnt skin	Edge	Normal skin
C1	**Ct** **diabetic** Burnt only			
C2	**Ct** **ND** Burnt only			
C6	**ND** + metformin			
C8	**ND** fat + vit B17			

Figure 36: photomicrographs from day 28 stained with haematoxylin and Eosin (H&E). Note that all wounds healed. Some have more epithelial layers like C8

Reactive Oxygen Species:

Oxidative stress is characterized by the increased production of reactive oxygen species (ROS). ROS are free radicals that bind to oxygen molecule and form biologically active substances such as superoxide (O2), hydrogen peroxide (H2O2) and hydroxyl radical (OH). The Quantification of ROS formation in the different Non- diabetic and diabetic conditions at all-time points is presented in figures (34-39) for all 10 conditions.

A very clear difference of ROS formation was detected between diabetic and non-diabetic conditions through all days, whereby ROS production varied approximately from (100 to 200) in non-diabetic and from (300-600) in Diabetic groups figure (37-42).

For the Diabetic conditions:

The ROS production was maximal for the diabetic control for day 3, 14 and 28. In parallel the 5 treatment conditions for diabetics showed a decreased rate of ROS production compared to the diabetic control. Moreover ROS production kept the same profile for all tested time points (day3, day14, and day 28), where the diabetic control scored the highest production of ROS , followed with diabetic treated with vitamin B17 only ,then diabetic with metformin and Vitamin B17. In other hand the 3 diabetic conditions with Fat relocation have almost the same rate of production with slight differences.

For the non-diabetic conditions:

ROS production kept also the same profile at day 3, after the second week, and arriving to the 4^{th} week. But the maximal production was seen for non-diabetic treated with Vitamin B17 alone or with Fat relocation.

Conclusion: In case of diabetes, ROS production is remarkably high compared to normal case, additionally vitamin B17 alone seems to have the less depressive effect on ROS production in diabetic condition, while it increase the ROS rate in non-diabetic.

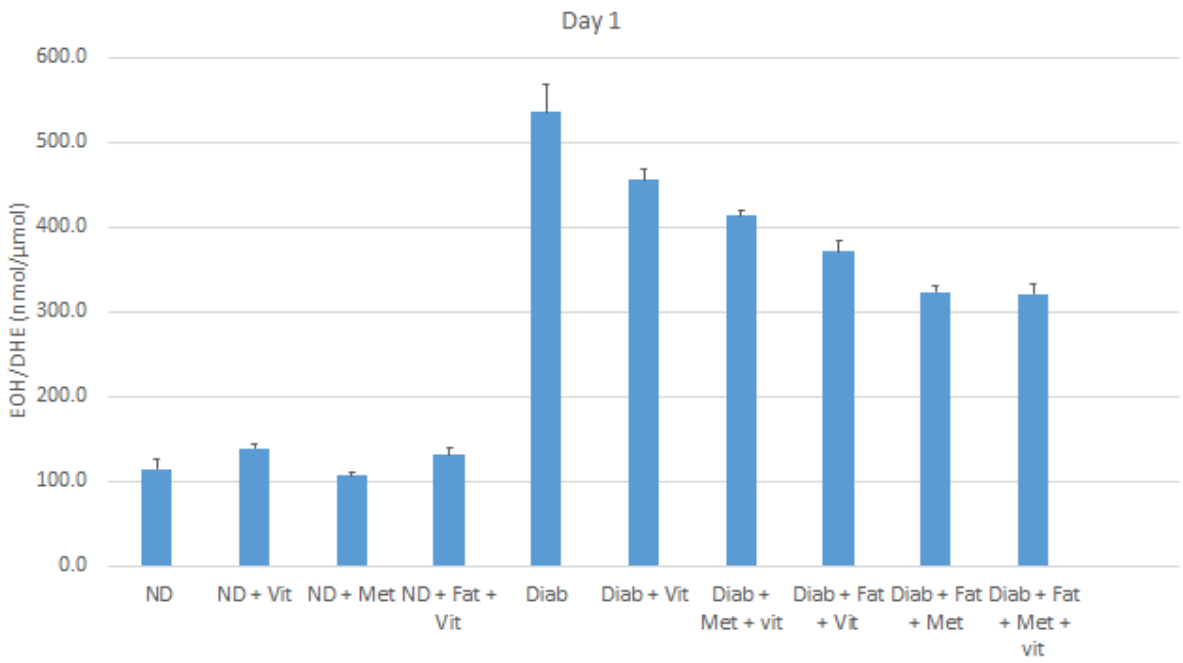


Figure 37: Quantification of ROS formation in the different Non- diabetic and diabetic conditions during Day1

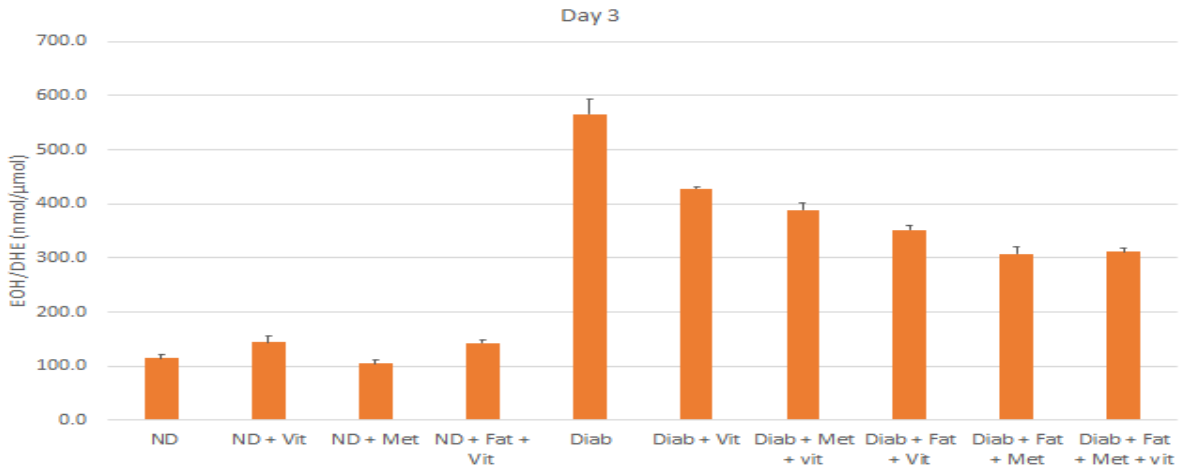


Figure 38: Quantification of ROS formation in the different Non- diabetic and diabetic conditions during Day3

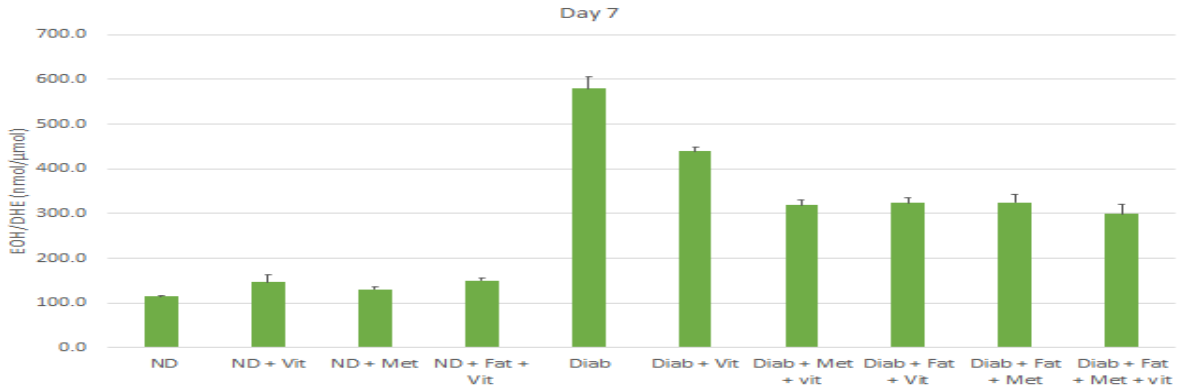


Figure 39: Quantification of ROS formation in the different Non- diabetic and diabetic conditions during Day 7

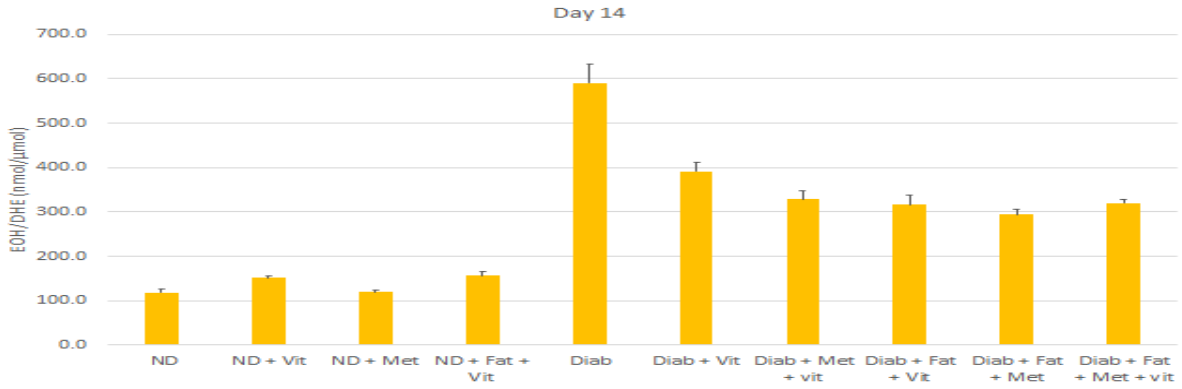


Figure 40: Quantification of ROS formation in the different Non- diabetic and diabetic conditions during Day14

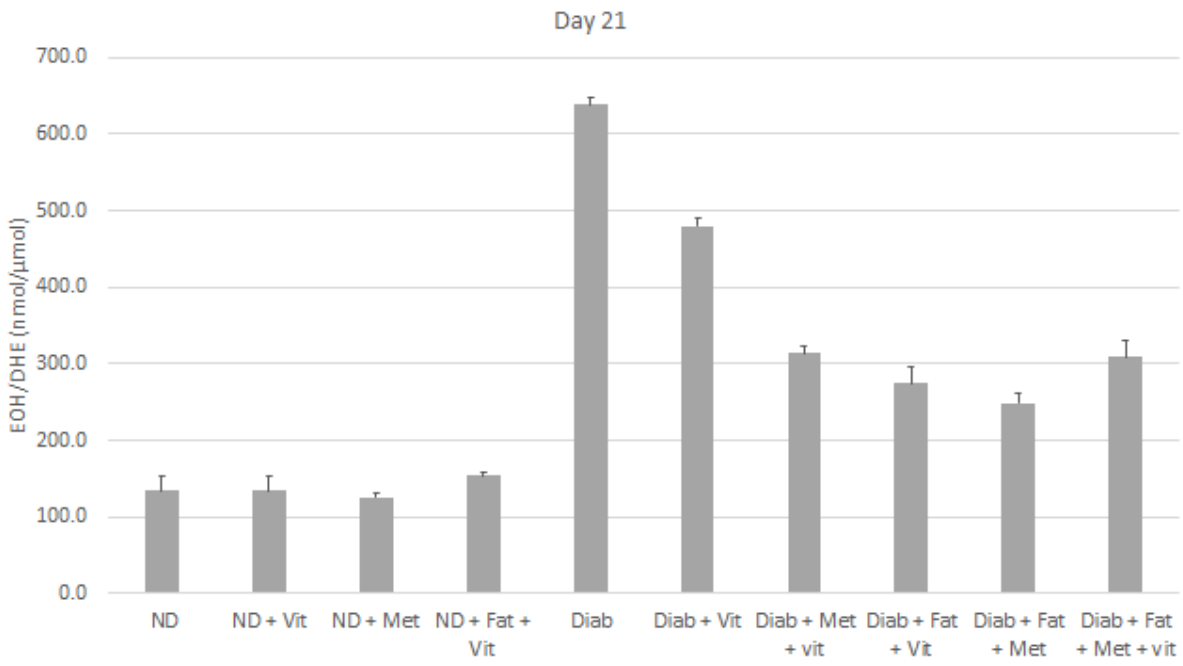


Figure 41: Quantification of ROS formation in the different Non- diabetic and diabetic conditions during Day21

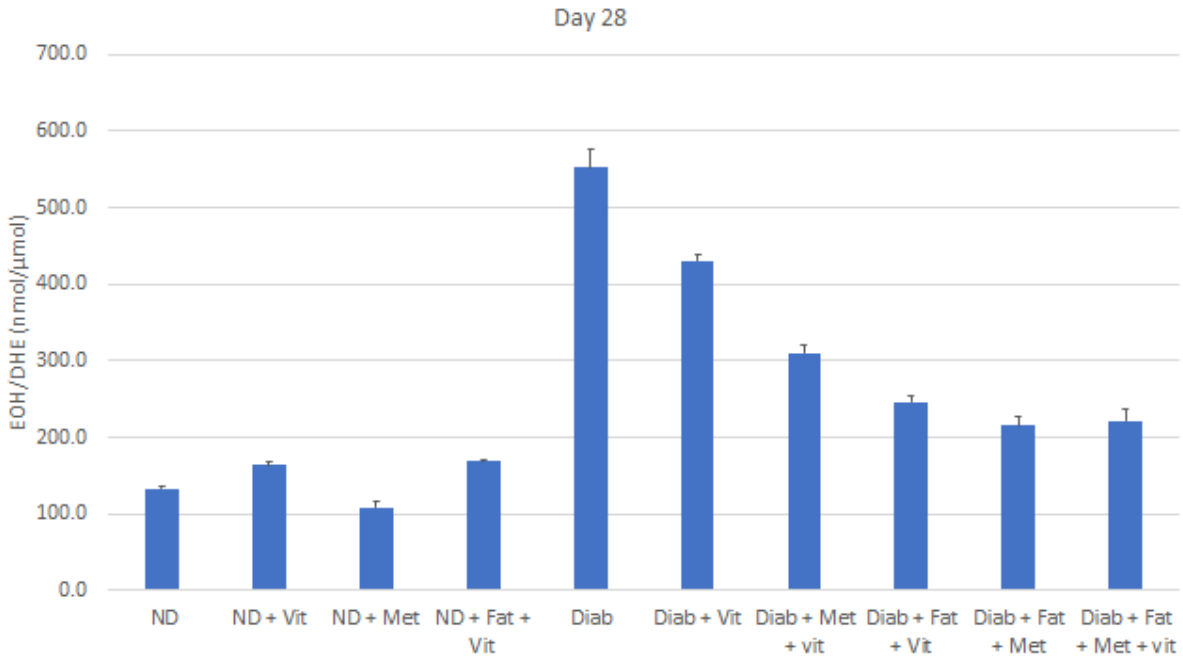


Figure 42: Quantification of ROS formation in the different Non- diabetic and diabetic conditions during Day28

A. Clinical Results by aims:

In this section, the data were analysed to depict their relation to the aims proposed and consequently hypothesis set at the beginning of the study.

Aim 1- *Main clinical differences in the burn wound healing process between diabetics and non-diabetics (Normal) groups:*

Diabetes induction was successful in all animals injected with STZ; the glycemia levels doubled after 7 to 10 days.

The 2 control subgroups; diabetic (condition 1) and non-diabetic (condition 2), were compared for this aim in order to detect the main differences in the burn wound healing process, between untreated diabetic and normal rat skins subjected to burns.

B.1- Clinical Observation:

Urination:

In general, the cages of diabetic rats needed to be changed daily due to an excessive urination (urine release), while the cages of non-diabetic animals were changed every 2-3 days.

Wound assessment:

At day1:

Daily observation of the animals showed that at day 1 (D1), the wounds were well delineated with an elevated rim and a high degree of redness (+3) in the **6** Diabetic burned conditions (subgroups) with no remarkable differences between the subgroups. On the other hand, the non-diabetic rats presented with a well delineated softer burn and less redness (+1); consequently less inflammation.

At day3:

On the third day (D3), (+3) redness continued for diabetic rats with several necrotic spots (figure 43). In the non-Diabetic conditions, no necrotic spots were seen and no other major changes were noted among the different conditions of the same group.

At day 7:

After the first week, on day 7-8, the wounds in the diabetic animals showed more necrotic spots, redness of rims (+2) with dryness and hardening of the crusts as well as partial elevation of the rims. On the other hand, the non-diabetic animals exhibited less hardening with elevation of the rims and less necrosis, as well as partial or decreased redness (+2).

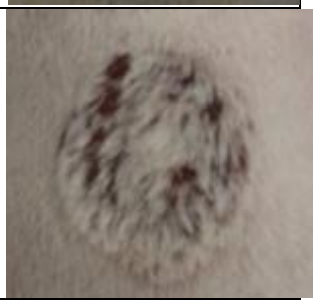
	Rat condition	D3	D7
1	**Control** **diabetic** Burnt only		
2	**Control** **ND** Burnt only		

Figure 43: gross wound comparison between diabetic and non-diabetic controls at D3 and D7

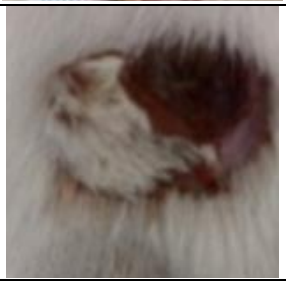
	Rat condition	D14	D21	D28
1	**Control diabetic** Burnt only			
2	**Control ND** Burnt only			

Figure 44: gross wound comparison between diabetic and non-diabetic controls after the first week (D14, D21, and D28)

At day 14:

For non-diabetic rats:

No partial or full skin detachment were detected for non-diabetic rats, the burn wounds showed hard elevated crusts but still in place with a rim of redness (+2) (figure 44); most likely new vascularization.

For diabetic groups:

All diabetic animals showed partial skin detachment after 2 weeks. The partial detachment left a good redness behind (+2).

At day 21:

After 3 weeks, sloughing of crust was partial for all animals of both conditions diabetic and non-diabetics. In addition vascularization in all groups was good.

At day 28, the burn wounds were checked for the last time on **day 28** before sacrifice. The Rats for diabetic control (Condition 1) showed 100% of total skin detachment at day 28. On the other hand, for non-diabetic rats only 75% of rats showed a total skin detachment (table 16).

1. **Wound Area:**

Table 18: Wounded skin area for diabetic and non-diabetic controls at day 1 and day 28.

Wounded skin area (Area=$A = \pi r^2$) ± 0.05		
	Condition 1	**Condition 2**
	Diabetic Control Burnt only	**Non Diabetic** Control Burnt only
D1	3.14	3.14
D28	2.93	2.82

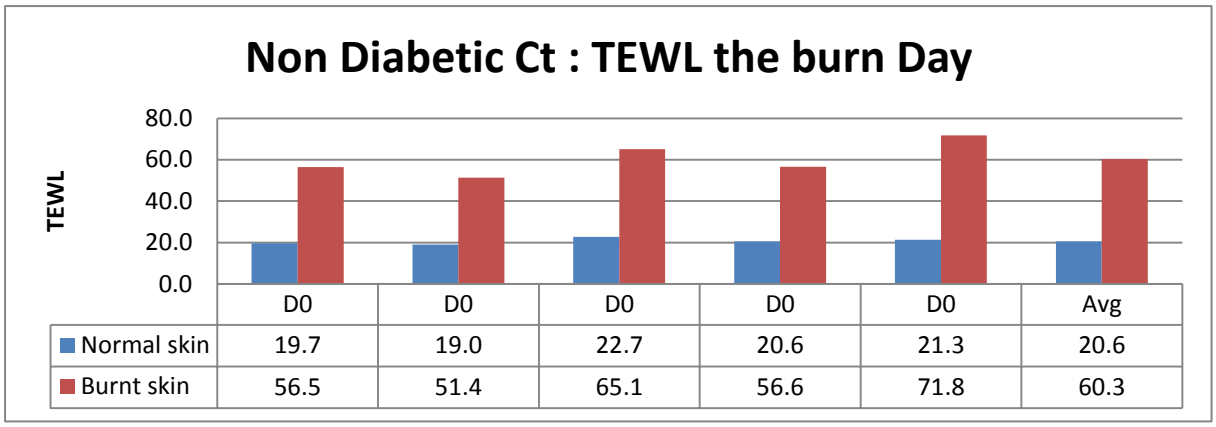
As shown in **Table 18**. There were no remarkable changes in the areas or diameters of the burns the first and second week. However, they significantly differed by the end of the third week once the skin was detaching. Moreover once detached after day 21, the average wound area decreased more remarkably in the non-diabetic group from D21 to D28. The wounded skin area decreased from (3.14 ± 0.05) at (day 1) to (2.82± 0.05) for non-diabetic rats and to (2.93 ± 0.05) for diabetic rats.

2. **Trans epidermal water loss (TEWL):**

The Trans Epidermal Water loss (TEWL) was measured at D0/D1/D3/D7/D14/D21/D28 both in normal and burnt skin.

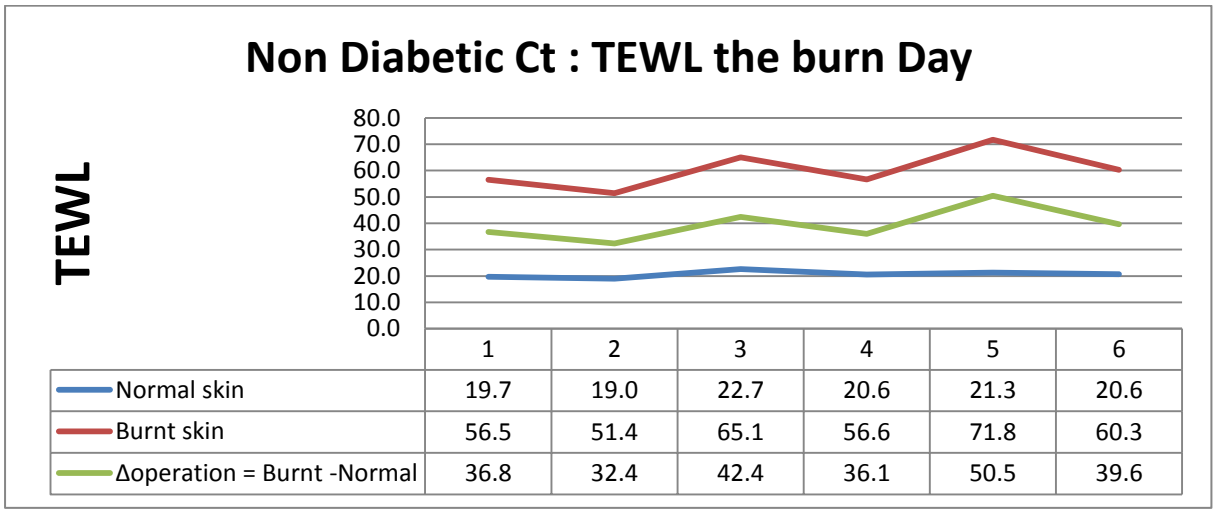
At day 0:

For the non-diabetic controls, the TEWL was almost the same for all non-diabetic rats in the normal (non-burnt) skin and range from (19 to 21) with an average of 20.6 (figure 45), while it ranged between (51.4 and 71.8) in burnt skin with an average of 60.3. The TEWL for non-diabetic rats scored a consistently increasing level of water loss (33.4-50.5) with an average about 39.6 at the level of burnt skin once compared to the normal (Δ) (figures 45 and 46).



	D0	D0	D0	D0	D0	Avg
Normal skin	19.7	19.0	22.7	20.6	21.3	20.6
Burnt skin	56.5	51.4	65.1	56.6	71.8	60.3

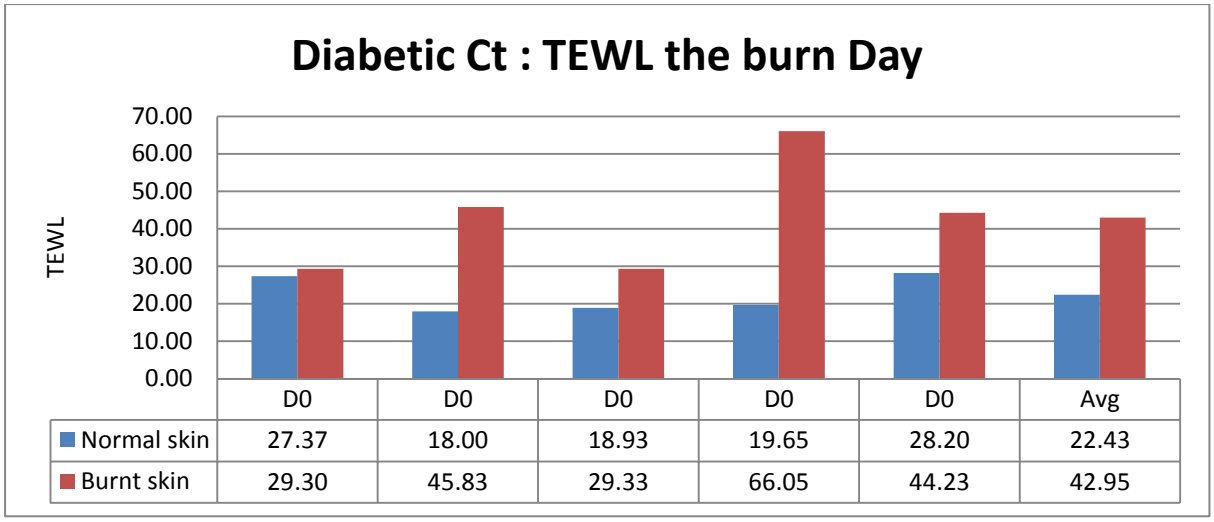
Figure 45: TEWL Records at day 0 for Control non-diabetic



	1	2	3	4	5	6
Normal skin	19.7	19.0	22.7	20.6	21.3	20.6
Burnt skin	56.5	51.4	65.1	56.6	71.8	60.3
Δoperation = Burnt -Normal	36.8	32.4	42.4	36.1	50.5	39.6

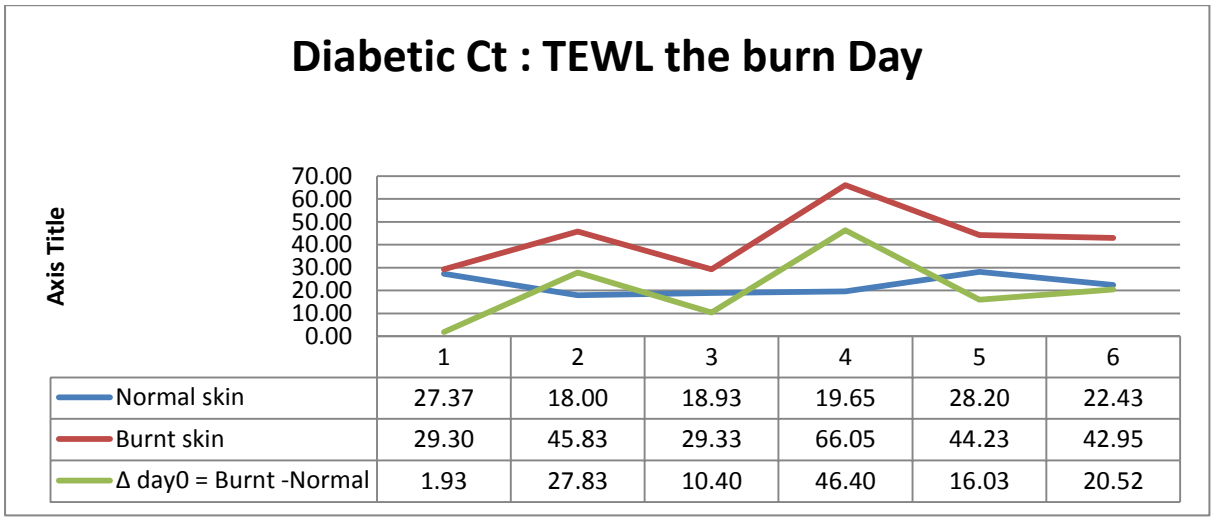
Figure 46: TEWL Records at day 0 for Control Non-diabetic

For the diabetic controls: the TEWL was variable for diabetic rats in the normal (non-burnt) skin and ranged from (18 to 28.2) with an average of 22.4, while it ranges between (29.3 and 66.05) in burnt skin, with an average of 42.95 (figure 44). The TEWL for the burnt skin scored a non-consistent increasing water loss which ranges between (1.93 and 46.4) with an average of 20.52 (figure 45 and 46).



	D0	D0	D0	D0	D0	Avg
Normal skin	27.37	18.00	18.93	19.65	28.20	22.43
Burnt skin	29.30	45.83	29.33	66.05	44.23	42.95

Figure 47: TEWL records at day 0 for Control Diabetic



	1	2	3	4	5	6
Normal skin	27.37	18.00	18.93	19.65	28.20	22.43
Burnt skin	29.30	45.83	29.33	66.05	44.23	42.95
Δ day0 = Burnt -Normal	1.93	27.83	10.40	46.40	16.03	20.52

Figure 48: TEWL records at day 0 for Control diabetic

TEWL variation at different time points:

As shown in the summary tables 20 and 21 as well as figure 49, there was consistently less TEWL in the normal skin of diabetics Vs non-diabetics. However, concerning the burnt skin the values were close, a little less for burnt skin till day 21. After day 21 TEWL course seemed to change but not significantly (figure 50).

Table 19: TEWL variation for non-diabetic controls

	burn day D0			sacrifice day				sacrifice – operation	
	Normal skin	Burnt skin	Burnt - Normal	day	Normal skin	Burnt skin	Burnt – Normal	ΔN N(sacrifice) - N(opp)	ΔB B(sac)-B(opp)
D0	19.7	56.5	36.8	D3	22.6	24.7	2.2	2.9	-31.7
D0	19.0	51.4	32.4	D7	35.7	29.3	-6.4	16.7	-22.1
D0	22.7	65.1	42.4	D14	24.8	32.8	8.0	2.1	-32.3
D0	20.6	56.6	36.1	D21	34.8	25.2	-9.6	14.3	-31.4
D0	21.3	71.8	50.5	D28	28.1	47.2	19.1	6.8	-24.5
Avg	20.6	60.3	39.6	AVG	29.18				

Table 20: TEWL record for diabetic controls

	burn day D0			sacrifice day				sacrifice – operation	
	Nrormal skin	Burnt skin	Burnt - Normal		Nrormal skin	Burnt skin	Burnt - Normal	ΔN N(sacrifice) - N(opp)	ΔB B(sac)-B(opp)
D0	27.4	29.3	1.9	D3	20.5	26.8	6.2	-6.8	-2.5
D0	18.0	45.8	27.8	D7	26.8	26.4	-0.4	8.8	-19.4
D0	18.9	29.3	10.4	D14	17.0	22.6	5.6	-1.9	-6.7
D0	19.7	66.1	46.4	D21	32.3	22.7	-9.7	12.7	-43.4
D0	28.2	44.2	16.0	D28	16.4	59.3	43.0	-11.9	15.1
D0	22.4	43.0	20.5	AVG	22.6				

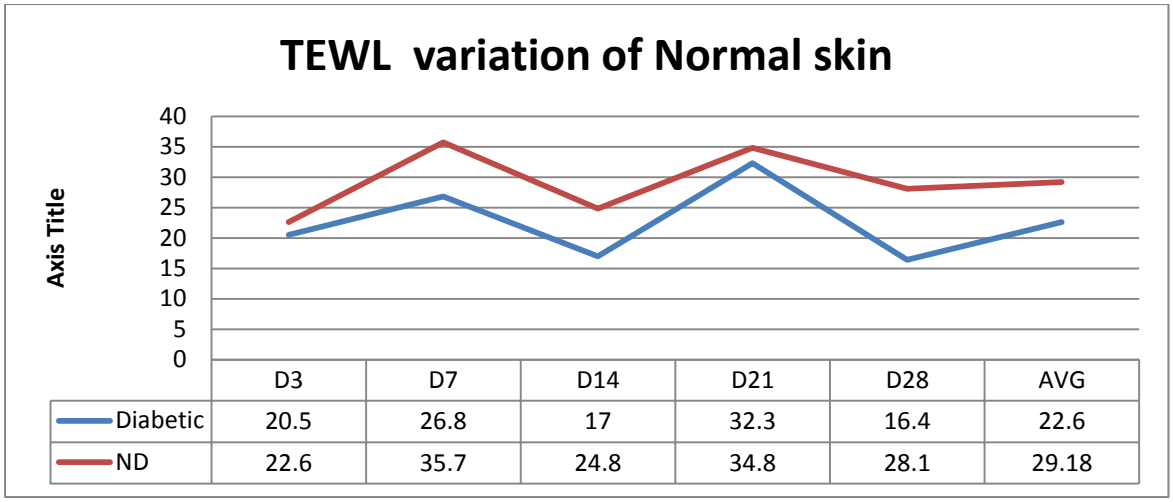


Figure 49: TEWL variation of normal skin in diabetics and non diabetics during the experimental period.

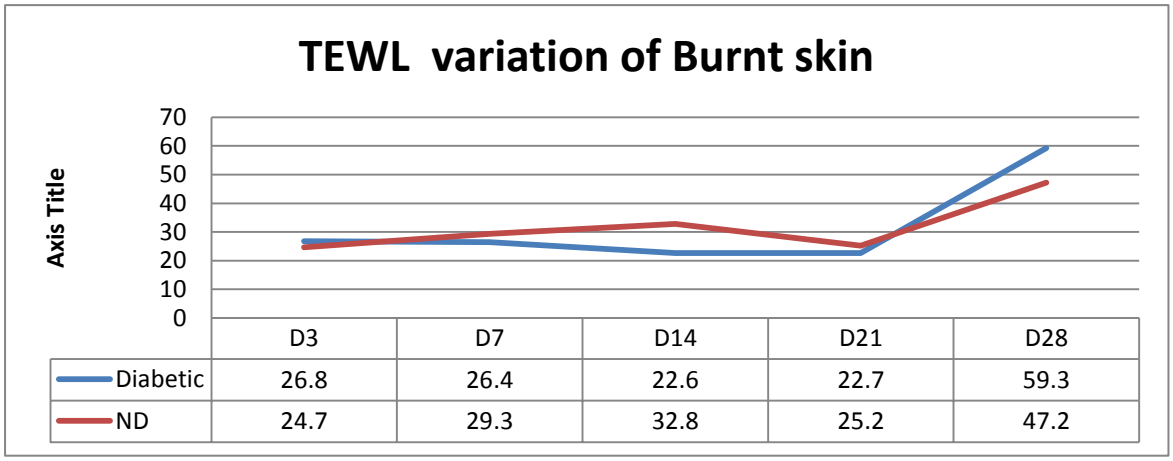


Figure 50: TEWL variation of burned skin in diabetics and non diabetics during the experimental period.

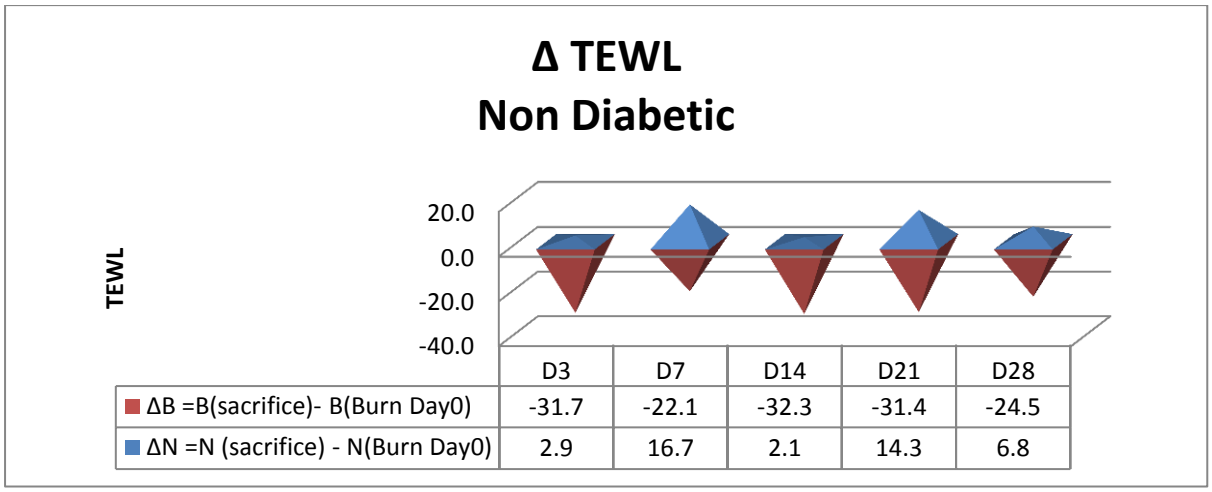


Figure 51: TEWL records at day 0 for Control Non-diabetic

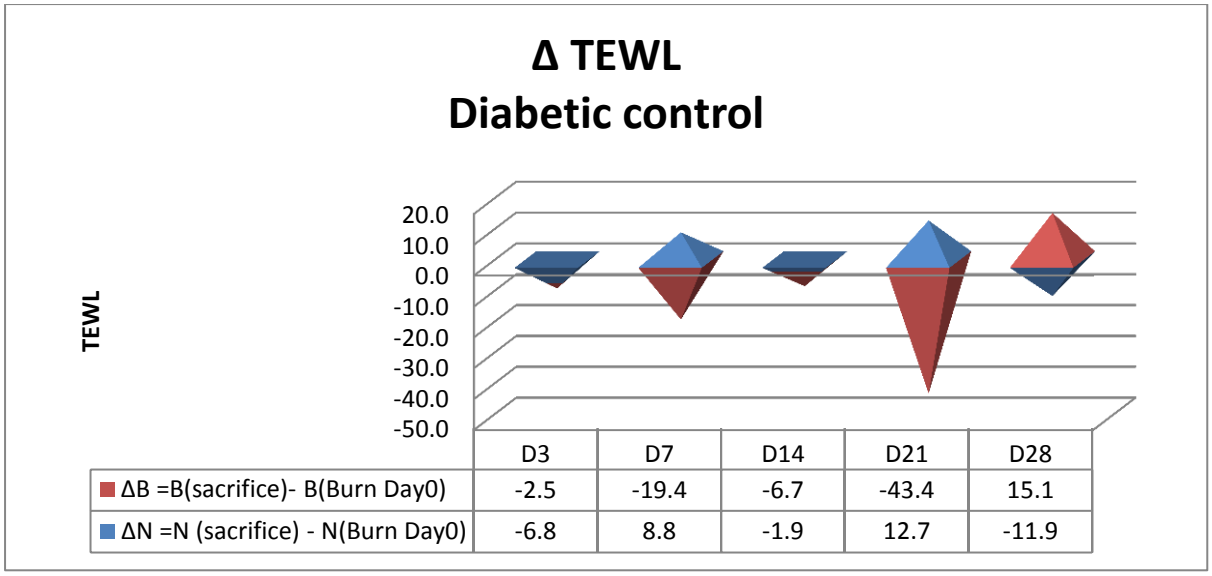


Figure 52: TEWL records at day 0 for Control

Aim 1 conclusion:

- Necrotic spots and redness (+3) dominate the diabetic rats.
- Both control groups start a total skin detachment only after the third week.
- Diabetic rats showed faster skin detachment signs and 100% of total skin detachment after 4 weeks.
- Non diabetic rats showed smaller wound surface left at day 28.
- TEWL changes were not consistent in the treatment animals.

***Aim 2**: to study the modulatory effect of vitamin B17 on burn wound healing in diabetic and non-diabetic rats:*

The wound assessment of both control subgroups: diabetic (condition 1) and non-diabetic (condition 2) were compared with Vitamin B17 injected groups in order to detect any changes in the burn wound healing process caused by vitamin B17 injection. The comparison consisted to analyse the data of (C1 Vs C9) for diabetic rats and (C2 Vs C5) reflects for normal non-diabetics.

The first week:

Normal animals treated with Vitamin B17 (condition 9) Showed almost the same wound appearance like the control groups (condition 2). On the other hand the necrotic spots and (+3) redness, seen at **day 3** and **day 7** in diabetic rats (condition 1 control) were absent in the animals treated with Vitamin B17 (condition 9); and the wound borders are well declined with only (+1) redness (figure 52).

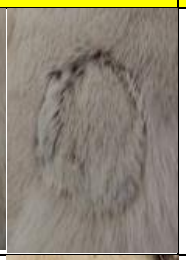
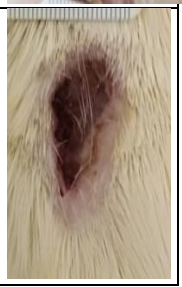
	Rat condition	D3	D7	D14	D21	D28
1	Ct diabetic Burnt only					
9	Diabetic + vit B17					

Figure 52: Gross wounds for control and Vitamin B17 injected diabetic rats

After two weeks:

- **<u>For diabetic rats:</u>**

At **D14** Diabetic animals treated with vitamin B17 started to show signs of wound skin detachment faster than control animals, 25% of rats had a total skin detachment (Table 22). At **D21** all diabetic rats treated with vitamin B17 had a completely shed away crust (100%). On the other hand control animal experienced (100%) total skin detachment only at **D28** and no cases of skin detachment were recorded before the third week **D21 (0%), table 21.**

➢ **For normal non-diabetic rats:**

At day 14, non-diabetics did not exhibit total skin detachment were detected in both control and vitamin B17treated animals at **D14. N**ormal animals treated with vit B17started to show signs of wound skin detachment faster than the control animals in addition to numerous necrotic spots (figure 51). At **D21 (**50%) of vitamin B17 treated rats had a total skin detachment (Table 22). After 1 month, rats treated with vitamin B17 had a completely shed away crust **D28** (100%). On the other hand, control animals experienced (100%) total skin detachment only at **D28;** there were no cases recorded before the fourth week at **D21 (0%)**, as seen in table 21.

	Rat condition	D14	D28
2	**Control** **ND** Burnt only		
5	**ND** + vit B17		

Figure 51: Gross wounds for control and Vitamin B17 injected normal rats

Table 21: skin total detachment percentage in diabetic and non-diabetic controls and vitamin B17 injected animals at day 14, 21 and day 28

Conditions				
	C1	**C2**	**C5**	**C9**
Condition Description	**Diab** CT Burnt only	**ND** CT Burnt only	**ND** + vit B17	**Diab** + vit B17
Skin Detachment percentage				
D14	0%	0%	0%	25%
D21	0%	0%	50%	100%
D28	100%	75%	100%	100%

Wound Area:

After 4 weeks, rats injected with Vitamin B17 showed a decreased wound area in both diabetic and non-diabetic conditions. In diabetic rats the area decreases from 2.93 to 2.35 and scored a difference of (0.58 cm²). On the other hand, the area was remarkably reduced (about 0.96 cm²) from 2.82 to 1.89, in normal rats, almost 60% more than those treated with Vitamin B17 (table 22).

Table 22: Wounded skin area in (cm²) for diabetic and non-diabetic controls and vitamin B17 injected animals at day 1 and day 28

Wounded skin area (Area=$A = \pi r^2$) ± 0.05				
	C1	**C2**	**C5**	**C9**
Condition Description	**Diab** CT Burnt only	**ND** CT Burnt only	**ND** + vit B17	**Diab** + vit B17
D1	3.14	3.14	3.14	3.14
D28	2.93	2.82	1.89	2.35

Aim 2 conclusions:

The main effects of vitamin B17 include:

- Speeding up skin total detachment in both diabetic and normal rats starting at D14 for diabetics and at D21 for normal rats
- Causing 100% total skin detachment for diabetics at day 21
- Reducing redness (+3) seen the first week in diabetic rats to (+1), as if delaying inflammation.
- Reducing the wound area after the third week, that is a better healing outcome.

Aim 3: *to evaluate the possible additive effects of metformin and vitamin B17 on burn wound healing*

Normal rats treated with metformin showed an increased number of necrotic spots and redness at day 3 and day 14 (figure 54), no other obvious effects of metformin alone were recorded on skin detachment which was 75% at day 28 in both control and metformin normal non-diabetic rats. Moreover the wounded area left after metformin treatment was slightly decreased.

	Rat condition	D3	D14	D28
2	Ct ND Burnt only			
6	ND + metformin			

Figure 54: Gross wounds for control and metformin treated normal rats

However, it's important to note that only condition 9 (Diabetic + metformin +Vitamin B17) showed a fluid retention at the burnt area over all the 4 replicates (which gave a shape of camel hump) at day 3 and 7 as illustrated before in (figure 19).

Aim 3 conclusions:

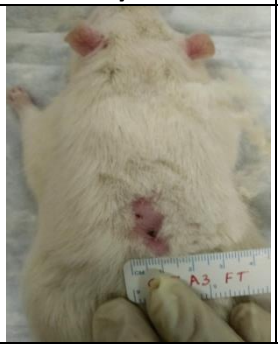
There seems to be no positive additive effect of metformin on wound healing, however on day 3 animals treated with vitamin B17 alone (diabetic or non-diabetic) showed less edema compared to those treated with metformin alone or metformin and Vitamin B17 combined together; the inflammatory response was delayed or reduced.

***Aim 4**: to assess the effect of added autologous adipose tissue on burn wound healing in normal and diabetic rats.*

The main findings were as follow:

- No death cases were recorded after fat relocation
- No complications were detected for the operated groups
- None of the burns showed any infection
- The skin around the burns was soft and looked normal and in a better shape for all groups except for the animals treated with metformin and vitamin B17 together.
- The quality of the scar was better when autologous fat was used. Actually, the scar formed after injection vitamin B17 alone or Vitamin B17 and autologous fat tissue were the best in quality compared to other conditions with faster regeneration of epithelium.
- The final size of the burnt area was the smallest with grown fur.
- The relocated fat improved the healing process wherever it was used.

Table23: illustration of the best wound healing for both conditions (7 and 8) at D21 and D28

Condition 7: **Diabetic** +Fat+ Vitamin B-17		**Condition 8:** **ND** +fat+ Vitamin B-17	
Day 21			
Day 28H	**Day 28 V**	**Day 28H**	**Day 28 V**
			
Day 28			
Day 28H	**Day 28 V**	**Day 28H**	**Day 28 V**
			

A sum up conclusion of all macroscopic and microscopic findings was as follow:

- Necrotic spots and (+3) redness dominate the diabetic rats during the first week.
- Both control groups start a total skin detachment only after the third week.
- Diabetic rats showed faster skin detachment signs and 100% of total skin detachment after 4 weeks.
- Non diabetic rats showed smaller wound surface left at day 28.
- TEWL changes were not consistent in the treatment animals.
- Vit B17 speeds up skin total detachment in both diabetic and normal rats starting at D14 for diabetics and at D21 for normal rats.
- Vit B17 causes 100% total skin detachment for diabetics at day 21.
- Vit B17reduces redness (+3) seen the first week in diabetic rats to (+1), as if delaying inflammation.
- Vit B17 reduces the wound area after the third week, that is a better healing outcome.
- There seems to be no positive additive effect of metformin on wound healing. However on day 3 animals treated with vitamin B17 alone (diabetic or non-diabetic) showed less edema compared to those treated with metformin alone or metforminand Vitamin B17 combined together; the inflammatory response was delayed or reduced.
- No complications were detected for the operated groups.
- None of the burns showed any infection.
- The skin around the burns was soft and looked normal and in a better shape for all groups except for the animals treated with metformin and vitamin B17 together.
- The quality of the scar was better when autologous fat was used. Actually, the scar formed after injection vitamin B17 alone or Vitamin B17 and autologous fat tissue were the best in quality compared to other conditions with faster regeneration of epithelium.
- The final size of the burnt area was the smallest with grown fur.
- The relocated fat improved the healing process wherever it was used
- The histological findings showed a better scar and smaller surface area of the wound with better fur growth where relocated fat was present, especially in the presence of vitamin B17.
- The presence of metformin in the combination has any added value, especially in non-diabetic rats

B. Molecular analysis and expressions:

1) P-AMPK protein expression:

Hyperglycemia levels reflected the success of transforming normal rats into diabetics by STZ injection. In addition, P-AMPK expression was performed in order to test the efficacity of metformin treatment during the experiment.

As expected **P-AMPK expression** increased remarkably with metformin treated animals for diabetics at Day14. These results indicate that the treatment of metformin worked properly since non- diabetic rats treated with metformin showed the same profile like normal controls while **P-AMPK** in diabetic rats treated with metformin scored high level of expression compared to the diabetic controls (figure55).

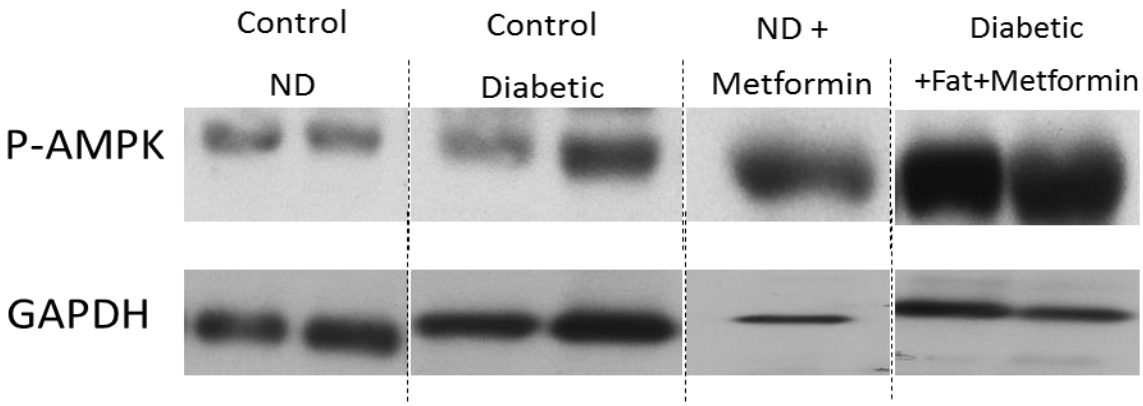


Figure 55: p-AMPK expression in control groups and in non-diabetic and diabetic rats treated with metformin only at day 14

Note: Concerning the modulation of interleukins IL-1 alpha, IL-6 and IL12, the presence of Vitamin B17 led to variable effects as follow:

In diabetics there was little increase of their gene expression on day 3; the acute phase, and a significant increase on day 21, however, in the level of cytokine expression was lowered when combined with metformin.

2) IL-1 alpha gene expression:

Interleukin 1 alpha (IL-1α) is a cytokine of the interleukin 1 family that in humans is encoded by the IL1A gene. This interleukin is mainly responsible for the production of inflammation; it promotes fever and sepsis. IL-1α is produced mainly by activated macrophages, as well as neutrophils, epithelial cells, and endothelial cells. It possesses metabolic, physiological, haematopoietic activities, and plays one of the central roles in the regulation of the immune responses. As expected its expression at the gene level was modulated during the 28 days experiment.

IL-1α expression in diabetic animals treated with vitamin B17:

IL-1α expression was masked during day 3, 7, and 14. After 3 weeks a very clear peak appeared at day 21 (8 times higher than day 1). Then at day 28 IL-1α showed remarkable drop again (figure 56).

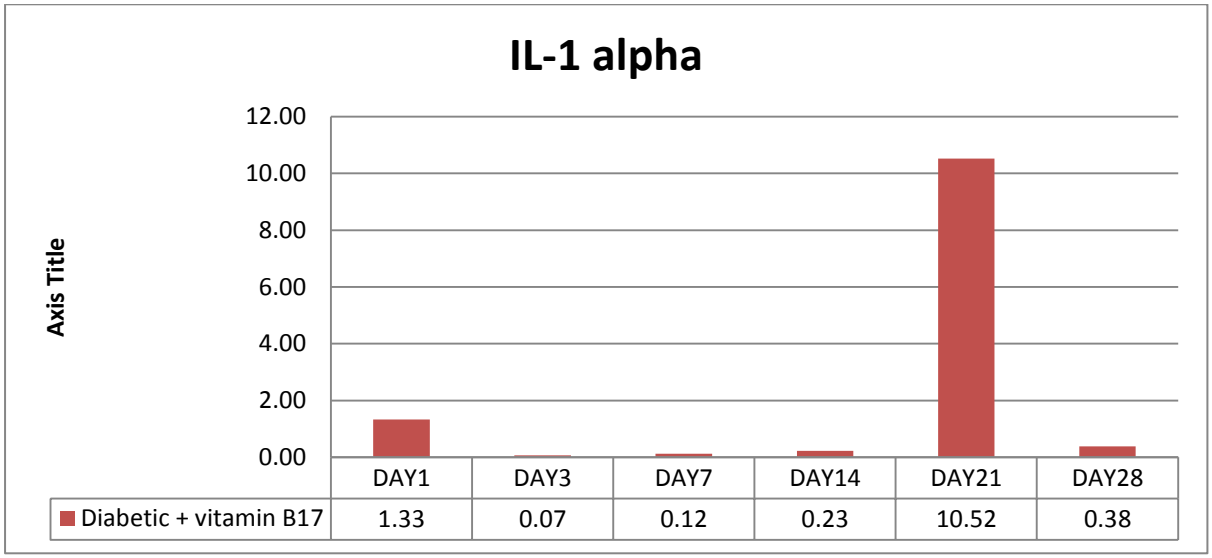


Figure 56: IL-1 variation of expression at all-time points for diabetic rats treated with Vitamin B17 alone

IL-1α expression in diabetic animals double treated with vitamin B17 and metformin:

Animals double treated with metformin and vitamin B17 showed a different profile of IL-1α expression, where a very clear peak was detected at day 7 (3.84), followed by a drop at Day 14 (0.66), then a progressive increase at day 21 (1.14) and day 28 (2.59) (figure57).

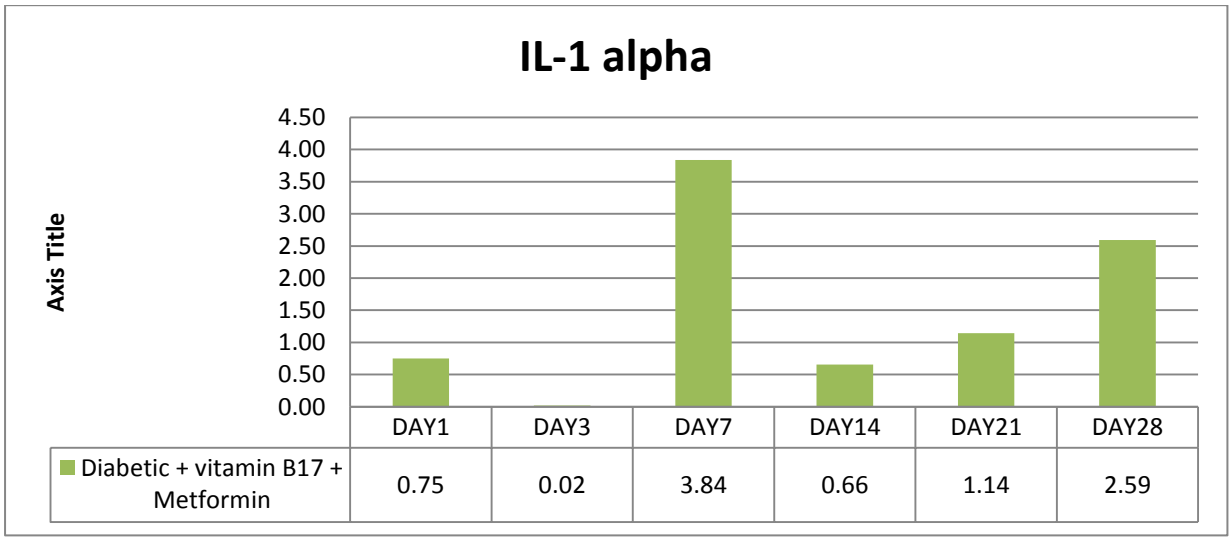


Figure 57: IL-1 variation of expression at all-time points for diabetic rats double treated with vitamin B17 and metformin

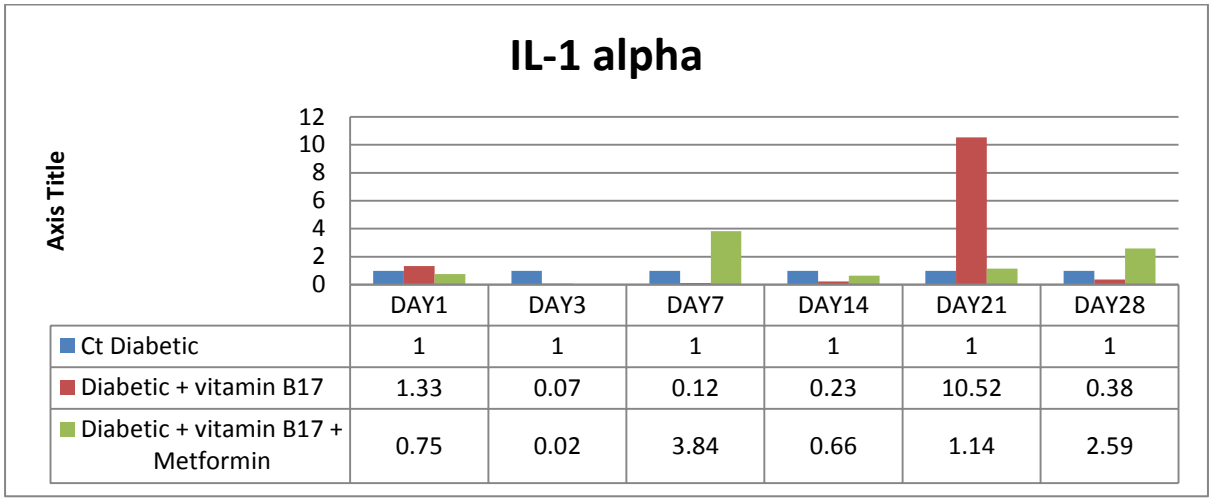


Figure 58: a comparative graph of IL-1 expression in diabetic rats treated with vitamin B17 alone and/or in combination with metformin at all-time points.

IL-1α expression in normal rats treated with vitamin B17 or metformin:

For normal rats IL-1α scored the highest expression at day 14 (16.8) in metformin treated rats, then it decreased to 3.12 on 28. However, in the presence of vitamin B17, IL-1α gene expression was also elevated to 10.35 and continued so till the end of the experiment day 28 reaching higher levels of 14.97 (figure59).

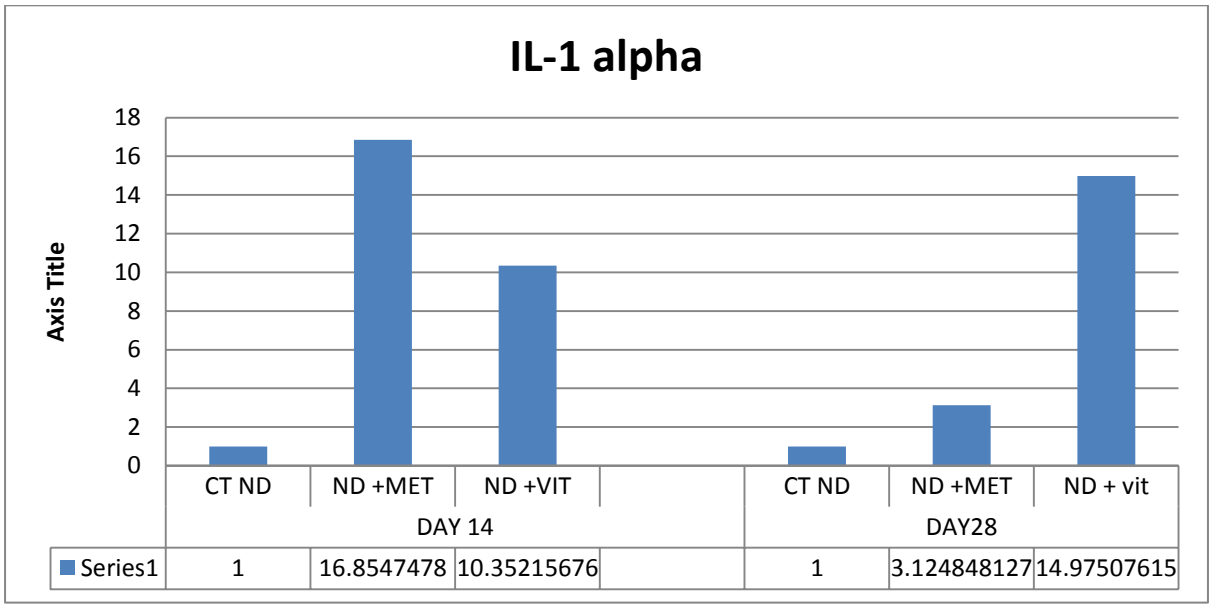


Figure 59: IL-1 variation of expression at day 14 and day 28 for normal rats treated with vitamin B17 or metformin

3) Gene expression of pro / anti-inflammatory cytokine: IL-6:

Concerning IL-6, which might play both a pro and an anti-inflammatory cytokine depending on multiple variables, its gene expression in non-treated diabetics was down-regulated at day 3, day 7 and day 24 by both metformin and vitamin B17 treatment, while it was up regulated the day 21 in both conditions (figure 60) and (figure 61). On other hand those treated with Vitamin B17 had an initial elevation in the acute phase day 1 (1.46) (figure 60) followed by a

peak (7.26) on day 21 and a tapering down on day 28 (3.37). The presence of metformin with vitamin B17 did not show any added value; on the contrary it lowered the values on day 21 to 2.03 and on day 28 to 0.78 significantly lower than the respective values in its absence (figure 62). On the other hand, the expression of IL-6 in non-diabetics scored a remarkable increase in the presence of metformin (36.44) and went down to very low expression (0.68) on day 28. A decrease of IL-6 by almost 50% was also noted on day 28 for the vit B17 treated non-diabetc rats compared to a peak (12.05) on day 14.

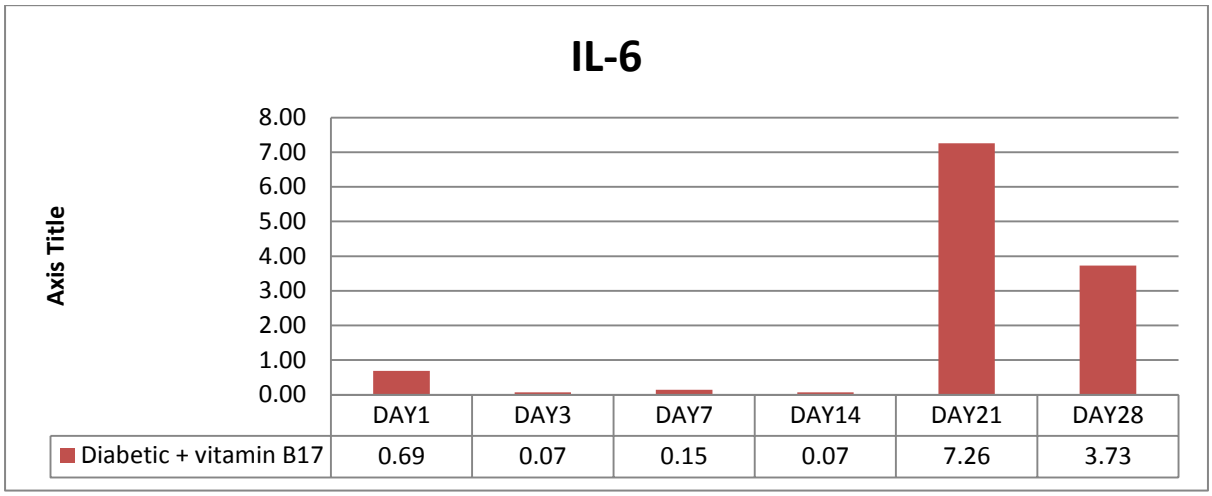


Figure 60: IL-6 variation of expression at all-time points for diabetic rats treated with Vitamin B17 alone

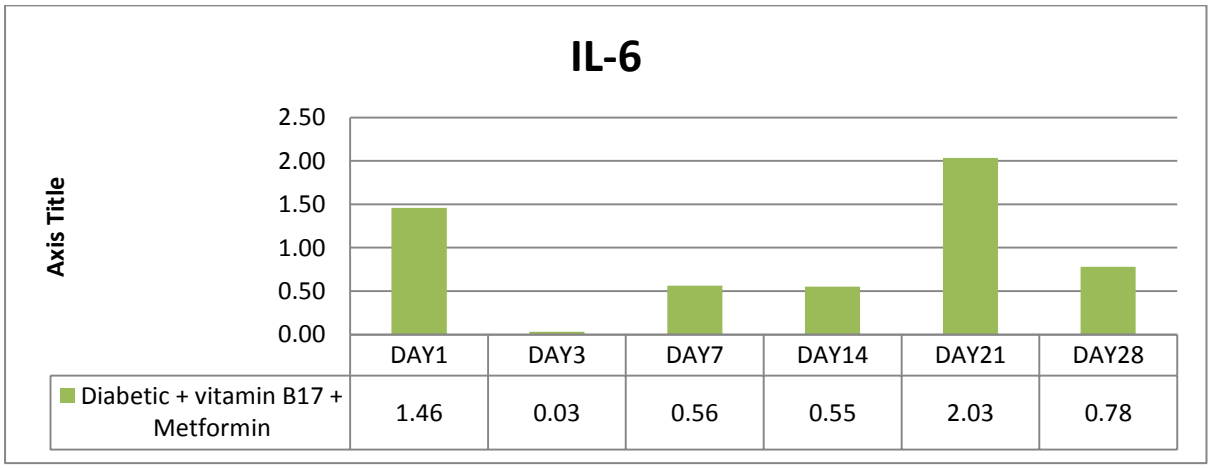


Figure 61: IL-6 variation of expression at all-time points for double diabetic rats treated with Vitamin B17 and metformin

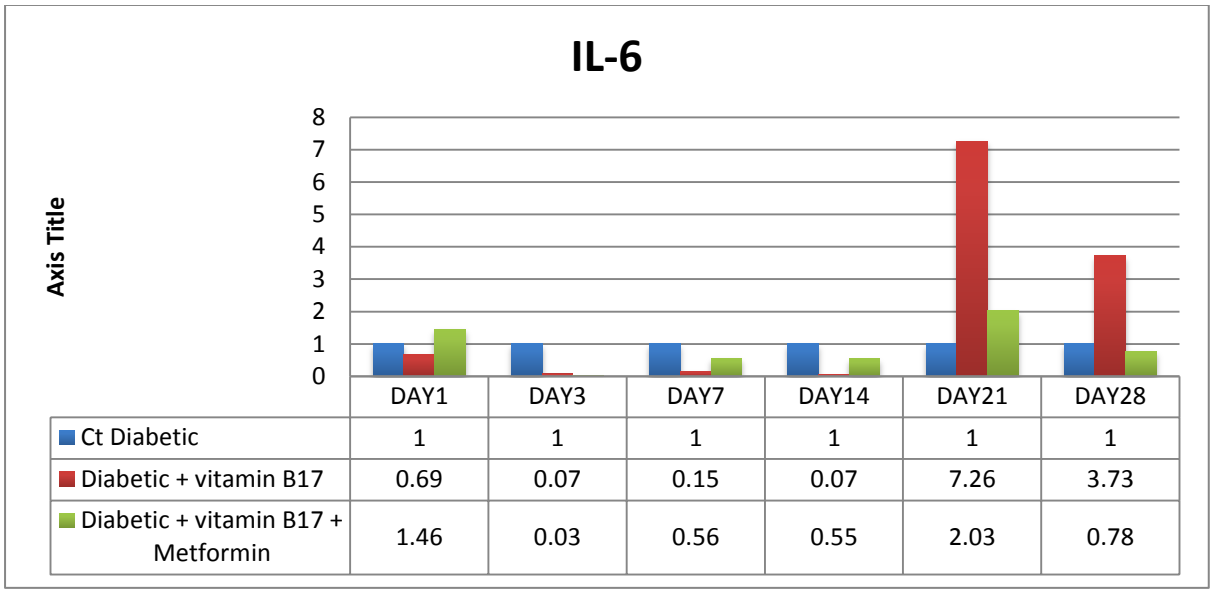


Figure 62: a comparative graph of IL-6 expression in diabetic rats treated with vitamin B17 alone and combined with metformin at all-time points.

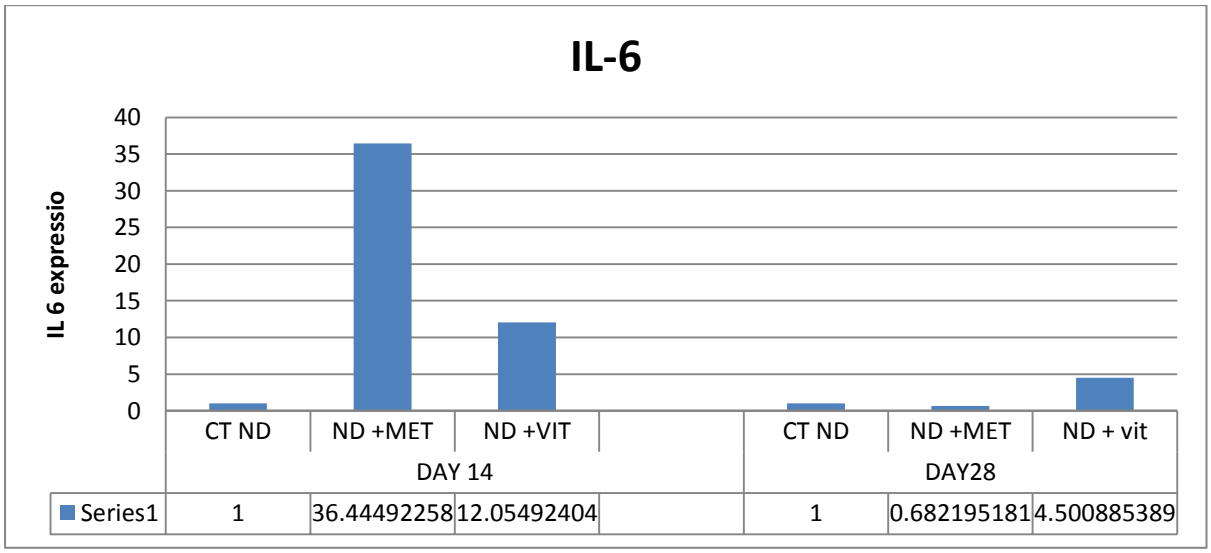


Figure 63: IL-6 variation of expression at day 14 and day 28 for normal rats treated with Vitamin B17 or metformin

4) Gene expression of pro / anti-inflammatory cytokine: IL-12:

IL-12 is naturally produced by dendritic cells, macrophages, neutrophils, and human B-lymphoblastoid cells in response to antigenic stimulation. IL-12 also has an anti-angiogenic activity, which means it can block the formation of new blood vessels during the wound healing process.

In the diabetic rats treated with Vitamin B17, the gene expression of IL-12 showed a little increase 1.33 compared to non-treated controls in the acute phase then a sharp increase (10.52) on day 21 and then back to 0.38 on day 28 (figure 64). However in the presence of a combination of Vit B17 and metformin, the expression showed a continuous presence of from day 7 on after an initial small expression on day 1. The peak was noted on day 7 (3. 84) followed by another peak on day 28 (2.59) (figure 65). On the other hand, in the non-diabetic, IL-12 behaved like IL-6 on day 14 but differently on day 28, whereby the presence of vit B17 alone led to another peak of 14.9 (figure 67)

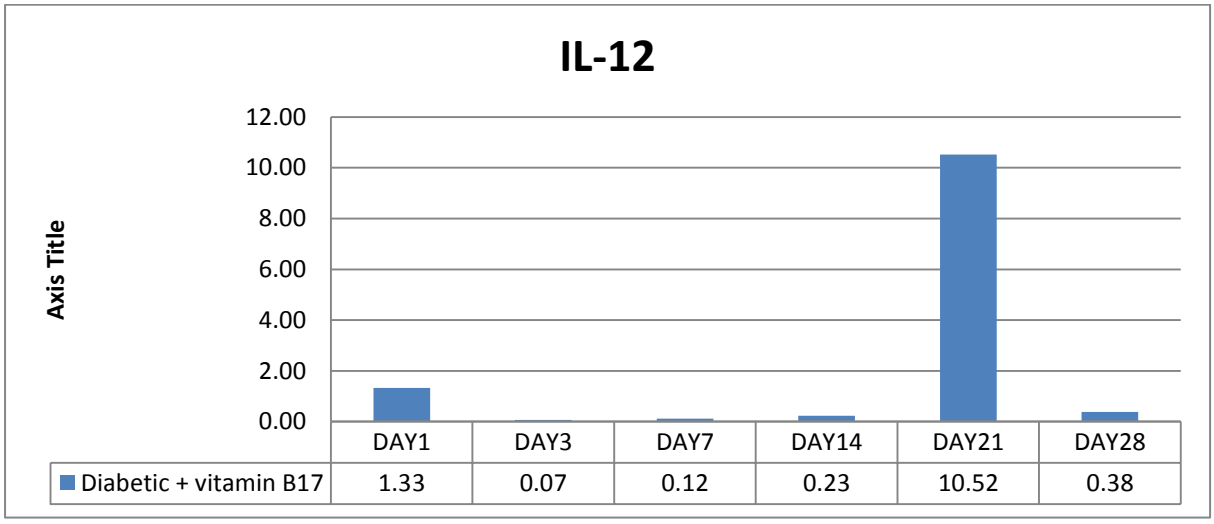


Figure 64: IL-12 variation of expression at all-time points for diabetic rats treated with Vitamin B17 alone

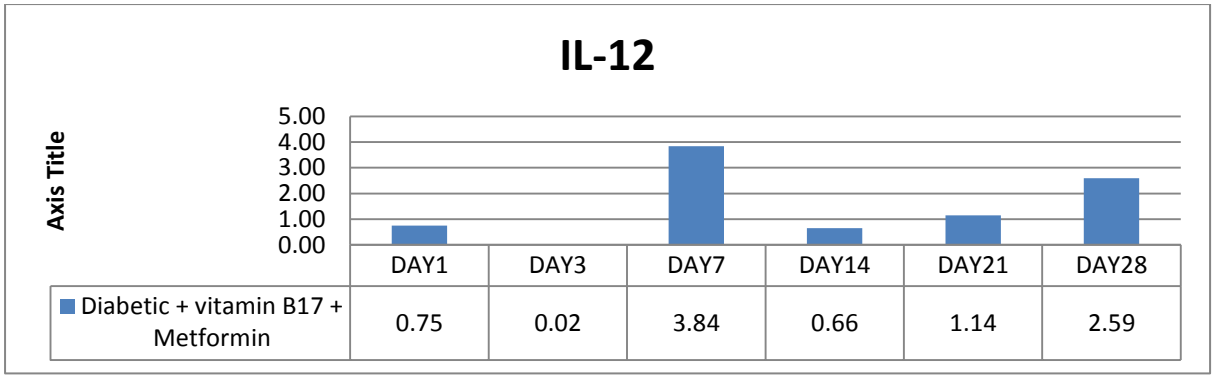


Figure 65: IL-12 variation of expression at all-time points for diabetic rats double treated with Vitamin B17 and metformin

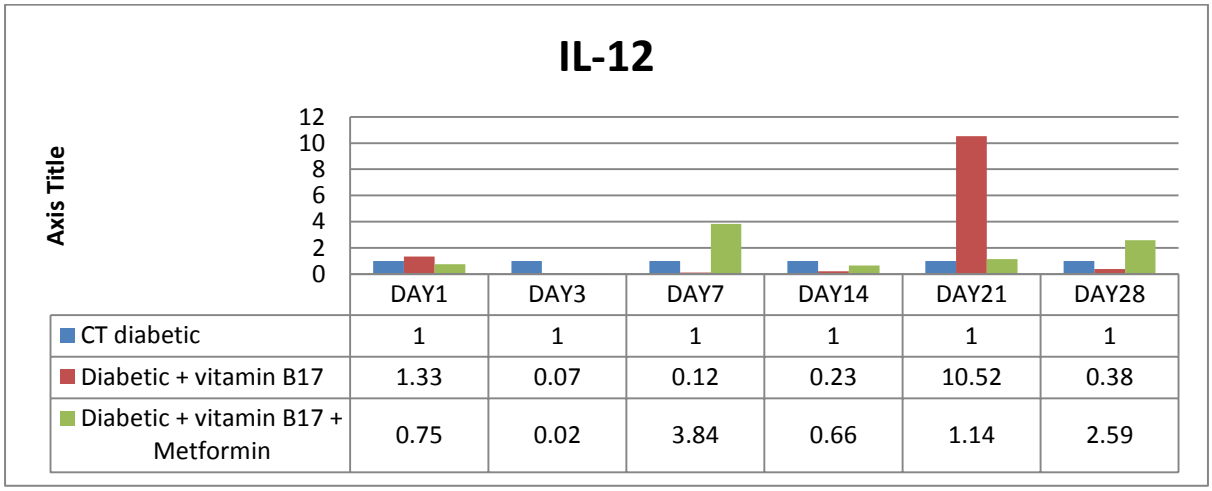


Figure 66: a comparative graph of IL-12 expression in diabetic rats treated with vitamin B17 alone and combined with metformin at all-time points.

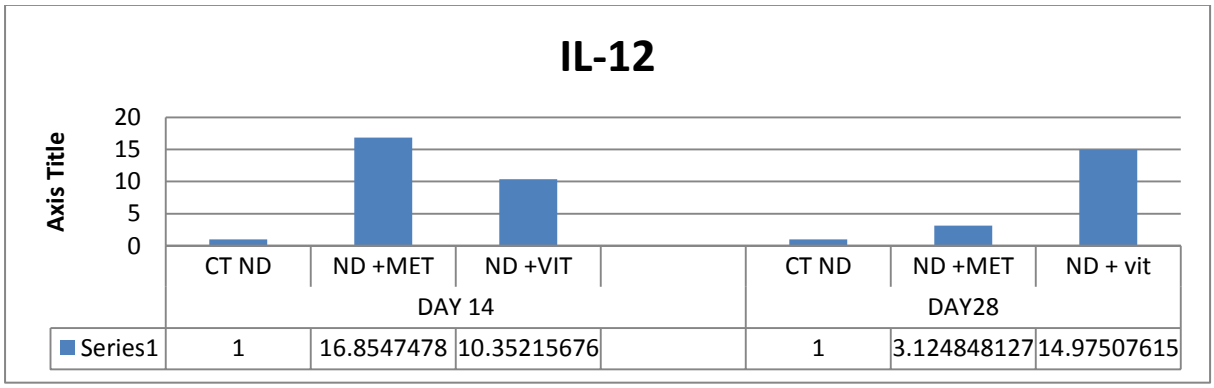


Figure 67: IL-12 variation of expression at day 14 and day 28 for normal rats treated with Vitamin B17 or metformin

5) HSP70 RNA expression:

70-kDa heat shock proteins (Hsp70s) assist a wide range of folding processes, including the folding and assembly of newly synthesized proteins.

The data showed that in diabetic rats treated with vitamin B17 **HSP70** expression on day 14 (6.68) (figure 68), and more importantly with a significant increase in the presence of metformin (139.1) (figure 70), on day 14 and also on day 7 (18.13). This increase probably corresponded with the speeding up of the healing process (figure 71), with an added value of metformin and vit B17 in promoting its expression.

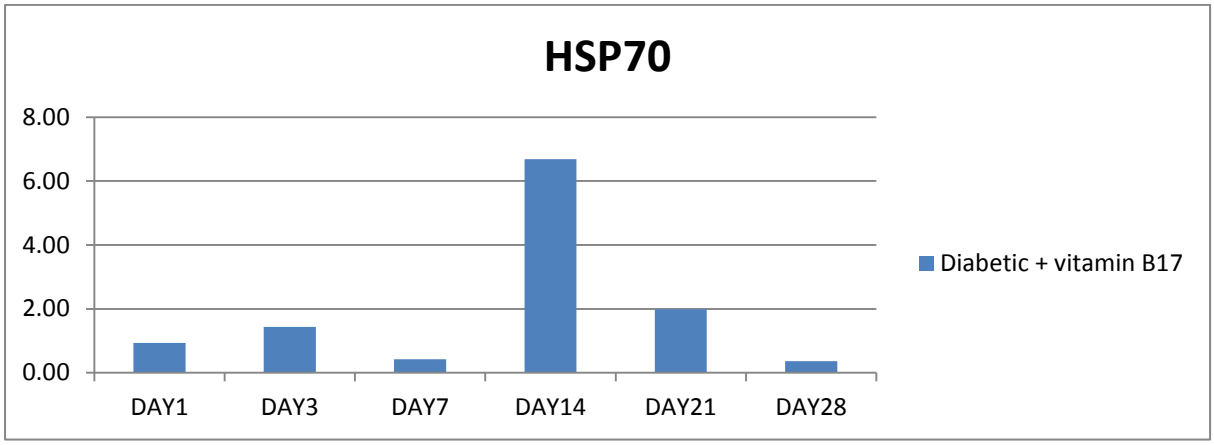


Figure 68: HSP70 variation of expression at all-time points for diabetic rats treated with Vitamin B17 only

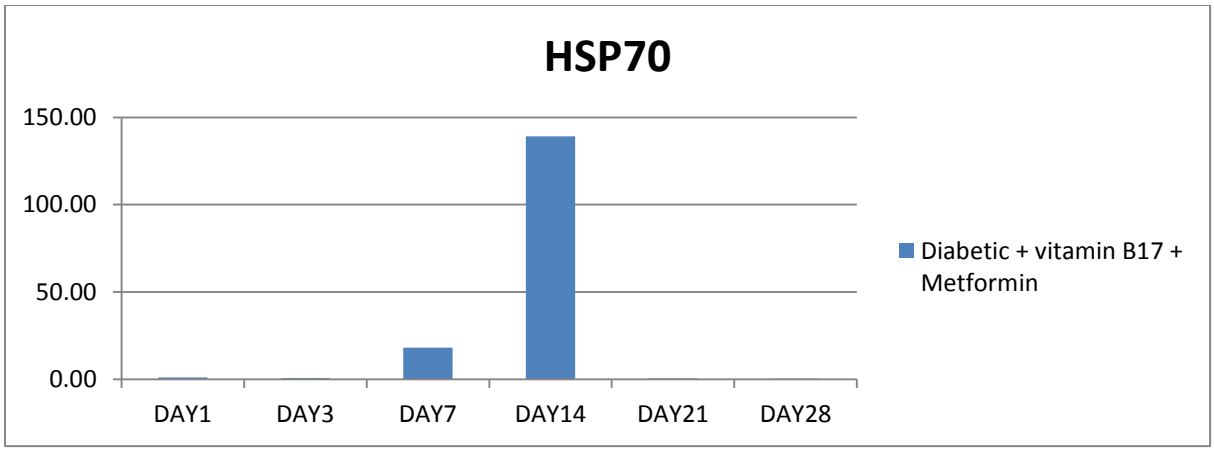
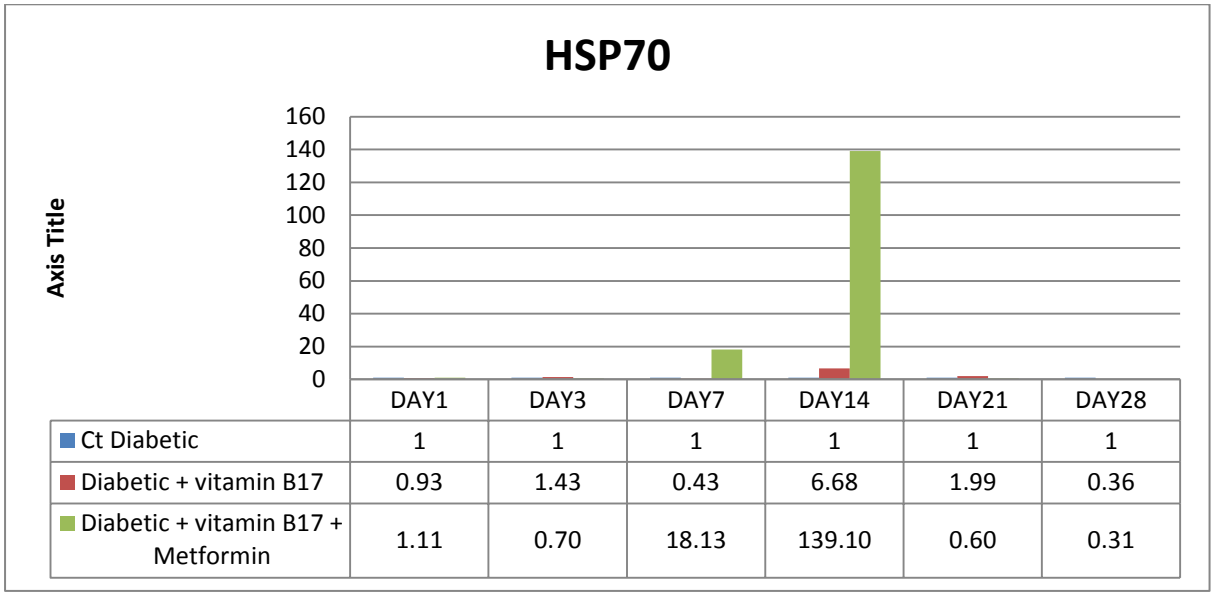


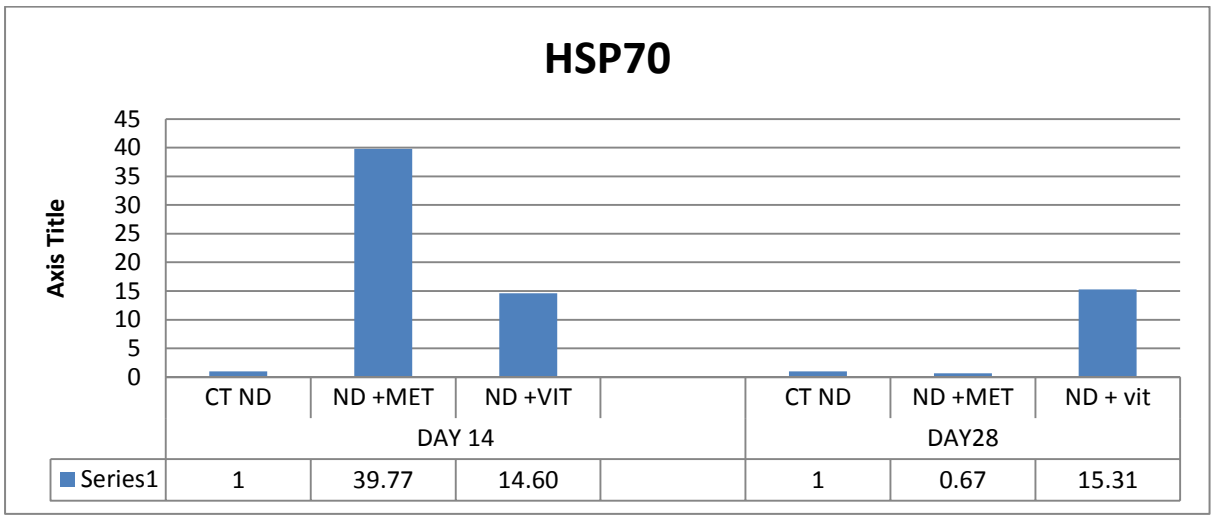
Figure 69: HSP70 variation of expression at all-time points for diabetic rats double treated with Vitamin B17 and metformin

In the normal non diabetic rats, the expression of hsp70 was very similar to IL-12 with stimulation by metformin and to a lesser extent by Vitamin B17 on day 14 and to a lesser extent on day 28 (0.067 and 15.3 respectively) (figure 71).



	DAY1	DAY3	DAY7	DAY14	DAY21	DAY28
Ct Diabetic	1	1	1	1	1	1
Diabetic + vitamin B17	0.93	1.43	0.43	6.68	1.99	0.36
Diabetic + vitamin B17 + Metformin	1.11	0.70	18.13	139.10	0.60	0.31

Figure70: a comparative graph of HSP 70 expression in diabetic rats treated with vitamin B17 alone and combined with metformin at all-time points.



	CT ND	ND +MET	ND +VIT		CT ND	ND +MET	ND + vit
	DAY 14				DAY28		
Series1	1	39.77	14.60		1	0.67	15.31

Figure71: HSP70 variation of expression at day 14 and day 28 for normal rats treated with Vitamin B17 or metformin

V. DISCUSSION

Cutaneous burn wound healing is a multistep complex process where no single cell or cellular mediator, growth factor, or gene can successfully accelerate the healing alone. In parallel, the field of burn and wound care is showing prominent advances by using stem cells (Ghieh et al., 2015) and new technologies in order to develop skin substitutes or biophysical and pharmacological molecules that could positively influence any or all stages of the burn wound healing process (Arno et al., 2011). Despite all available biological resources, products and techniques, the diabetic patients have their own challenges. It is important to take in consideration their complicated physiopathology (Kahn, Cooper, & Del Prato, 2014) in this domain since diabetic persons need different approaches which can guarantee wound reconstruction and minimize the acute and chronic diversified side effects.

Data emanating from this study, based on a validated animal model for diabetes and burn wound healing (Abdallah et al., 2012); have confirmed the uniqueness of the behaviour or burn healing in diabetics. All 3 stages of the healing process were affected to different extents. In the diabetics, the inflammatory reaction in stage one was more extensive and the wound accumulated more fluids than non-diabetics which could have resulted in the influx of more inflammatory cells into the site. Such as active acute inflammation could have delayed the regeneration process of keratinocytes as well as neovascularization in stage two of healing, thus leading to a retarded or reduced formation of myofibroblasts in the diabetics. Such as phenomen might have resulted is a defective contracture and consequently, a larger surface wounds and a delay in the crust formation and shedding in the diabetics.

The main aims of this study were to evaluate the effect of the most controversial vitamin B17 also called amygdalin, on skin burn wound healing, and to tag any additive effect of metformin and / or fat tissue on the healing process for diabetic and normal rats in a well described animal model. In brief, it is a new topic not investigated before.

The modulatory effect of Vitamin B17 on burn wound healing:

The use of amygdalin or vitamin B17 dates back a minimum of 2 centuries (since 1803), when traditional Chinese medicine (TCM) described it as an effective component in bitter almond and suggested its use as an auxiliary medicine of cough (Song & Xu, 2014). Moreover, several publications and in vitro studies have reported the efficiency of amygdalin in treating several types of cancer and the possible supportive role in asthma, atherosclerosis, immune suppression, leprosy and other diseases (Song & Xu, 2014) (Amira Nour, Basel Azar, Anas Rabata, & Ahmad Manadili). Despite the different roles of amygdalin, this vitamin became famous and largely used for its implication in cancer therapy(Moertel et al., 1982), a popular anti-tumor vitamin, due to its effectiveness in inhibiting the growth of cancer cells. However, it was not fully adopted by different professional societies and committees and it has been a subject of considerable controversy(Bromley, Hughes, Leong, & Buckley, 2005), since other researches proved some physiochemical and biochemical complications caused by amygdalin (Moertel et al., 1981). In pharmacology, the term amygdalin is a cyanogenic compound. However, amygdalin itself is not toxic but after oral administration, it gets decomposed into hydrocyanic acid (HCN) and benzaldehyde; HCN decomposition by some enzymes results in poisonous substances and induces an anti-neoplastic effect while benzaldehyde is believed to be able to induce an analgesic effect(Qadir & Fatima, 2017).

Advanced research reported several interesting roles played by amygdalin and its different analogues at the molecular level which led to its capacity to treat cancer. In parallel with its anti-tumor effect, amygdalin was described as an apoptosis promoter, immune enhancer, apoptosis regulator, and an antiulcer(Qadir & Fatima, 2017).

After a deep look onto the different mechanisms involved and the proposed molecular roles among available studies, we hypothesized that amygdalin may improve burn wound healing in diabetic rats, a subject not explored before, based on the apoptotic and immune activities of the compound. Most of previous studies were in vitro based research applied on isolated cells while in our experiments we tested the in vivo direct application on the wound healing process which was affected by the drug both in normal and in diabetic rats.

During the last 2 decades, thorough research on vitamin B17 improved our understanding of its main roles. Researchers highlighted the different mechanisms involved, altered, or improved by amygdalin use(Song & Xu, 2014). Their main focus was to target the regulation of the 5 essential processes: apoptosis, inflammation, cell proliferation, cell cycle and angiogenesis. One of the focused studies about the effect of amygdalin on apoptosis was performed on a Cervical cancer cell line the HeLa cells (Chen et al., 2013). The cells and after being treated with metformin, showed an increased ratio of Bax protein to Bcl-2 protein suggesting the involvement of amygdalin in apoptosis. Moreover, amygdalin was able to suppress TGF-β1 secretion in lymphocytes in a study that targeted the treatment of renal intestinal fibrosis and chronic kidney disease(Makarević, Rutz, Juengel, Kaulfuss, Tsaur, et al., 2014). Further studies proved that tumor cells treated with amygdalin undergo early apoptosis after long term amygdalin exposure. Another important aspect was that the inhibitory effect on proliferation caused by amygdalin was not caused by its toxic effect (Makarević, Rutz, Juengel, Kaulfuss, Reiter, et al., 2014). In parallel amygdalin play an important role in influencing mitosis in 3 different bladder cancer cell lines, since at the molecular level, amygdalin was proved to reduce the phosphorylation of Akt and the m-TOR that are play an important role in signalling in diabetes and in bladder carcinogenesis. Additionally, a study in 2005 was the first to suggest that amygdalin has an anticancer effect via its down regulation of cell cycle-related genes in human colon cancer cells, by comparing the gene expression profile of these cells after amygdalin treatment(Park et al., 2005). The downregulated genes belong to categories of cell cycle-related genes, they are related to cell growth, response to stress and transcription. The results obtained in this study on normal and diabetic rats showed various effects of vitamin B17 in line with reducing inflammation, especially in early stages of the burn, and the decrease in ROS production in response to the burning stress to various degrees in all treated groups.

In addition, other studies showed that amygdalin treated animals have comparatively higher level of TGF-β (MiSung & Aree, 2002), an essential cytokine with multiple functions including burn wound healing enhancement. Modulating TGF-β activities could happen through various mechanisms including stimulating secretions of other growth factors or the smad mechanism or signalling pathway. Moreover, additional studies ended suggesting that amygdalin improved microcirculatory disturbance and reduce inflammatory factors production in pancreatic fibrosis(Zhang et al., 2018). In line with the modulation of inflammatory factors, our data showed that vitamin B 17 caused a delay or a reduction of

inflammation in the initial acute stage of burn wound healing. In parallel the proinflammatory cytokines tested IL-1α, IL-6 were suppressed in the first four days, a situation that helped the healing process to progress with less necrosis, better scar and better wound contraction leading to a smaller surface wound and early shedding.

Moreover, vitamin B17 could have modulated TNF-α which increases production of pro-inflammatory molecules (IL-1, IL-6, IL-8, and NF-Kappa B), and adhesion molecules. Additional studies highlighted that amygdalin analogues could inhibit interferon signalling and reduce the inflammatory response in human epidermal keratinocytes. Such works come in support of our data in the reduction the inflammatory reaction in epidermal cells, thus leading to a better outcome. The suppression or inhibition of such mediators favors a faster healing with a smaller wound area and a better scar. Early limitation of the acute inflammatory response and decrease in ROS production favour an optimal progression events in the extracellular matrix favouring some adhesion molecules (integrins) to promote epithelial replication and growth from stem cells available in the wound surroundings. The extracellular micro environment created partly by vitamin B17 could have led to an optimal development of myofibroblasts, thus leading to a better contraction and remodelling of the wound. In addition, the stimulation of the anti-inflammatory cytokine. IL-12 secretion in the last 2 weeks by vitamin B17 could have contributed positively to stage 3 of the healing process by improving the necessary elements for a better keratinization and appropriate wound contraction. All these elements could have had an orchestrated mechanism leading to a better scar and a smaller wound area in vitamin B17 treated animals.

The animals treated with amygdalin reported an earlier shedding of the crust which may have been caused by an increased rate of apoptosis and better vascularization compared to the non-amygdalin treated animals. On the other hand, vascularization and sloughing of the crust were delayed for almost 2 weeks in conditions with no Vitamin B17 injection, which reflects a faster apoptosis in the vitamin B17 treated rats as described in literature. Moreover, Vitamin B17 delayed the inflammation process, by masking the cytokines (IL-1α, IL-6,) towards the end of the third week (D21), at the same time increased the anti-inflammatory IL-12 cytokine. At the stage of remodelling cytokines become less harmful and may be enhancing for the proliferative process in the skin. In addition, the burn surface was reduced significantly when fat was relocated under the burnt groups, especially when combined with vitamin B17, there could have been an additive effect in improving the outcome. The autologous fat with all what it contains of cytokines, potential stem cells and cushioning

could have interacted with vitamin B17 resulting in the creation of a better healing environment for the burn. On the other hand, metformin in presence of vitamin B17 did depict an additional remarkable positive effect on the wound healing process, days 14 and 21, beside lowering the glucose level in the diabetics, which is by itself a beneficial factor for wound healing.

Concerning ROS molecules, it is well known that they are generally produced during oxidative stress, play a central role in cellular physiology and take part in various biological processes such as proliferation, senescence, apoptosis and autophagy. They are also related to TGF-β and TNF-α pathways. ROS production is enhanced in various tissues through enzymatic and non-enzymatic pathways including glucose autoxidation, the polyol pathway, Advanced Glycation End Products (AGE/RAGE System), metabolites of arachidonic acids, NADPH oxidases, uncoupling of nitric oxide and others. In addition, oxidative stress has emerged as a critical pathogenic factor associated with early development and progression of diabetic microvascular dysfunction. Moreover, the production of ROS has been linked to dysregulation of mesangial contraction, endothelial dysfunction and matrix expansion. Furthermore, oxidative stress has also been shown to induce epithelial injury and apoptosis. In our experiment ROS production increased, most likely due to the stress introduced by the burn, and consequently was harmful to the smooth progression of the healing process in all its stages, especially in the early stages, and more so in the diabetic rats. Injecting vitamin B17 seemed to have lowered or reduced ROS production, especially in diabetics. Such and effect favoured a better environment for the orchestrated functions of many cells involved, ranging from the decrease of inflammatory cells in stage 1 to enhancement of epithelialization, neovascularization, in stage 2 and a better functioning of myofibroblasts for an adequate contraction, as well as a better apoptosis for remodelling in stage 3 of the healing process.

From among this complicated mix of factors, underpinning a seemingly common pathophysiologic mechanism of healing, HSP70 emerged as one of the putatively important drivers of events in the burn wound healing process. In the diabetic rats treated with B17 only, there was a continuous presence of HSP70, low but it was there, which peaked up at day 14 of remodelling contraction and epithelialization of the wound. Such an effect was greatly potentiated in the presence of metformin moving from 7 units to 140 units on that day and then fading away slowly till day 28.

In the non-diabetic rats, again vitamin B17 lead to an increase of HSP70 on days 14 and to a lesser extent on day 28. Metformin one more time increased the expression of HSP70 on day 14, thus playing a possible role in enhancing, indirectly in this case, the healing process which resulted in a smaller wound area and a better scar. It would be important to note that the peaks of HSP70 correlated with a similar behaviour of IL-12, the anti-inflammatory cytokine. Such parameters induced by B17 were, mostly likely, behind creating the appropriate environment for the improved healing process outcome.

The diagram below illustrates the possible mechanisms occurring in response to vitamin B17 treatment. Further exploration of these mechanisms may help us to understand how normal epithelialization occurs and why in chronic wounds this control is lost.

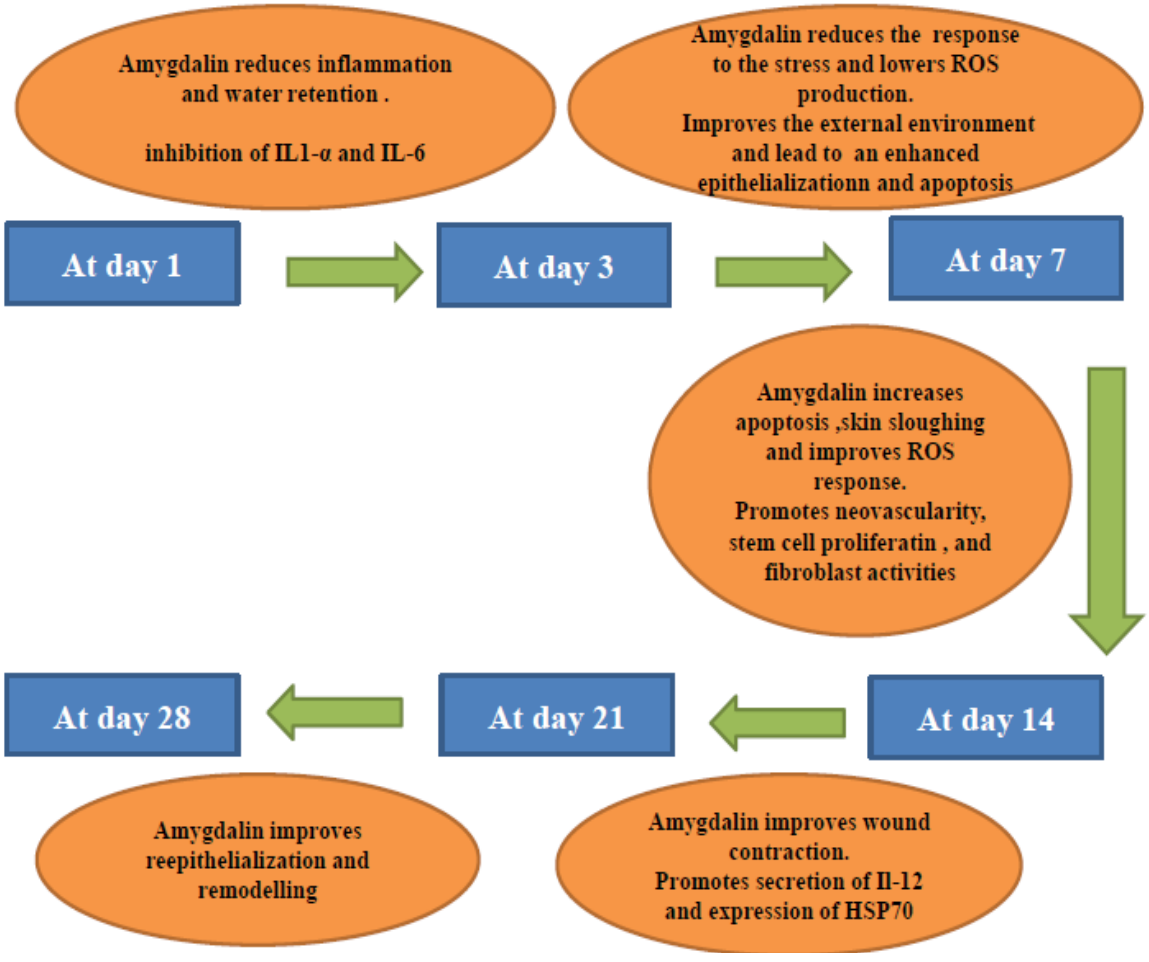


Figure72. Schematic representation of the possible effects of vitamin B17 and the related mechanisms taking place during wound healing.

V. CONCLUSION AND FUTURE PERSPECTIVES

In conclusion, our data demonstrate that amygdalin may be able to improve the burn wound healing process in both diabetic and non-diabetic rats by speeding up the sloughing time, masking and delaying inflammation, improving reepithelization, and decreasing the wound surface. On the other hand, fat relocation created a better environment for healing of the burned wounds leading to a faster and better wound contraction and better scar reduction. This analysis is based on the results found in both conditions 7 and 8 which showed the fastest wound healing time and the smallest wound area, after 4 weeks. Such data indicate that Vitamin B17 treatment and fat relocation linked together improved the healing process. Moreover, Vitamin B17 delayed the inflammation and masked the expression of IL1-alpha, IL6, and IL-12 until day 21 in diabetics and regulated positively HSP70 response.

It will be important to study the mechanism by which vitamin B17 could lead to a delayed inflammation by reducing the proinflammatory cytokines and Ros or by increasing the anti-inflammatory factors IL-12 and HSP70. Moreover, a focus on the necrosis and apoptosis signal pathway and the screening for targeted caspases protein expression can be important in order to shed more light on the molecular role of vitamin B17, since there are two pathways mediating the development of apoptosis, the intrinsic and extrinsic pathways. In addition, another essential aim can be developed regarding the use of autologous stem cells where by choosing the right stem cell type that will aid in the regeneration of a functional skin will be important and it can support the results of our research and may optimize better tissue repair and regeneration.

Therefore, more research is required to further explore the long-term effects of using the autologous fat stem cell tissue and provide safer and more effective therapies for future clinical application. In addition, in this study, we didn't use a control group for fat relocation alone since the study was focusing essentially on the Vitamin B17 effect on the healing process, while the role of the stem cells presented in the fat tissue was introduced as an additional factor. Based on our data, it will be so informal and helpful to study the effect of autologous fat tissue relocation alone, as a simple microsurgery, improving the healing process in diabetics. Briefly, we suggest that amygdalin subcutaneous injection under burnt area may serve as a prominent and efficient metabolic therapy for skin burn wound healing in diabetics.

In our experiment we were using a very low dose of vitamin B17 (5 mg), this dose was expected to be nontoxic in most publications; the use of different doses in order to study the dose-dependent effect of vitamin B17 will be also important.

In conclusion, the burn wound healing process is confirmed one more time as a multifactorial complex process triggered and monitored by an array of factors that contribute to the skin homeostasis and to a balance between regulatory mediators of inflammation.

In this study data generated were in favour of a potential role of vitamin B17 interfering favourably, through its interactions with multiple parameters involved in the process (IL-1β, IL-6, IL-13, ROS, HSP70 and others). Moreover, some light was shed on vitamin B17, autologous fat, and metformin in diabetics and non-diabetics, however, a deeper knowledge of the molecular mechanisms underlying the overall positive outcomes from using vitamin B17 is still needed before making any final conclusion.

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