AMERICAN UNIVERSITY OF BEIRUT

AUTOLOGOUS FAT PROMOTES SECOND DEGREE BURN WOUND HEALING IN DIABETIC RATS: A MECHANISTIC APPROACH

by
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ABSTRACT
OF THE THESIS OF

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Title: Autologous fat promotes second degree burn wound healing in diabetic rats: A mechanistic approach

Introduction: Adipocytes have several roles in different tissues that go beyond simple energy storage to include metabolism regulation and growth. There is evidence that adipocytes have immunological and stem cells like properties when it comes to tissue regeneration and vascularization. In addition, transplanted fat improve facial and body proportions and the surrounding tissue as well as the quality of aging skin and scaring especially in damaged skin conditions.
In a study in drosophila, skin wound healing with fat cells transfer, it was observed that adipocytes have motility and migration properties which aid in wound repair and prevention of infection. Hence, this study was conducted, in order to gain a better understanding of the role of autologous fat tissue transferred in second degree burn wounds in diabetes.

Methods: A total of 80 adult male rats, 200-250g weight, were used according to IACUC guidelines. The interscapular region was selected as a site of burn and fat transplantation. Diabetes was induced by a 60mg/kg intravenous injection of Sterptozotocin (STZ). Glucose serum levels and body weights were monitored regularly. The burn was performed at 80°c for 40 sec and 4 animals were sacrificed on days 3-7-14-21-28 post-burn from each group.
The clinical status of the burn was checked on daily basis. Routine histology was performed as well as the levels of interleukins IL-6, IL-12, and IL-1**α** were determined by qRT-PCR.

Results: Redness around the rim of the burn was reduced in the presence of transferred fat in both diabetic and non-diabetic rats on days 3 and 7. Crust formation started at day 7 post-op in all subgroups. however, on day 14 the edges of the crusts started to peel off in the diabetics treated with fat as well as non-diabetic with fat alone to a more advanced degree. On day 21, the crust has fallen first in the groups having fat both diabetic and non-diabetic and the wound surface area got smaller than the controls. At the last time point, day 28, groups with fat healed better with a smaller surface area and hairs in most of the burned surface, almost 90% in non-diabetics,70% in diabetics, and 50% in the absence of fat in non-diabetics.
The histological studies showed a movement of the fat deep to the dermis with accumulation of myofibroblasts, inflammatory cells and collagen fibers, as well as earlier reepithelialization and early foundation of skin adnexa. The molecular analysis of IL-1α, IL-6, and IL-12 showed a distinct modulation of the interleukin levels in the

presence of fatty tissue by increase till day 14 then a decrease to day 28 corresponding to the progress in healing.

Conclusion: Our preliminary results have shown that fat transfer improved healing of the burned wounds, leading to a faster and improved wound recovery with reduced scaring, especially in the diabetic rat model. The mechanism was explored further with the completion of the molecular part of the study, which indicated control of inflammation in early stages with induction of more myofibroblastic activities leading to early contraction of the wound and improved scar.

Keywords: wound healing, burn wound, scar formation, autologous fat

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CHAPTER I

INTRODUCTION

Wound healing is a complex process where a multitude of cells cooperate in order to reestablish the integrity of the affected area or tissue. On the other hand, wound healing in diabetes needs special attention and care, as this process is particularly delayed due to the pathophysiology of the disease. Multiple approaches have been investigated and implemented to improve the burn wound healing in diabetes. However, the application of these methods might be hindered by the abnormal manifestations caused by diabetes. Therefore, the use of innovative practices is of fundamental importance in order to facilitate diabetic wound healing. Among these tactics, autologous fat transplant represents a promising path whose specific contribution has not been fully clarified.

This work is conceived in a way to clarify the exact role of autologous fat tissue in the burn wound healing process in rats. Multiple experimental processes are being implemented to elucidate the multiple facets of the burn wound healing process.

CHAPTER II

LITERATURE REVIEW

A. Burns, a worldwide burden

Burns are defined as a destructive injury of skin or other organic tissue. Their main etiology corresponds to exposure to harmful thermal elements, such as hot liquids or solids, fire, radiation, and electricity (Peck, 2011).

Burns pose a global public health issue as they affect around 11 million individuals per year around the world. Additionally, they are the cause of mortality of approximately 180 000 deaths annually (Greenhalgh, 2019).

Moreover, even when burns are non-fatal, they may impact the quality of life of affected people in various manners. The degree and extension of the burn wounds in addition to the healing and scarring process leave many with life-long disabilities (Brewin & Homer, 2018). On one hand, many burn survivors experience several psychological disturbances during their hospital stay and post discharge, amongst those disturbances we mainly note anxiety, depression, low self-confidence and self-esteem, and Post-Traumatic Stress Disorder (PTSD). All these could lead the survivors to withdraw from society and enter isolation (Gilboa, 2001; Taal & Faber, 1998). On the other hand, numerous survivors have to live with their scars, even with the best treatment scars are not completely avoidable. Some survivors may suffer from permanent damages to appearance and or function (Herndon, 2007).

Furthermore, they possess a devastating financial load on burn patients and their families. For example, in Norway, burn management performed in a hospital setting costed more than €10.5 million in 2007. Whereas, in South Africa, it is estimated that 26 million USD per annum are spent on burn care caused by paraffin cook stove accidents alone (World Health Organization, 2018).

Nonetheless, in the past few decades, there has been a significant increase in survival of burn patients with extensive and aggressive scarring, this is mainly attributed to the improved surgical and wound management techniques (Brewin & Homer, 2018).

Treatment modalities of burn wounds vary according to degree and depth of the burn injury, they range from simple application of topical creams to surgical interventions such as skin grafting.

One of the most ancient treatments of burn wounds is the application of dressings, in the intent of infection prevention, promotion of re-epithelialization, and preserving water at injury site (Chester & Papini, 2004). Examples of such dressings include, vaseline gauze, silicone sheets, Mepilex, Tegaderm, and hydrocolloids, as well as silver containing dressings (Khundkar et al., 2010; Silverstein et al., 2011; Wasiak et al., 2013).

More recently, advancement in bioengineering has enabled researchers to create dressings and gels whose composition is derived from nature, which can contain substances such as glycosaminoglycan and chitin (Ribeiro et al., 2009; Singh et al., 2008). This type of dressing has been shown to enhance fibroblast proliferation and angiogenesis while also inhibiting the growth of microorganisms (Dai et al., 2009, 2011; Do Nascimento et al., 2009).

Burned skin regions are highly susceptible to infections by various microbes such as, viruses, fungi, as well as gram-positive and gram-negative bacteria. The use of systemic antimicrobials offers an adequate solution to the infections, however, their administration needs to be carefully considered in order to prevent the emergence of resistant organisms (H. F. Liu et al., 2017). Consequently the development of topical antimicrobials designed to specifically target burn wounds associated infections has been the focus of multiple studies (H. F. Liu et al., 2017).

With large total body surface areas (TBSA) involved in burns, the risk of hypovolemic shock increases. Hypovolemic shocks are life threatening, damages at cellular levels happen with the increase in transmembrane potential, leading to a buildup of intercellular sodium and water. Adequate fluid resuscitation done using the golden standards such as the Evans, Brooke and Parkland Formulas, which calculate the needed amount of fluids according to the patient's weight and TBSA, is vital to maintain proper end-organ perfusion and thus prevent the detrimental consequences of hypovolemic shock. The first 24-48 hours post-burn require continuous monitoring and adjustments in fluids administration according to clinical status (Baxter & Shires, 1968; EVANS et al., 1952; Parks et al., 1977; Pruitt et al., 1971; Reiss et al., 1953).

Partial-thickness and full-thickness burns require early excision and grafting of the affected areas, mainly at days 2 through 21 post-burn which are considered of utmost importance for the patient's general survival and outcome. Eschars and blisters if left untreated, no excision or openings done, could lead to further damage due to the ongoing inflammatory cascade. Also to note, the necrotic tissue is a perfect environment for opportunistic gram-positive bacteria to cause cellulitis and hence hinder the healing process by slowing it, causing physiological impairments, contractures and functional

deficits. Furthermore, studies have proven that early excision and grafting of the affected areas has lead to a decrease in the hospital length of stay and to a speedier recovery. It has also been noticed that a delay period (24 to 28 hours post-burn) before starting the process of wound excision and grafting, presented improved resuscitation and rectification of physiologic imbalances that enhanced results, in addition to offering time cells at the center of the wound to become necrotic or exhibit sustainability (viability) (Ahuja et al., 2016; Edmondson et al., 2018; Israel et al., 2017).

Another treatment option for wound scars that has been in development since the 1800s and has bloomed in the recent years with more implication and usage in cosmetic and reconstructive surgeries is fat grafting, also referred to as lipofilling. Adipose tissue is a type of connective tissue that is filled with different types of cells, which includes mesenchymal stem cells (MSCs). These fat MSCs possess as principal functions, regeneration (self-renewal) and the capability of differentiating into multiple specialized cell types (Marco Klinger et al., 2020). The availability and abundance of fat cells have made them particularly prevalent particularly in subcutaneous tissue or hypodermis. This topic is discussed in more details below.

B. Classification and staging of burns

Accurate assessment and time sensitive treatments have been proven to importantly diminish morbidity and mortality rates emanating from the burn injuries. To decide the most appropriate course of treatment to be adopted for each patient, properly identifying and staging the burn wound extent is vital. From a clinical point of assessment, the depth of burn wounds is examined based on visual appearance, extent of bleeding, capillary refill, and the amount of sensitivity still present in the burn area

(Papini, 2004). Based on that, burn wounds have been categorized differently over the time, there is an old categorization system and a new one, as seen and characterized in Figure 1(Abazari et al., 2020).

Partial thickness burn wounds progress over time, their depth can change. Some wounds that start as superficial partial or deep partial burn injuries may become deep partial or deep burns respectively, in a period between 2 and 4 days after sustaining the injury. Studies have shown that burn injury is a dynamic process, it all peaks around 3 days from injury (Gravante et al., 2006). This progression in the wounds in mainly attributed to the inflammation and necrosis that is happening in the zone of stasis. The zone of stasis is mostly found in mid to deep dermal burns, it is the area of vascular stasis and ischemia, and will be discussed further in the next section (Evers et al., 2010; Jackson, 1953).

Superficial and partial-superficial burn wounds require 1-2 weeks of healing in general, with basic interventions, such as antimicrobial creams application, topical analgesics if needed and monitoring of the injury itself for signs of healing or deterioration in state. Whereas deep partial to full-thickness burn wounds would require invasive surgical interventions for possible excision of tissue and skin grafting (Rangaraju et al., 2019).

In our experimental model the burn reached the dermis with loss of epidermis and superficial part of the dermis reaching to deep part of dermis. It is classified as deep partial according to various criteria in the literature.

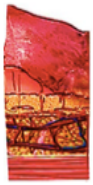
New system (old system)	Superficial (first degree)	Superficial partial-thickness (second degree)	Deep partial-thickness (second degree)	Full-thickness (third degree)	Full-thickness (fourth degree)
Etiology	Ultraviolet light, very short flash (flame exposure)	Scald (spill or splash), short flash	Scald (spill), flame, oil, grease	Scald (immersion), flame, steam, oil, grease, chemical, high-voltage electricity	Cause as for deep partial-thickness burns
Histology	Epidermis only	Epidermis and papillary dermis, skin appendages intact	Epidermis and reticular dermis, most skin appendages destroyed	Epidermis and dermis; all skin appendages destroyed	Involves fascia and muscle and/or bone
Clinical features (appearance)	Erythema, dry and pink or red; blanches with pressure	Erythema, blisters; moist, red and weeping; blanches with pressure	Blisters (easily unroofed); wet or waxy dry; variable color (patchy to cheesy white to red); does not blanch with pressure	Waxy white to leathery gray to charred and black; dry and inelastic; does not blanch with pressure	Black (dry, dull, and charred)
Sensation	Painful	Painful to air and temperature	Perceptive of pressure only	Deep pressure only (insensate)	Deep pressure only (insensate)
Healing time	3 to 6 days	7 to 20 days	More than 21 days	Never (no spontaneous healing)	Never (no spontaneous healing)
Scarring	None	Unusual; potential pigmentary changes, moist, elastic	Severe (hypertrophic) risk of contracture	Very severe risk of contracture, eschar tissue (hard and inelastic)	Eschar tissue (hard and inelastic)
Treatment	Cleaning, cooling with running water or a cold compress, return of full function	Cleaning; cooling with running water or a cold compress, topical agent; sterile dressing, the return of full function	Cleaning; topical agent; sterile dressing; possible surgical excision and grafting; earlier return of function with surgery	As for deep partial-thickness burns plus surgical excision and grafting at the earliest possible time, functional limitation more common if not grafted	Healing requires surgical intervention, functional impairment
Complication	Increase the risk of skin cancer	Local infection, cellulites	Possible skin grafting	Possible amputation	Amputation, gangrene, death
Schematic appearance					

Figure 1 – The Old and New system classification of Burn wound based on Depth (Abazari et al., 2020).

C. Healing

It is well established that when the body sustains an injury, normally, the process of lesion healing starts immediately. It is a complex and dynamic process whereby multiple elements work in an orchestral manner in order to achieve fast recovery and regain ordinary function. It is conceived that wound healing consists of 4 phases which are highly interconnected and might even overlap at times. These phases in chronological order are: hemostasis, inflammation, proliferation, and remodeling, as observed in Figure 2. Each phase is characterized by the predominance of specific cell types, cytokines, growth factors, and extra cellular factors (Kirsner & Eaglstein, 1993;

Oryan et al., 2017; Weaver et al., 2018). The complete healing of injuries relies highly on the correct sequence of the phases mentioned above, their occurrence at the accurate timings and their completion/continuation at an optimal intensity as illustrated in figure 2 (Abazari et al., 2020).

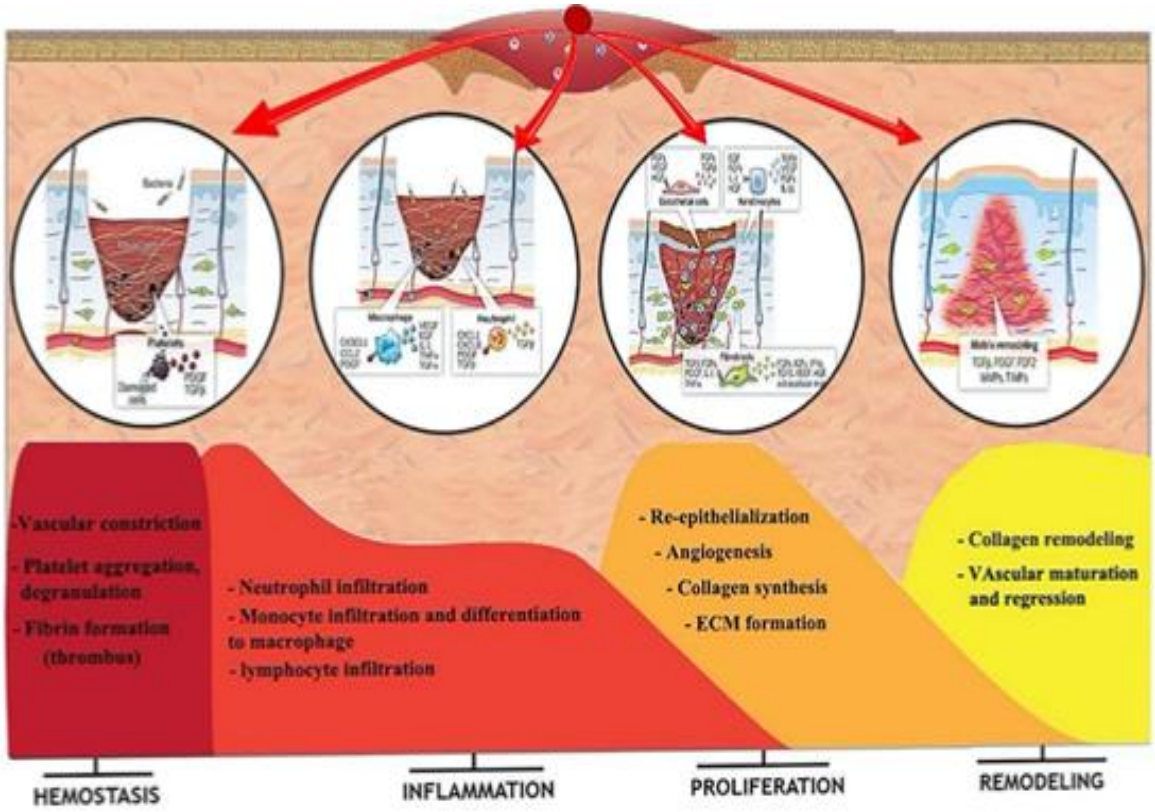


Figure 2 – General process for wound healing with the different processes shown in text for burn wounds (Abazari et al., 2020).

1. Hemostasis

Hemostasis is the first phase of the healing process, it is the is shortest phase and denotes to the mechanisms that ultimately halt the hemorrhage. It starts instantly after the injury and it is also referred to as the pro-inflammatory phase (Abazari et al., 2020).

Vasoconstriction is the first reaction in this phase, it is directed by the release of thromboxane A2 and prostaglandin 2-α from tissue injury to the wound bed which leads to vasoconstriction of small vessels that exist in the wound to constrict resulting in vasoconstriction. The vasoconstriction portion of this phase has an approximate duration of 5 to 10 minutes, it is followed by the vasodilation portion that has its strongest effect around 20 minutes after the injury. Platelets along with other cells released factors result in the vasodilatory effect such as: epidermal growth factor (EGF), fibronectin, histamine, and platelet-derived growth factor (PDGF) (Broughton et al., 2006; Foncerrada, Guillermo; Capek, Karel D; Herndon, David N; Lee, Jong O ; Sirvent, Ramon Zapata; Finnerty, 2017; Landén et al., 2016).

When subendothelial collagen is exposed to platelets, it leads to platelet aggregation, degranulation and eventually the initiation of the coagulation cascade. Ultimately the degranulation process of platelets results in activation of the complement cascade. All these steps transpire in the activation of the clotting cascade which in turn causes the platelets to gather together and adhere to connective tissue resulting in the fibrin clotting formation also known as thrombus (Broughton et al., 2006; Foncerrada, Guillermo; Capek, Karel D; Herndon, David N; Lee, Jong O ; Sirvent, Ramon Zapata; Finnerty, 2017; Landén et al., 2016).

The Hemostasis phase is not orthodox when it comes to burn wounds, since no real bleeding happens; therefore, there is no need to stop it. Nonetheless, in burn wounds, instantly post-injury, capillary permeability considerably increases, this effect is most likely attributed to the local release of histamine, and thus micro vessels lose their capacity to retain fluids away from the interstitial area (Friedl et al., 1989). The loss of fluids and proteins are typically severe and result in the ionic modification of the cells

(ischemia) (Kamolz et al., 2012; Tiwari, 2012). These changes lead to systemic hypotension which in turn causes end-organ hypoperfusion (Abazari et al., 2020).

Of course the gravity of these consequences and symptoms depends on the type, location, depth and severity of the burn wounds and the burn zones. Those were first described by Jackson in 1959 and are still referenced to in current literature (Hettiaratchy & Dziewulski, 2004; Kamolz et al., 2012).

Jackson in his research has classified the local changes in burn wounds, into three zones as depicted in figure 3 (Jackson, 1953). The central zone, named zone of coagulation, at the center of wound, mainly consisting of devitalized tissue, complete coagulative necrosis with irreversible tissue loss. The third zone, outermost area, the zone of hyperemia, where vasodilation and inflammatory changes occur, tissue in this zone recovers completely except if there were infections or severe hypoperfusion complications that occurred. The region around the zone of coagulation, in between both zones mentioned above, is the zone of stasis. This zone, is of extreme clinical importance, as it is the area that has the prospective of healing or the contrary, the potential of progressing to a full thickness lesion. The best chance of rescuing this region, is by achieving revascularization within merely a few days, to avoid irreversible tissue death. If this procedure is delayed, there is a significant risk of reperfusion injury, where intracellularly accumulated biproducts of ischemia are converted into toxic reactive oxygen species (ROS), upon re-exposure to high amounts to oxygen (Collard & Gelman, 2001). The resulting oxidative stress can cause detrimental effects in the affected area, mainly the spread of tissue damage due to apoptotic cell death (Jackson, 1953).This phenomenon is seen post major burns of partial thickness degrees. Some studies have

found that inhibition of inducible NO synthase in partial thickness burn wounds has an effect in reducing the rate of apoptosis (Evers et al., 2010).

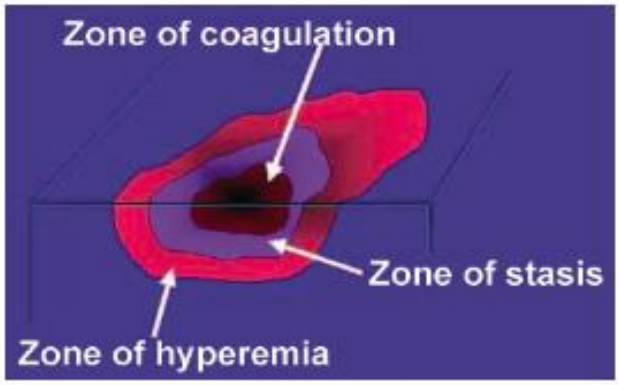


Figure 3 – The three zones in burn wounds as described by Jackson (Jackson, 1953).

2. Inflammation

Following the hemostasis phase, inflammatory cells migrate to the wound site, this is facilitated by the increased vascular permeability that is mainly due to local histamine release as mentioned above. This phase, known as inflammation, starts directly post infliction of the injury; its general duration is between 1 to 4 days. Although this stage is crucial in wound healing, its exaggeration and prolongation could lead to the arrest of proper regeneration (Landén et al., 2016; Nguyen et al., 2016).

Alterations in this process might occur from the presence of foreign organisms in the wound. Such event typically leads to the activation of the C3 complement cascade which eventually amounts to bacterial lysis. Moreover, more local inflammatory mediators such as cytokines are continuously released at the wound site, where they function as chemoattractant to neutrophils, which in turn act as phagocytes for present pathogens (Broughton et al., 2006; Landén et al., 2016; Sinno & Prakash, 2013). Macrophages which arise from monocytes that are stimulated by fragments of the extracellular matrix (ECM) protein, secrete growth factors in addition to enzymes and

cytokines, that have a main role of promoting bacterial killing and phagocytosis. Additionally, macrophages assume the major function of production of EGF and PDGF, which in turn begin the formation of granulation tissue, hence initiating the proliferative and regeneration phases (Abazari et al., 2020).

During later stages of the inflammatory phase, lymphocytes infiltrate the wound site where they might reside even during the remodeling phase. Both cytotoxic and helper T-cells have been proved fundamental in the wound healing process as they produce a plethora of lymphokines linked to the success of wound healing, with particular effects on cellular subsets being in charge of fibroblast replication and collagen synthesis (Agren, 2016; Fishel et al., 1987).

The above mentioned process is not identically followed in burn wounds, whereby histamine acts as a vasoconstrictor consequently increasing blood pressure and vascular permeability at injury site. This leads to decrease in intravascular fluids which in turn could cause hypovolemic shock. Additionally, histamine could activate the kinin system which in turn stimulates the kallikrein-bradykinin system, while also triggering a multiple cascades, mainly to note the complement cascade, coagulation fibrinolytic cascade, and the arachidonic acid cascade (Foncerrada, Guillermo; Capek, Karel D; Herndon, David N; Lee, Jong O ; Sirvent, Ramon Zapata; Finnerty, 2017). The upregulation of metabolites of the latter cascade, specifically prostaglandins, prostacyclin, and thromboxane A2, help in the orchestration of local ischemia as well as edema formation and vasoconstriction. Whereas the instigation of the complement system permits it to control the function of polymorphonuclear leucocytes. Mast cells contribute to the secretion of chemoattractants which are directly responsible for the recruitment of leukocytes to the burn wound sites (Pastar et al., 2014; Rowan et al.,

2015). In addition, monocytes even change their role when it comes to burn wound, beside their main function as active macrophages, they secrete IL-1 and tumor necrosis factor-α (TNF-α). They also regulate the synthesis of other important factors, such as IL-6 and acute phase proteins (Pastar et al., 2014; Rowan et al., 2015).

3. Proliferation

Proliferation is the phase that follows inflammation, it starts at day 4 post injury and lasts till approximately 2 weeks (14 days) post injury. It continues with the beginning of the maturational phase also known as remodeling phase. In this phase, there is release of multiple types of cells that direct several migration and proliferation processes (detailed below), with the main aim of restoring the vascular network and granulation tissue formation. This phase is made possible by the capability of regeneration and healing of the epidermis, due to it being originated from the ectoderm (Lau et al., 2009).

First the keratinocytes present at the basal layer of the wound edge along with epithelial stem cells from local hair follicles and sweat glands, migrate to the surface of the wound where they differentiate into epithelial cells under the influence of multiple stimuli. Thereafter, these cells begin to divide and proliferate forming a granulation tissue that separates the wound from the surrounding environment. This process is known as reepithelialization. At a deeper level in the wound, fibroblasts proliferate and begin to secrete collagen which in turn becomes a scaffold for further fibroblasts proliferation and the subsequent ECM production (Tracy et al., 2016; M. Xue & Jackson, 2015).

A vital part in wound repair is the availability of oxygen and nutrients, this is made possible by proper blood supply which is guaranteed by formation of new blood vessels (angiogenesis). This event is initiated by the migration and proliferation of endothelial cells under the influence of multiple growth factors (Broughton et al., 2006; Sinno & Prakash, 2013).

Unlike the phases mentioned previously, this phase of the wound healing is almost the same for burn wounds when compared to other wound types. The reepithelization occurs within a few hours of the injury, this stage depends on the depth and severity of the burn injury. For example, in first degree burns where the basement membrane preserves its integrity, the cells undergo normal upward migration, thus a normal layer of the epidermis is restored within 2 to 3 days from injury due to its replenishment by intact progenitor epithelial cells. Conversely, in deeper burn injuries, where the basement membrane sustained damage, it is the cells from the appendages and the wound peripheries that migrate to insure reepithelialization of the wound. The restoration of connective tissue post reepithelialization takes place due to the proliferation of fibroblasts, which begin to deposit collagen. There is also an initiation of angiogenesis and fibrogenesis, which are integral components in the reconstruction of the dermis. Finally, the wound edges start to contract and continues to do so during the maturation phase, nevertheless scars and scabs could still be formed during this phase (Foncerrada, Guillermo; Capek, Karel D; Herndon, David N; Lee, Jong O ; Sirvent, Ramon Zapata; Finnerty, 2017; Hettiaratchy & Dziewulski, 2004; Landén et al., 2016).

This migration is the basic mechanism by which interstices meshed split-thickness skin grafts work, they are filled in by keratinocytes coming from the skin

bridges. The biological properties of the epidermis and its renewal characteristics are currently of large interest in research, with possibilities of translating this into technology that can be used in the clinical context, improving wound healing outcomes (Lau et al., 2009).

4. Maturation

The last phase of wound healing is called maturation or remodeling, and it can extend from months to years. The matrix becomes more organized due to fibroblast cells migrating out of the wound bed and the remodeling of collagen. Scar tissue arises from granular tissue which could happen two weeks after injury.

The increased cross linking between the collagen fibers leads to a rise in the mechanical tension at the level of the wound. Besides myofibroblasts that differentiate from fibroblasts under the influence of cytokines, begin to secrete and remodel ECM components through enzyme secretion. An example is the substitution of collagen III by collagen I, which leads to further enhancement of the tensile strength. The resulting cumulative tensile strength is responsible for wound contracture. Ultimately, when healing is complete, myofibroblasts undergo apoptosis (Sinno & Prakash, 2013). Angiogenesis at this stage declines, allowing the establishment of an avascular and acellular environment. The depth or wound severity controls the degree in which appendages are recovered, such as hair follicles and sweat glands in the skin, complete healing is not always achievable (Broughton et al., 2006; Darby et al., 2014; B. Li & Wang, 2011).

As Gabriel stated in his research in 2009, the primary molecular mechanisms directing the progression of the burn wound include, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and transforming growth factor-ß

(TGF-ß). In the first stage of wound healing TGF-ß is vital for the activation and proliferation of fibroblasts.

Prolonged activity of TGF-ß is connected with hypertrophic scars and wound contraction that lead to deformity. In Gabriel's experimental models, the procedure of using TGF-ß neutralizing antibodies showed a decline in scar development without undesirably disturbing wound healing (Gabriel, 2009).

However, depth and severity are still important determinants of the outcomes and the progression of remodeling, contraction and scar formation. In superficial burns, for example, hyperpigmentation due to overreaction of melanocytes is observed, whereas hypopigmentation is seen in deeper wounds due to the death of melanocytes. Furthermore, in deep burns it is established that remodeling takes more time, while skin contraction and scar formation are more probable (Carr-Collins, 1992; De Chalain et al., 1998; Foncerrada, Guillermo; Capek, Karel D; Herndon, David N; Lee, Jong O ; Sirvent, Ramon Zapata; Finnerty, 2017; Greenhalgh, 2015; Grover & Morgan, 1996; Landén et al., 2016; Tiwari, 2012).

D. Involvement of mast cells in wound healing

Mast cells have hematopoietic precursors which migrate into tissues and differentiate into their mature forms characterized by numerous electrodense cytoplasmic granules. They are found distributed throughout the body with concentration at the level of the dermis and the epithelial lamina propria. They are typically present in the epidermis under pathological conditions (Artuc et al., 1999; WEBER et al., 1995).

The granules contained inside mast cells house a variety of molecules, which can be released upon stimulation from the microenvironment that surrounds the cell. Among these chemicals lie important messengers that partake in wound healing, such as cytokines, chemokines, growth factors and histamine. These mediators might be responsible for multiple steps that occur during wound healing, such as vasodilation and influx of inflammatory cells (Czarnetzki et al., 1995; Möller et al., 1998; Rumsaeng et al., 1997). Besides, these granules might be phagocytized by fibroblasts and endothelial cells, leading to the continuously increased levels of histamine at the site of injury, which in turn results in the effects of histamine described above (Seibold et al., 1990).

Moreover, mast cells participate in the formation of granulation tissue, alongside fibroblasts and macrophages (Bolton & Montagna, 1993), where they might enhance the release of interleukins, namely IL 1 (Bechtel et al., 1996). Under the influence of this cytokine and many others, mast cells synthesize and secrete nerve growth factor (NGF) (Di Marco et al., 1991). This pleiotropic factor is a chemoattractant to neutrophils and an enhancer to their phagocytosis (Kawamoto et al., 1995). It stimulates nerve ends to secrete substances such as the neuropeptide VIP (vasoactive intestinal peptide), which in turn acts as mitogen for keratinocytes (Wilkinson et al., 1994).

On the other hand, mast cells located at proximity to capillaries may in fact contribute to the improvement of angiogenesis, by releasing angiogenic factors like bFGF (basic Fibroblast Growth Factor) and TGF-β (Transforming Growth Factor) and VEGF (Vascular endothelial growth factor) (Grützkau et al., 1998; NILSSON et al., 1995; Qu et al., 1995). Furthermore, mast cells have been correlated with the maintenance of the long lasting integrity of vascular endothelium (Watanabe et al.,

1997). Additionally, mast cells could release proteases which are involved in regulating the process of angiogenesis (Gruber et al., 1989).

The mast cells proteases might have other roles in wound healing. For example, tryptase could induce fibroblast mitosis, thus empowering collagen synthesis and secretion, while also modifying extracellular matrix components (F. Levi-Schaffer & Rubinchik, 1995; Francesca Levi-Schaffer & Kupietzky, 1990; Ruoss et al., 1991; Schwartz, 1990). However, this enzyme might also inhibit the proliferation of keratinocytes (Harvima et al., 1994; NADEL, 1991). Another instance is chymase, which can enhance the formation of angiotensin II, an inducer of keratinocyte proliferation (Muscha Steckelings et al., 1996; Sanker et al., 1997).

E. Important roles of selected interleukins

As mentioned before, a plethora of cytokines, chemokines and growth factors interplay in the burn wound healing process. In this review we selected specifically IL-12, IL-6 and IL-1α.

IL-12 is considered a pro-inflammatory interleukin that enhances the differentiation of naïve T-cells into T effector cells (Matias et al., 2011; Trinchieri et al., 2003). It has also been shown to have effect in neutrophil recruitment into the wound bed (Gillitzer, 2001), and possesses an anti-angiogenic activity, through the increase of interferon gamma (IFN-g) secretion (Voest et al., 1995). On the other hand, its absence could achieve an accelerated reepithelization process as its roles in regulating early inflammation responses and angiogenesis would be eliminated (Matias et al., 2011).

IL-6 is also considered as a pro-inflammatory interleukin, it acts as a chemoattractant substance to neutrophils, and has powerful mitosis inducing effects on

keratinocytes (Werner & Grose, 2003a). Several studies have shown that IL-6 has a particularly important role in the early phase of inflammation, it is suggested that IL-6 may be a regulator of leukocyte recruitment to the inflammatory sites (Akira & Kishimoto, 1992). In addition, there is evidence that it also has a crucial role in angiogenesis via inducing VEGF expression. It also enhances collagen deposition at wound sites, by inducing collagen production and/or procollagen gene expression in multiple types of cells including dermal fibroblasts, making IL-6 an important factor in remodeling not only in inflammation (Duncan & Berman, 1991; Greenwel et al., 1993; Lin et al., 2003). Moreover the lack of IL-6 may lead to the delay of re-epithelization indirectly through a decrease in TGF-1β (Lin et al., 2003). Furthermore IL-6 upregulates the expression of another fundamental interleukin implicated in wound healing, which is IL-1.

The latter regulates the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICMA-1), important regulators of leukocyte extravagation (Lin et al., 2003). This pro-inflammatory cytokine instigates the migration and proliferation of keratinocytes, while also stimulating the production of fibroblast growth factors (Werner & Grose, 2003a). IL-1 and IL-6 are irreplaceable in wound healing, as their absence would lead to a slower healing process (Eming et al., 2007; Ishida et al., 2004).

F. Diabetes

In the past 25 years, there have been many advances in the research about diabetes, which have led to better understanding of the disease, its pathophysiology, and its increasing complications. In the past, diabetes type 1 was sought to be only a

juvenile disorder, affecting children and adolescents, new studies have shown that diabetes type 1 cases occur with up to 50% in adulthood (Thomas et al., 2018).

Diagnosing diabetes type 1 in children and adolescents relies primarily on the classic trio symptoms, polydipsia, polyphagia and polyuria, in addition to overt hyperglycemia. However these diagnostic hallmarks are less reliable in the adult cases. Another trademark of this disease is the immediate need for exogenous insulin substitution, this treatment is life lasting and could be lifesaving (Atkinson et al., 2014).

Diabetes type 1 was once considered to be a single autoimmune disorder, which rises from T-cell mediated attacks on the insulin-producing β cells, now it is contemplated as being the outcome of intricate interactions among environmental factors and microbiome, genome, metabolism, and the immune system that differ amid singular cases (Dimeglio et al., 2018).

Even though there are genetic predispositions and underpinnings for diabetes, a person's DNA sequence dose not dictate that they must end up with diabetes, even when having the utmost risk combination of HLA (human leukocyte antigen) alleles, which has made primary prevention of this disease quite difficult (Dimeglio et al., 2018).

Diabetes is now known as a multisystem disorder, the complications that arise from it cause physiological changes at the level of cells and tissues, these ultimately lead to an impaired wound healing process. This interaction is quite complex, and little is known about it. It is obvious that diabetic wounds become arrested in the inflammatory phase of the healing process, which is described extensively previously. It is important to note that the continuous influx of neutrophils and inflammatory mediators produce large collateral damage to the surrounding wound tissue. Wound

healing is delayed primarily due to the disturbance in the healing process, where the overproduction of free radicals stimulates oxidative stress to the tissue (Dissemond et al., 2002; Soneja et al., 2005; V, 2000). Besides, diabetic wound healing is characterized by decreased angiogenesis, due to a decline in the number of endothelial progenitor cells (EPC) (Loomans et al., 2004; Tepper et al., 2002). (98)

Therefore, to improve wound healing in diabetics, the introduction of anti-inflammatory agents might be beneficial as it could halt the constant inflammation process (Dissemond et al., 2002). On the other hand, diabetes is marked by a remarkable decrease in the growth factors secreted at wound site such as VEGF (Frank et al., 1995), which have a main role in stimulating keratinocyte proliferation and correct wound closure (Pastore et al., 2008; Peplow & Chatterjee, 2013; Werner & Grose, 2003b). Finally, a decrease in collagen formation is observed accompanied by a delay in the process of change of fibroblast into myofibroblast (DiPietro & Burns, 2003).

Moreover, it has been shown that neutrophil functions are impaired in diabetic patients. As these cells in collaboration with other polymorphonuclears are fundamental for the formation of an inflammatory band, and the secretion of an abundance of growth factors and cytokines, their hindrance results in dampening the healing process (Collison et al., 2002; Shah et al., 1983; Wetzler et al., 2000). This phenomenon has been correlated with an increase in the formation of AGE (advanced glycation end products) in diabetes. AGE acts as a ligand for its receptor RAGE, whose expression has been shown to be increased in diabetes, on the surface of neutrophils. Additionally the blockage of their interactions does in fact enhance wound healing in diabetic mice (Basta et al., 2002; Collison et al., 2002; Hudson et al., 2003; Nelson et al., 1975; Shah et al., 1983; Stern et al., 2002). Furthermore, a study has demonstrated that in diabetic

rats inflammatory cells failed to form the inflammation band at the site of the injury, even though these cells were accumulated all over the injury site. They also secreted elevated amounts of inflammatory cytokines, namely IL-1α and TNF-α (Tian et al., 2016). Their persistent high level can be closely related to the delay in granulation tissue formation (Roth et al., 2006; Wetzler et al., 2000). Other than PMNs, diabetes has also been correlated with a deviation of macrophage activation during wound healing, resulting in abnormal sequence of events (Miao et al., 2012).

G. Role of fat in wound healing

Burn injury management mechanisms have been newly focusing on the reduction in the inflammatory response in the initial phase of the injury and the acute influx of cytokines. Additionally, these new approaches try to regulate the fibroblast-myofibroblast activities, hence decreasing scar formation and wound contractures without hindering the wound healing and repair process (Holland et al., 2008). In order to accomplish these goals, stem cells have become a widespread option these days.

Stem cells have been already examined for their potency in skin regeneration and wound healing, however their use as a novel treatment approach for burn wounds is being currently investigated. Studies have found that collagen–glycosaminoglycan constructs planted with mesenchymal stem cells increase healing and keratinization, reduce wound contracture and improve vascularization. Therefore, pluripotent stem cells could be used in the development of skin replacements, specifically in burn wounds (P. Liu et al., 2008). Among these, mesenchymal stem cells (MSCs) have shown the capacity to enhance the repair process at cellular and matrix levels. They also may secrete chemicals that can regulate the activity of other cells (paracrine activity).

Human MSCs seem to be particularly potent in ameliorating wound healing (Rosique et al., 2015; Singer & Caplan, 2011).

Lipofilling, a practice described since the 1980s, is the use of fat transferred from one area of the body to another in cosmetic surgery procedures for body remodeling. This method increases the volume of the area to correct a contour deficit. Currently, its use has gone beyond only cosmetic surgery, it is in fact used for several clinical applications including wound healing (M Klinger et al., 2008).

In a study that used adipose tissue for remodeling of severe old burn scars, histological sections from punch biopsies after the fat injections showed remarkable patterns of new collagen deposition, and local hypervascularity along with dermal hyperplasia. In addition, the use of fat tissue had improved the formation of normal annexal structures, with better structural and architectural preservation of the tissue when compared to scar tissues from burns (M Klinger et al., 2008).

Moreover, in a study in drosophila concerning skin wound healing with fat cells transfer, it was observed that adipocytes have motility and migration properties which aid in wound repair and prevention of infection (Franz et al., 2018).

Some studies have also focused on the use of lipostructure technique to treat chronic vascular sores and radiodermatitis. A positive outcome was seen in the target area (Rigotti et al., 2007).

This recent exploration of fat in multiple medical fields is due to its microstructural properties. Adipose tissue which has an extracellular matrix containing collagens, laminin, fibronectin and growth factors along with its cellular components are being considered as stem cells sources. Besides, adipose stem cells have shown the capacity to differentiate not only into fat cells, but also to cartilage, bone, muscle, and

even nerve. Therefore, these cells are multipotent, capable of regeneration and responsive to gene therapy Furthermore, their collection is performed in a less aggressive way than their bone marrow counterparts (P. A. Zuk et al., 2001; Patricia A. Zuk et al., 2002).

What is particularly attractive about fat cells is that they are abundant in the body, expendable and can very easily be harvested in a safe technique with minimal complications (liposuction), which yields a high number of cells (De Ugarte et al., 2003; Tholpady et al., 2006).

CHAPTER III

AIM

This study was conducted, in order to gain a better understanding of the role of autologous fat tissue transfer in burn wounds in diabetic rat model. As fat contains pluripotent stem cells, their mobilization and differentiation into cell types that participate in wound healing, might enhance and accelerate this process. Therefore, we aimed to identify the effects of untreated autologous fat transplant at different stages of the burn wound healing. We explored its role on the morphology of the wound and then, we tried to unravel the effects of the applied treatment at histological level, with focus on reepithelization, movement of the fat cells and the presence of mast cells at the site of injury. Afterward, we dove into the molecular changes taking place with specific focus on some interleukins that are typically involved in wound healing, IL-6, IL-12, and IL-1α.

CHAPTER IV

MATERIALS & METHODS

The experimental design included both in-vivo and in-vitro methodologies.

A. Animal work

A total of 80 Sprague-Dawley adult male rats,200-250g weight, were used according to the guidelines of the Institutional Animal Care and Use Committee of the American University of Beirut (IACUC). The rats were kept in a controlled environment in the animal care facility with a day night cycle 12/12 and free quantities food and water.

The rats were divided into 4 groups, 20 rats in group 1 diabetic rats with burn only, 20 rats group 2 diabetic rats with burn and autologous adipocyte tissue (Fat) transposition, 20 rats in group 3 non diabetic rats with burn only, 20 rats in group 4 non diabetic rats with burn and autologous fat transposition.

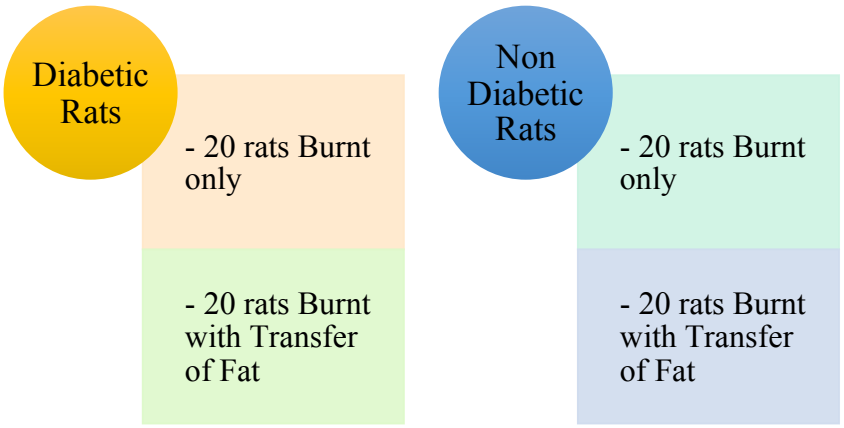


Figure 4 – Diagram of the experimental design.

Once rats were received at the animal care facility grouping was done according to approximate weights, 4 rats were placed in the same cage at first and marking for

differentiation was done for all the rats, along with recording of initial weights and blood glucose levels.

B. Diabetes Type I induction

Once the rats reached the desired weights between 200 and 250 gr the Sterptozotocin (STZ) injections for the diabetic group started. Diabetes was induced, by a 60mg/kg intravenous (in tail vein) injection of STZ once, within 3 days of injections. Blood glucose levels were measured at days 3 and 7 post injections to confirm Diabetes induction. Some of the rats did not become diabetic from the first injection of STZ, they thus required a second injection with the same dosage to induce Diabetes at day 7. Additionally, glucose serum levels and body weights were monitored for all rats at all set time points of the experiment.

C. Burn and Surgery

Operations were performed between day 7 and day 10 post injections. The interscapular region was selected as a site of burn and fat transplantation, since it is protected, unreachable from the rats themselves.

Anesthesia was performed by intraperitoneal injections of a mixture of Ketamine with a dose of 80mg/kg and Xylazine with a dose of 10 mg/kg. The operation was done in a clean environment with sterilized surgical equipment.

All animals were shaved for the procedures, on the dorsal side (animal's back) the intrascapular region was shaved using a commercial electric shaving machine, this was done post induction of sedation. Only the animals that underwent the fat transfer

operation had been shaved on their ventral side as well, as a source for the autologous fat.

A second degree burn was done (deep partial-thickness wound) was performed at 80°c for 2 mins to achieve second degree burns for all the rats in all the groups alike, using an ordinary soldering iron (20W) retrofitted with 2.5 cm diameter aluminum stamp.

As for the rats in the fat transfer groups, harvesting of autologous fat was done through an incision of 0.5 cm in the right lower quadrant of the animal's abdomen, once a sample of fat of size at least 0.5 cm width and length was harvested it was placed in a dish with 0.9% NSS sterile solution until it was transplanted in the animal's interscapular region deep to the burn wound. Then the incision in animal's abdomen was sutured. An incision was done at the edge of the burn wounds and the fat was placed deep to the wound. Suturing of the incision was done after that. For the groups of Animals that received fat transposition the burning and operation were done consecutively on the same day. Both incisions on dorsal and ventral were sutured using 3-0 Monofilament Nylon C-14.

Four animals from each of the different four groups were sacrificed on each of the experiment's time points post operatively, days 3-7-14-21-28 post-burn.

Blood glucose levels, Weight, and pictures with measurements of the wounds were done for all the animals at the procedure day and at the 5 time points of the experiment including the animals that were sacrificed on each of the time points. The clinical status of the burn was checked on daily basis.

Clinical monitoring was done by observing the rats feeding and water drinking habits, in addition to wound progression. Wound size was measured at each time point with a cm ruler, and the surface area was calculated by multiplying width by length.

Sacrifice was done by inhaled anesthesia, through a continuous flow of Isoflurane to the rats. Once the samples from the wounds were taken while the rats were still under effect of the anesthesia, cervical dislocation was done.

On the days of sacrifice the wound was excised into 4 different sections, the first section was placed in a cassette and into formaldehyde, for sectioning and staining. The second and third sections were placed in Eppendorf tubes and directly put into liquid nitrogen later on transferred to the -80 °C Fridge, these sections were destined for PCR and Western Blot analysis. And the fourth section was placed in aluminum foil paper and put into liquid nitrogen then was also placed in the -80 fridge, this section was later destined to be used for ROS.

D. Histological studies

Biopsies from wounds destined for routine histology were fixed in 10% formalin and embedded in paraffin, sectioned into 5 μm thick sections. 6 slides were taken from every sample, with 2 sections per slide. Each 2 slides received a different type of stain: either Hematoxylin-Eosin (H&E), or Toluidine Blue (TB), or Periodic Acid-Schiff (PAS).

H&E slides were used to better understand at a tissue level the progression of the healing process. Images of selected fields were taken of the slides by Olympus E330 camera connected to a CX41RF Olympus light microscope. Mast cells count was done

on the histological slides stained with TB, using Olympus light microscope, at magnification 400x.

E. Molecular studies

1. Protein extraction and quantification

For protein extraction, the frozen samples were crushed and transferred to a tube containing 980 microliters of Laemmli buffer with 10 microliters Protein inhibitor and 10 microliters Phosphatase inhibitor. The samples were then centrifuged at 13000 rpm at 4 degrees Celsius for 15 mins, and then the supernatant was transferred to a new tube and placed at -80 °C.

Proteins concentrations were determined using LOWRY assay (Bio-Rad DC Protein Assay Reagents A (#500-0113), B (#500-0799) and S (#500-0115)) using a BSA/Bovine Serum Albumin (Amresco #9048-46-8) gradient as a standard.

2. Quantitative real-time polymerase chain reaction (PCR)

For RNA extraction, the frozen samples were crushed and transferred to a tube containing 1 ml of Trizol (Sigma-Aldrich). After mixing the samples well with the Trizol, 250 microliters of Choloroform were added to each tube. The contents of the tube were then vortexed and centrifuged at 1200 G at 4 degrees Celsius for 15 mins. Supernatant was removed and placed in new tubes, then 500 microliters of Isopropanol were added, mixed gently by inversion, and placed in freezer at -20 degree Celsius for 10-15 mins. Then they get centrifuged again at 1200 G at 4 degrees C for 15 mins. The supernatant was then discarded and palette was kept and washed twice by 75% ethanol with a volume of 750 microliters.

The washes were performed as follows: after adding ethanol samples were inverted gently and centrifuged at 7.5 G for 10 mins at 4 degrees C, supernatants were discarded and the pellets were kept to air dry a few mins, then 20 microliters of RNAse free water were added.

Complementary DNA (cDNA) was synthesized using QuantiTect Reverse Transcription Kit (Qiagen # 205311) with a final volume of 20 microliters. The c-DNA was then diluted with a dilution factor of 10.

Quantitative real-time Polymerase Chain Reaction (PCR) was performed in a 96 well plate. In each well, 2 microliters of c-DNA were placed and 8 microliters of the mix corresponding to the desired gene were added. The plate was then placed in Real-Time Bio-Rad CFX96.

The relative gene expression was normalized against the expression of a housekeeping gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). The relative gene expression was then normalized compared to the control and expressed as delta delta CT.

- IL-1α mix:
    - 0.5 microliters of IL-1α Forward
    - 0.5 microliters of IL-1α Reverse
    - 5 microliters Sybr green
    - 2 microliters RNAse Free water
- IL-6 mix:
    - 0.5 microliters of IL-6Forward
    - 0.5 microliters of IL-6 Reverse
    - 5 microliters Sybr green
    - 2 microliters RNAse Free water
- IL-12 mix:
    - 0.5 microliters of IL-12 Forward
    - 0.5 microliters of IL-12 Reverse
    - 5 microliters Sybr green
    - 2 microliters RNAse Free water

Table 1 – Different pairs of primers used in the RT-qPCR

Gene Name	Sequence Forward Primer	Tm °C	Sequence Reverse Primer	Tm °C	Ta °C
GAPDH	5'- AGACAGCCGCATCTTCTTGT -3'	60	5'- CTTGCCGTGGGTAGAGTCAT-3'	60	60
IL-1 α	5'-AGGGAGTCAACTCATTGGCG-3'	59.9	5'-GGACAGTCGAGGAGCAAACA-3'	59.9	59.9
IL-12	5'- ATCATCAAACCGGACCCACC-3'	60	5'-CAGGAGTCAGGGTACTCCCA-3'	60	60
IL -6	5'-CTGGTCTTCTGGAGTTCCGTT -3'	60	5'- ATGAGAGATGGGGACGCACT -3'	60	60

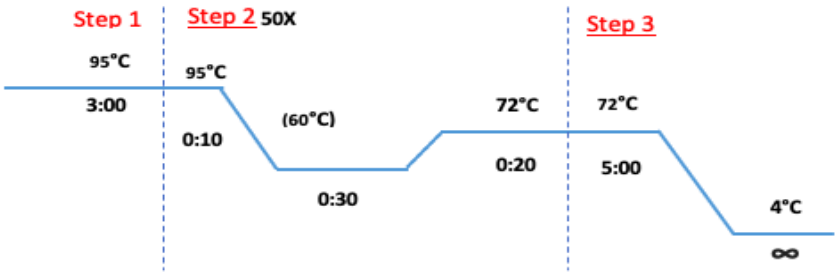


Figure 5 – Scheme of PCR conditions

F. Statistical analysis

Data were expressed as the mean ± SEM and analyzed with t-tests via the GraphPad Prism 7 software as described in the figures' legends.

Significance was recorded using: * for p value < 0.05, ** for p value < 0.01 and *** for p value < 0.001.

CHAPTER V

RESULTS

A. Effect of fat transfer on the wound area

To study the effect of fat transfer on the wound size, the surface area of each burn wound was calculated for each rat at each time point (days 3,7,14,21, and 28). The wound area of each group was compared to the control group Non-Diabetic Burn (ND B) by a t-test. Representative images of each group at different time points are seen in figures 6 to 9 and histogram 10.

As seen in figure 10, the wound areas were similar for all 4 studied groups at day 3. However, starting at day 7, differences could be observed. The wound area for the groups treated with fat both diabetic and non diabetic (D B+F and ND B+F) decreased significantly compared to the control (p-values < 0.001), regardless of the diabetic status of the rat. While, the wound area of the diabetic burn group (D B) in the absence of fat increased significantly compared to the control (p-value < 0.05), as seen in figure 10.

Progressing to day 14, the fat-treated groups (D B+F and ND B+F) continued to show significant decrease in wound area compared to the control (p-values < 0.001), as seen in figure 10. Moving to day 21, only the D B+F group showed significant decrease compared to the control, with a p-value < 0.05 (figure 15). Similar observations were made at day 28 (figure 18).

The D B group stopped showing any significant changes compared to the control from day 10 onwards, while the ND B+F group showed no significant changes compared to the control starting at day 21, meaning early improvement.

Therefore, the fat transfer appears to accelerate the wound contracture and healing process. Additionally, the fat transfer exerts its most observed effect on wound size day 14 and onwards. This observation could be noticed when comparing the wound area of each group to itself while taking the wound area at day 3 as a baseline (figures 6 to 10).

In brief, autologous fat decreased wound areas at all time points compared to absence of fat (figure 10).

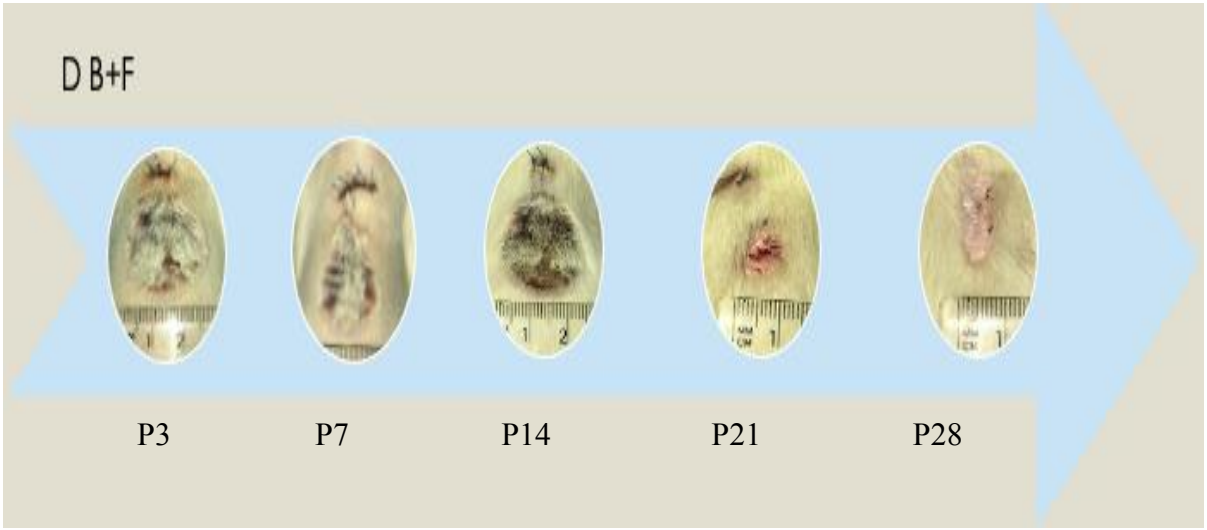


Figure 6 – Representative images of the wounds of the 4 rats of the group D B+F throughout the 5 time-points, starting from day 3 to day 28.

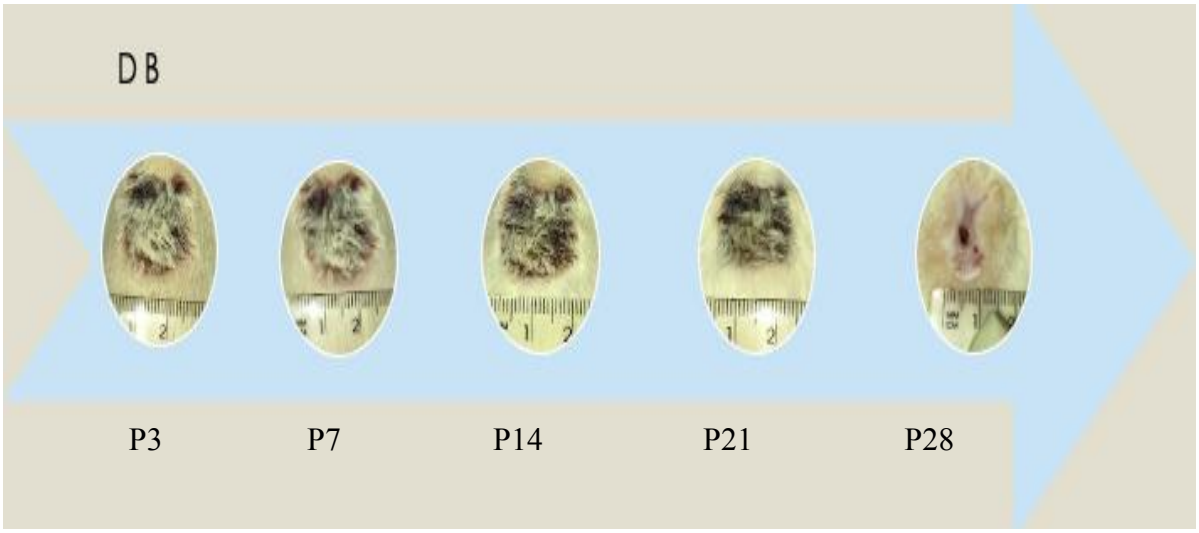


Figure 7 – Representative images of the wounds of the 4 rats of the group D B throughout the 5 time-points, starting from day 3 to day 28

Figure 8 – Representative images of the wounds of the 4 rats of the group ND B+F throughout the 5 time-points, starting from day 3 to day 28

Figure 9 – Representative images of the wounds of the 4 rats of the group ND B throughout the 5 time-points, starting from day 3 to day 28

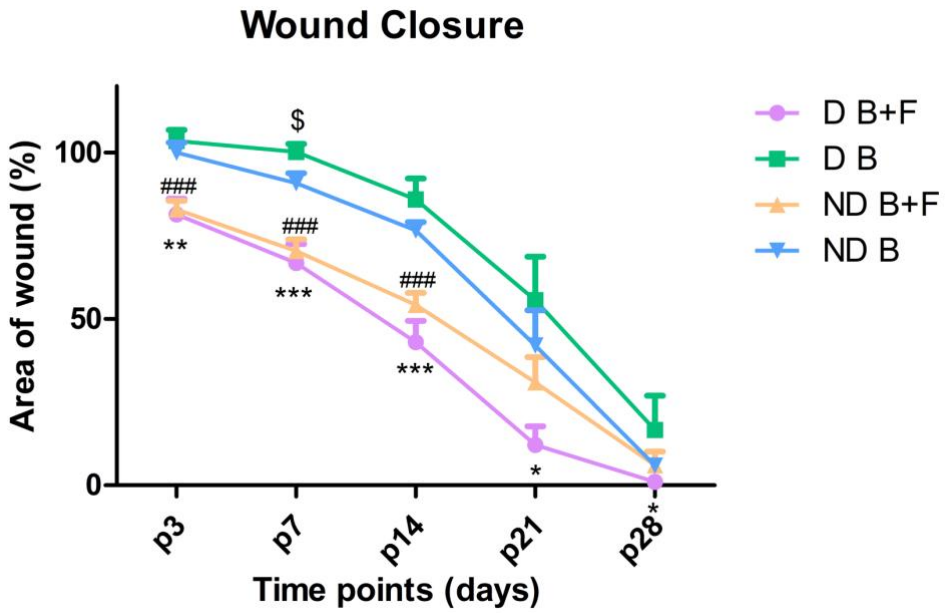


Figure 10 – Wound closure for all groups at different time points. Wound areas obtained from the 4 different sacrificed rats at each time point were calculated. Data were expressed as percentages of the mean of the ND B group at p3. At each time point, each group was compared to ND B of the same time point via t-test. For D B+F *** for $P < 0.001$. ** for $P < 0.01$. For ND B+F ### for $P < 0.001$. For D B $ for $P < 0.05$.

B. Effect of fat transfer on the wound healing at the histological level

To study the effect of fat transfer on wound healing at the tissue level, the H&E-stained and TB- stained histological sections obtained at different time points were

studied thoroughly. Representative images of each group at different time points are seen in figure 11.

At day 3 post operatively (post-op), all groups showed almost a similar histological state of healing. One could clearly see the burnt area with inflammation and disorganized tissue. However, the groups that include fat transfers (D B+F and ND B+F) showed fat cells migration throughout the tissue and less inflammation reaction.

At day 7 post-op, groups D B+F, ND B and ND B+F all show the beginning of formation of an epithelial lining at the edges. The ND B group is also characterized by the existence of more granulomatous tissue, which are masses of immune cells and other tissue (necrotic) that form at sites of infection or inflammation. The ND B+F group also displayed formation of blood vessels, which entails vascularization of the area, that is part of the healing process. Inflammation was well noted especially at the site where the fat had been placed. The transferred fat cells now have lost their fat lobules and are behaving differently, this could be due to them losing their direct blood supply due to the surgical procedure. We found in that area mostly aggregated nucleated cells, showing dark nuclei, arranged next to each other; these could be helping in the healing process through secreting various products. Whereas in the D B group, the burnt skin was still prominent, no growth signs were noted, no epithelial lining beginning its formation was seen, and no skin appendages could be observed. However, the inflammatory process is obvious in the dermis/hypodermis area with granulation tissue and fibroblast.

At day 14 post-op, groups D B+F, ND B+F and ND B all displayed the formation of more than 2 layers of epithelial cells lining, with some blood vessels and hair bulb formations. In addition, the D B+F group demonstrated hair follicles and skin

appendages which were notably formed myofibroblasts were increased. The transferred fat cells have changed form, and lost their lobules. There was an abundance of inflammatory cells in that area with an ongoing inflammatory reaction where the fat cells were placed. While the D B group exhibited disorganized epithelial lining beginning to form, with some primordial skin appendages. Inflammation was also well noted. Mast cells degranulating or ghost cells and blood vessels could be seen with some blood cells formation. Collagen fibers were also present in an unorganized manner along with some myofibroblasts.

At day 21 post-op, all groups demonstrate multiple epithelial layers formed. The D B+F group had in addition well-formed sebaceous glands with their respective ducts. Hair follicles and hair shafts were present. There was no visible scar tissue. There was also an abundance of collagen fibers that are aiding in the healing process. While the ND B+F group still had some slight residue of scar tissue.

Besides, the ND B group displayed some more granulomatous tissue. Furthermore, the D B group still had scar tissue attached. There was also an abundance of collagen fibers, that were not well organized, noted with multiple inflammatory cells and on TB staining there were numerous mast cells and fibroblasts which probably helped in wound contraction and collagen production. These findings contribute to the assumption that healing is still ongoing at Day 21 in this group of animals, which can be labeled as relatively delayed or prolonged healing process taking place.

At day 28 post-op, groups D B+F, ND B+F and ND B, all had clear and well-formed skin layers and skin tissue, with all its appendages, hair follicles, and sebaceous glands. No more inflammatory processes were noted. Similarly, the D B group now had better organized tissue with less inflammation and better organized collagen fibers.

Nonetheless there was still ongoing a process of collagen formation whilst it was less prominent than at day 21 post-op.

Therefore, the fat transfer appeared to enhance and accelerate the wound healing process at cellular and tissue levels, with influences on the inflammatory process and epithelial layers formation as well as fibroblast differentiation and migration.

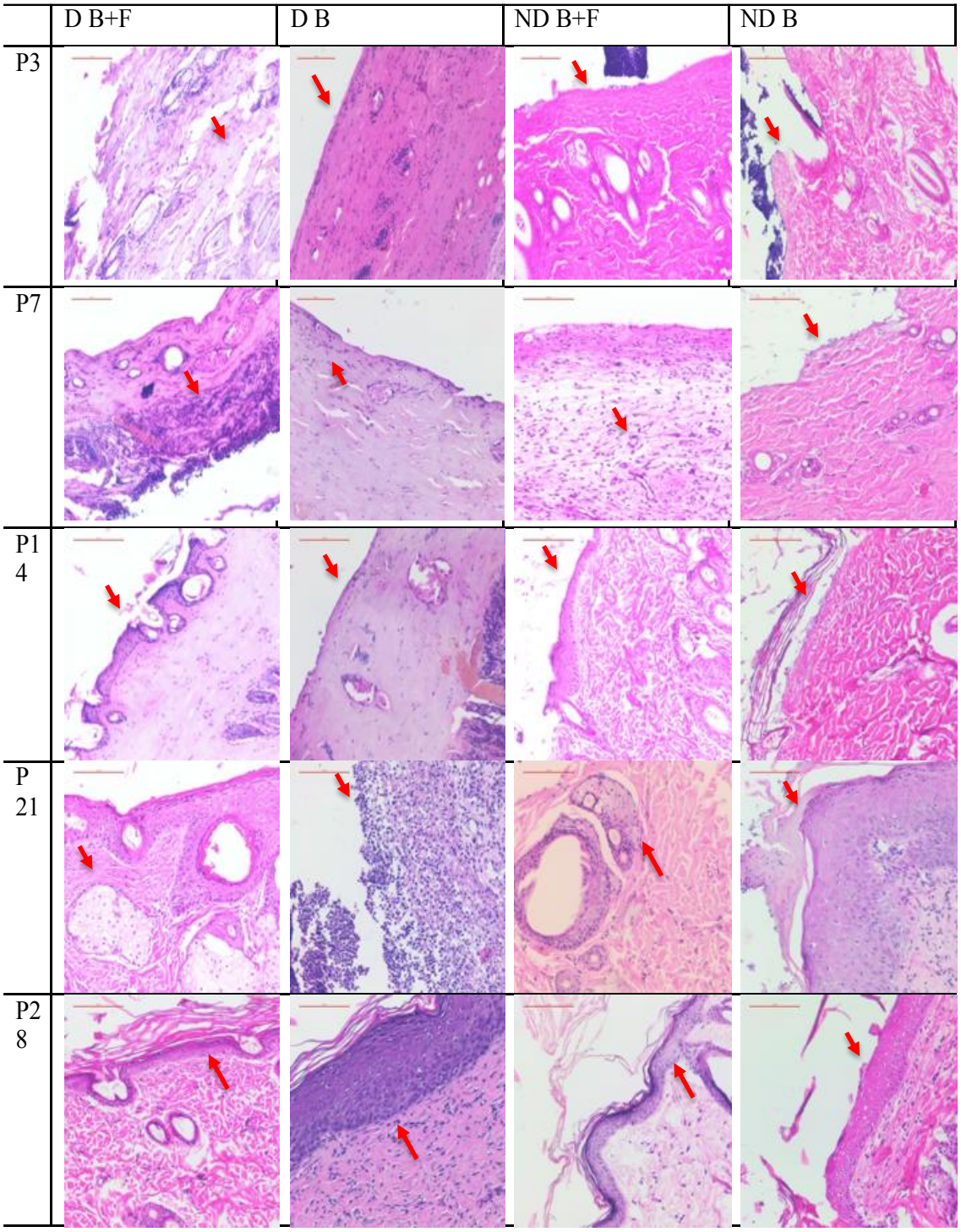


Figure 11 – Representative images of H&E-stained histological sections obtained from the 4 rats of each group at days 3, 7, 14, 21, and 28. Arrows pointing to important structures mentioned in text above.

C. Effect of fat transfer on the mobilization of mast cells in the burnt area : active participants

Based on growing evidence supporting the role of mast cells in tissue repair and scar formation, the effect of fat transfer on the mast cells abundance and activity was studied. The mast cells count was done on the histological slides stained with toluidine blue (TB) for each rat at different time points (days 3,7,14,21, and 28). Each slide had 2 main sections, and in each section there were burn and the non burn areas. In each area, counting was performed at the surface level and at the subcutaneous level. Data are expressed as averages of mast cells counts throughout the different time points. At each time point, the presence of mast cells and their degranulation state in the wound area of each group was compared to the control group Non-Diabetic Burn (ND B) by a t-test.

On day 3, at the site of the non-burn area, the mast cell count increased significantly for the D B group compared to the control group ND B at both dermis and the subcutaneous levels with p-values of < 0.01 and < 0.001 respectively. Minor increases in mast cell count were observed at both the dermis and the subcutaneous levels for the ND B+F and D B+F groups, with the latter (the diabetic) having a significant upsurge at the dermal level compared to the ND B group (p-value < 0.05) (Figures 14 -15). However, at day 3, at the site of the burn area, less mast cell counts were seen at the subcutaneous level for all the different treatments compared to the ND B group (Figure 13). Whereas, at the dermal level, all the different groups showed decreases in mast cell counts compared to the control, with significance (p-value <0.05) observed for the ND B+F group . Therefore, the presence of fat on day 3 controlled the number of mast cells.

On day 7, the presence of fat significantly decreased the mast cell count at the site of the non-burn areas at the subcutaneous level with p-values of < 0.05 and < 0.01 for D B+F and ND B+F respectively (Figure 15). No significant changes of the mast cell count were seen at the dermal level of the non burn area, nor at the dermal or the subcutaneous levels of the burn area at day 7, as all groups had similar mast cell counts. This trend continued on day 14 for both the dermal and subcutaneous levels at both the burn and non-burn areas, where all groups showcased alike mast cell counts (Figures 12 to 15).

On day 21, at the site of the non-burn area, only the D B group showed a considerable upsurge from the control ND B group at the dermal level. Nevertheless, at the subcutaneous level, all different groups showed a substantial rise in mast cell count compared to the ND B group, with ND B+F and D B groups showing significance (p-values of <0.05 and <0.01 respectively) (Figure 15). Moving into the burn area, all treatments showed considerable increases in mast cell counts compared to the control group, with the groups D B+F and D B showing significance at the dermal level while the ND B+F and D B groups showed significance at the subcutaneous level (Figures 12 – 13).

On day 28, all the groups showed comparable mast cell counts at the subcutaneous level of the non-burn area. While group D B distinctively showed a significant increase in mast cell count compared to the control group at the dermal level of the non-burn area (Figure 14). At the site of the burn area, the ND B+F and D B groups showed significant upsurges in mast cell counts at the dermal and subcutaneous levels when compared to the control, whereas the D B+F demonstrated lower increases at both levels (Figures 12 - 13).

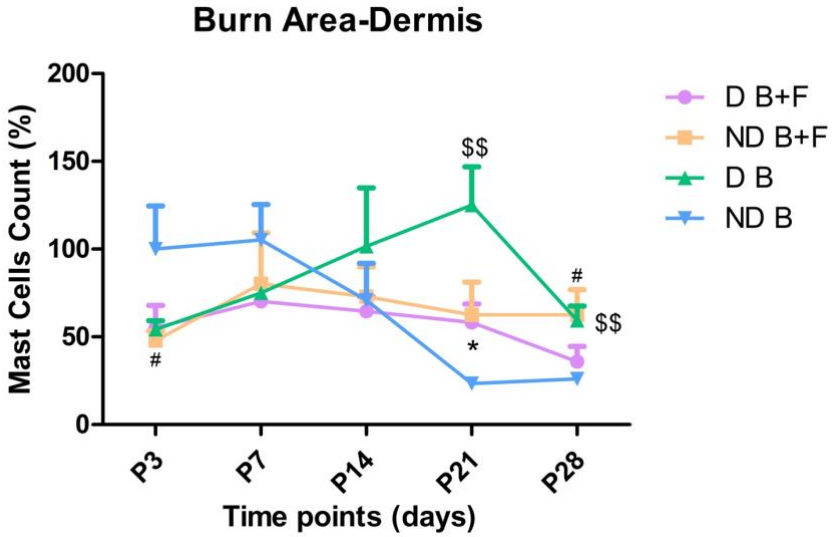


Figure 12 – Mast cell count at dermis level in the burn area for all groups at different time points. Mast cell count obtained from the 4 different sacrificed rats at each time point were calculated. Data were expressed as percentages of the mean of the ND B group at p3. At each time point, each group was compared to ND B of the same time point via t-test. For D B+F * for $P < 0.05$. For ND B+F # for $P < 0.05$. For D B $$ for $P < 0.01$.

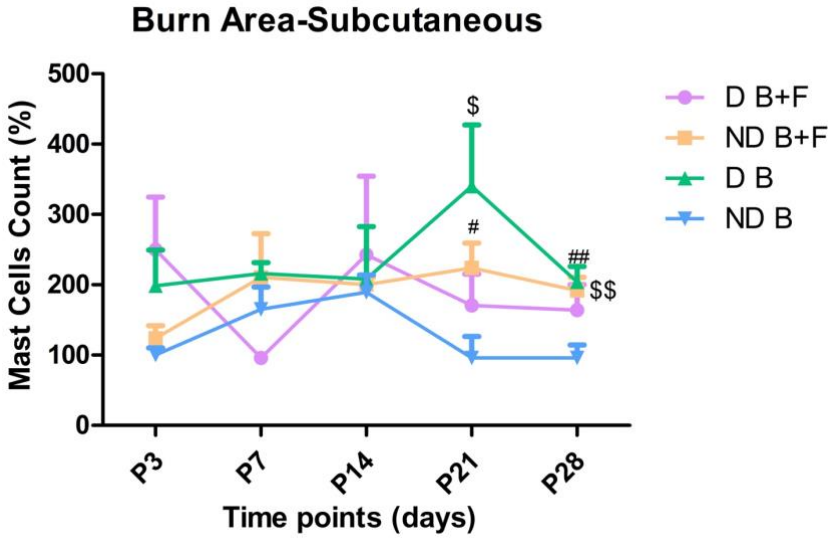


Figure 13 – Mast cell count at subcutaneous level in the burn area for all groups at different time points. Mast cell count obtained from the 4 different sacrificed rats at each time point were calculated. Data were expressed as percentages of the mean of the ND B group at p3. At each time point, each group was compared to ND B of the same time point via t-test. For ND B+F ## for $P < 0.01$. # for $P < 0.05$. For D B $$ for $P < 0.01$. $ for $P < 0.05$

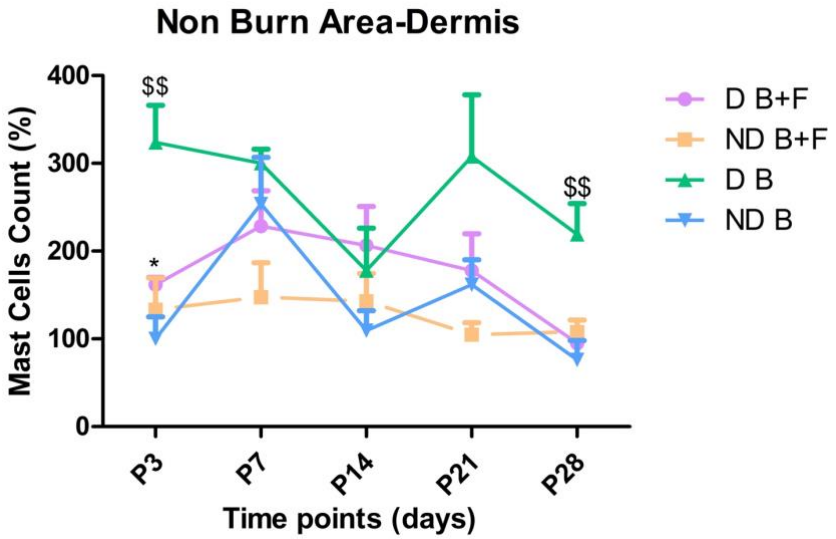


Figure 14 – Mast cell count at dermis level in the non burn area for all groups at different time points. Mast cell count obtained from the 4 different sacrificed rats at each time point were calculated. Data were expressed as percentages of the mean of the ND B group at p3. At each time point, each group was compared to ND B of the same time point via t-test. For D B+F * for $P < 0.05$. For D B $$ for $P < 0.01$.

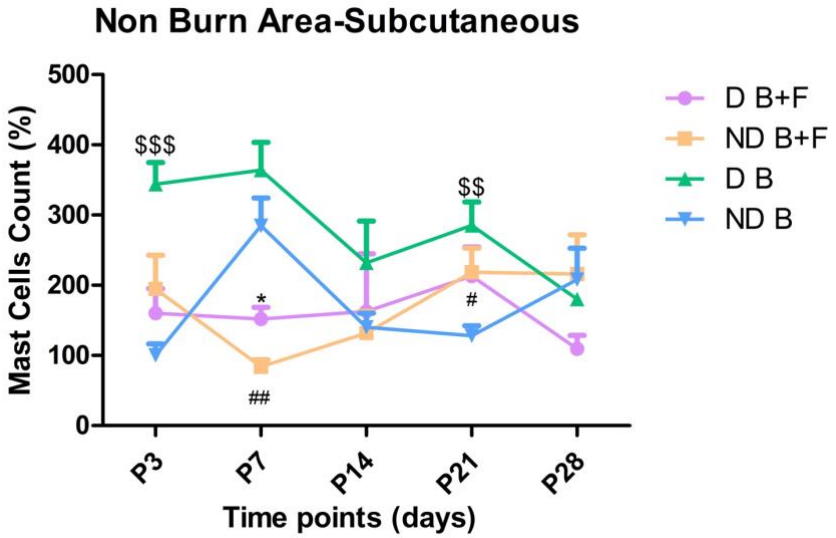


Figure 15 – Mast cell count at subcutaneous level in the non burn area for all groups at different time points. Mast cell count obtained from the 4 different sacrificed rats at each time point were calculated. Data were expressed as percentages of the mean of the ND B group at p3. At each time point, each group was compared to ND B of the same time point via t-test. For D B+F * for $P < 0.05$. For ND B+F ## for $P < 0.01$. # for $P < 0.05$. For D B $$$ for $P < 0.001$. $$ for $P < 0.01$.

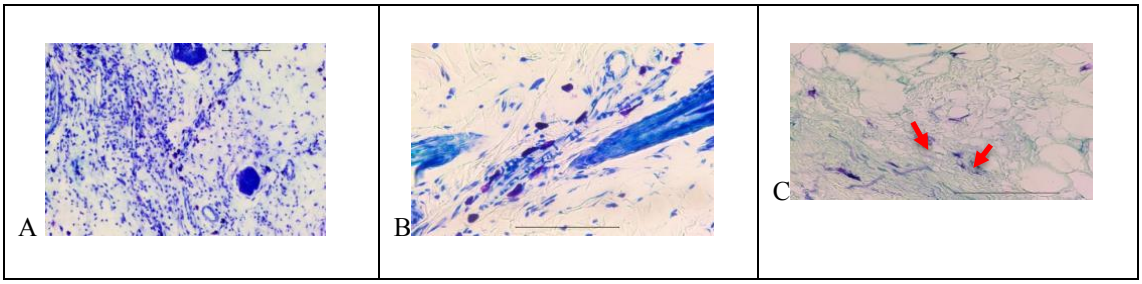


Figure 16 – Representative images of mast cells various activities, at different time points. (A) at 100x magnification, (B) at 200x magnification, (C) at 200x magnification (Red arrows pointed to ghost cell).

D. Effect of fat transfer on the molecular level: transferred autologous fat modulates inflammatory cytokines in the burnt site

To study the effect of fat transfer on gene expression of interleukins at the wound site, a real time reverse transcriptase PCR was performed on tissues taken from the wound area of animals of different groups at different time points. Data are presented as fold change compared to the control which is ND B P3.

1. IL-6

At day 3 all groups had to a great extent similar levels of IL 6 transcripts (figure 17). However, at day 7 a considerable increase in IL 6 expression was seen for the groups with fat D B+F and ND B+F, and this increase was of statistical significance ($p < 0.01$) for the latter group when compared to the group ND B (considered as the control group). While the group D B had no change in IL 6 expression compared to the group ND B as seen in figure 17. The levels of IL 6 mRNA were not significantly different for all groups at day 14. However, there was a slight rise in the IL 6 gene expression for the non diabetic groups (ND B and ND B+F) compared to the diabetic groups (D B and D B+F) as observed in figure 17.

On day 21, this trend continued for all groups except the D B group which now showed a considerable increase in the expression of IL 6 compared to the control (figure 17), probably a continuation of the inflammatory process causing the delay in the progress towards healing. Afterwards at day 28 the levels of IL 6 transcripts were again almost equivalent for all 4 groups, with a slight enhancement for ND B group (figure 17).

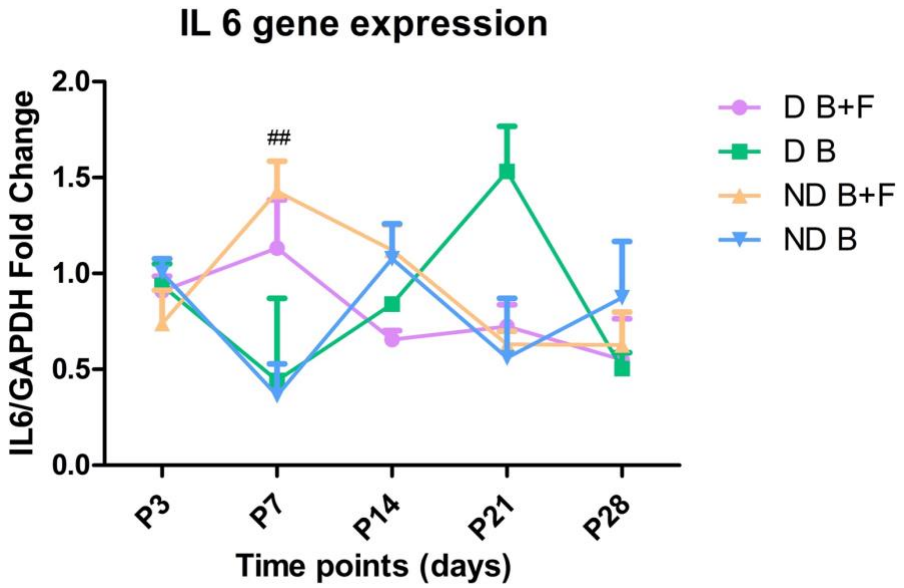


Figure 17- IL-6 gene expression. Data are expressed as fold change relative to the control ND B P3. Data are compared to the ND B group at each time point via t-test. For ND B+F ## for $P < 0.01$

2. IL-12

At day 3, IL 12 mRNA were expressed in all groups to variable extents, but not significantly different, with relatively lower levels in diabetics (figure 18). Whereas at day 7 a significant rise ($p<0.05$) of IL 12 expression was noted in the group ND B+F compared to the control (figure 18), or to diabetic animals. Also, at day 14, this surge of IL 12 transcripts was still significant ($p< 0.05$) for the ND B+F group, and the D B+F

group which started to show a small but significant increase in IL 12 expression (figure 18). At day 21 the increase of IL 12 expression in the group D B+F becomes impressively significant ($p< 0.001$), moreover the group ND B+F maintains its significant expression of the IL 12 gene ($p< 0.01$) as seen figure 18. Similar observations were recorded at day 28, nonetheless the group D B starts to show a small rise in IL 12 expression (figure 18).

Therefore, the fat transfer appears to enhance IL 6 and IL 12 genes expression both in diabetic and non-diabetic rats tissue.

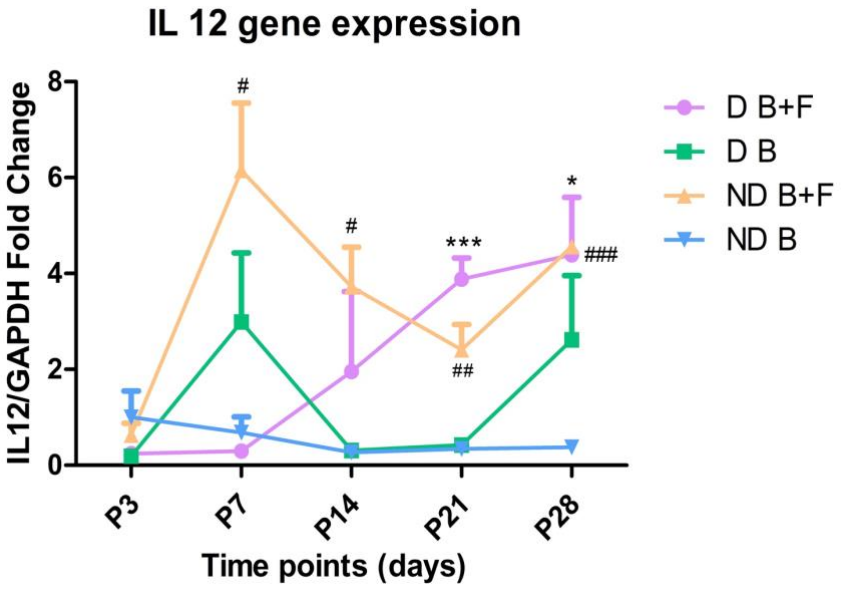


Figure 18- IL-12 gene expression. Data are expressed as fold change relative to the control ND B P3. Data are compared to the ND B group at each time point via t-test. For D B+F *** for $P < 0.001$. * for $P < 0.05$. For ND B+F ### for $P < 0.001$. ## for $P < 0.01$. # for $P < 0.05$.

3. IL-1α

At day 3, all groups with or without fat showed a lower expression of the gene IL-1α compared to the control group ND B (figure 19). At day 7, all groups continued to display lower expressions of the gene, notably the D B+F group was significantly lower ($p< 0.05$) compared to the control (figure 19). Similar results were also documented at day 14, with the groups D B+F and D B displaying a significantly lower expression of the IL-1α gene compared to the control, with p values lower than 0.01 and 0.05 respectively (figure 19). Conversely, on day 21, the levels of IL-1α transcripts in the aforementioned groups became identical to those of the control group, while the ND B+F group witnessed a significant increase ($p< 0.01$) compared to the control group (figure 19). At day 28 all groups exhibited low values compared to the control ND B (figure 19).

Thus, both the diabetes and fat transfer appear to reduce IL-1α gene expression, however, fat transfer showed an enhancing effect on IL-1α expression at later stages to variable extents, days 21 and 28.

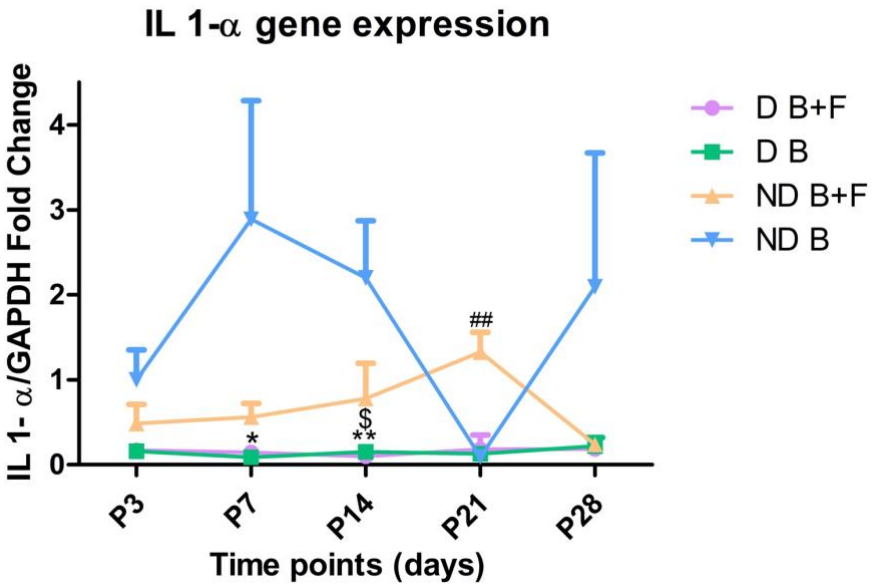


Figure 19- IL-1α gene expression. Data are expressed as fold change relative to the control ND B P3. Data are compared to the ND B group at each time point via t-test. For D B+F ** for $P < 0.01$. * for $P < 0.05$. For ND B+F ## for $P < 0.01$. For D B $ for $P < 0.05$

CHAPTER VI

DISCUSSION

Every year, millions of people are harmed by burn injuries of whom many cope with serious health problems like diabetes. In this context, foot ulceration is the most frequently recognized complication leading many times to amputations. Most remedies rely on topical application. In this study, we explored the therapeutic promise of autologous fat in the management of second-degree burn wounds in an experimental diabetic rat model.

Diabetes has been well associated with delayed wound healing primarily due to an ongoing exaggerated inflammatory process, as well as an impaired cellular proliferation and a fundamental resistance to growth factors which lead to deficient epithelialization abilities (Goren et al., 2006; Tuhin et al., 2017). In addition, the delay in wound healing could be related to the inhibition of new vessel formation due to compromised macrophage migration and defective release of signaling molecules among other factors (Maruyama et al., 2007). For burns, it is presumed that a prolonged and more intense inflammatory phase contributes to delaying healing and production of more scarring.

The results of this study have shown to a great extent the important role that fat cells play in the healing process, this effect was highlighted through the observation of the various studied parameters.

First, the wound areas of all the animal groups were studied and differences in its progression were analyzed. Beginning day 7 the differences between the groups started showing. Both groups that had fat transferred underneath their wounds showed a

significant ($p<0.001$) decrease in the wound size, while the non-fat treated D B group had a significant ($p<0.05$) increase in the wound area. This outcome emphasized how the fat has significantly contributed to the contracture of the burn wound in the various stages of healing. We could notice that the progression of the wound closure throughout the time points was consistently more significant and visible for the groups with fat, accentuating the ongoing positive effect that the fat cells had on this healing process. On the other hand, wound closure was delayed in the D B group, which goes in line with the characteristic diabetic traits of hindered healing, by maintaining a sustained inflammatory reaction. However, thanks to the D B+F group, we could observe that the effect of the fat overshadowed the effect of diabetes.

The positive outcomes of fat were also better highlighted when the histological aspects were studied. The D B group had shown throughout the time points an ongoing inflammatory process, which was highly prominent up until day 21 post-op as seen in the H&E-stained sections. The inflammatory process for the groups D B+F, ND B+F, and ND B was the most prominent on day 7 post-op, with some inflammation still being visible in the area of fat transfer up until day 14 post-op. The beginning of epithelial lining formation had started for the three aforementioned groups on day 7, and by day 14 more than 2 layers were well-formed. Conversely, for the D B group, the healing was delayed and it was until day 21 post-op that it seemed to catch up with the rest of the groups in terms of epithelial lining formation. However, it had still a visible scar formed, and unorganized morphology with an abundance of disorganized collagen fibers and multiple inflammatory cells. These observations show that the fat cells play a substantial role, specifically in the diabetic group, in both the proper initiation of the healing process and the correct formation of structures at the burn site, in terms of skin

appendages, sebaceous glands and ducts, and hair bulbs. Therefore, fat transfer has in fact improved and speeded up the wound healing process at both the tissue and cellular levels, with influences on the inflammatory process, the formation of epithelial layers and an organized dermis.

These findings correlate with evidence from the literature suggesting that adipose tissue plays an enhancing role in wound healing.

Adipose tissue is considered as a good source of stem cells, which are multipotent and could differentiate into several lineages. On this basis, adipose tissue has been studied for its effects in wound repair. Multiple analyses have found that adipose tissue and adipose-derived stem cells (ADSCs), have the capacity to induce wound repair through increasing mean collagen secretion and deposition. This hypothesis was mainly supported by the findings of improved extracellular matrix (ECM) deposition and granulation tissue thickness in the fat cells treated groups (Kim et al., 2011; Nambu et al., 2009).

In addition, researchers have noted that the groups treated by ADSCs exhibited amplified levels of TGF-β1 and TGF-β3 in wound environments. Both these cytokines play important roles in wound healing, TGF-β1 for example has a part in matrix deposition, as well as in cellular migration, in addition to wound contraction, whereas TGF-β3 is an anti-scaring factor, that contributes to collagen organization, while it also could regulate wound reepithelization (Shen et al., 2013; Zografou et al., 2013).

A study by Nie et al. related ADSCs-stimulated tissue repair to the increased epithelialization granulation tissue accumulation and natural site-specific differentiation of ADSCs into epithelial cells. They also confirmed that ASDCs had the capacity of secreting cytokines with angiogenic properties such as VEGF, hepatocyte growth factor

(HGF), and fibroblast growth factor-2 (FGF2) both in-vitro and in-vivo (Nie et al., 2011). Likewise, Ebrahimian et al. found the same results in their study involving the mouse chronic wound model (Ebrahimian et al., 2009). Furthermore, Maharlooei et al. in their study concerning adipose tissue-derived mesenchymal stem cell in wound healing in diabetic rats noted a release of VEGF and HGF which was correlated with enhanced survival (Maharlooei et al., 2011).

In a study on ischemic wound healing using ADSCs, Kim et al. mapped the healing process by having at first an acute inflammatory response occurring, for instance, perivascular leakage of red blood cells or neutrophils, followed by the formation of granulation tissue. They also noted early recovery of denervated tissues and improved cell survival. The latter was thought to be performed through controlled apoptosis and protection from oxidative stress. Besides, ADSCs were found to elevate VEGF levels in plasma and tissues, which in turn contributed to local angiogenesis and to triggering a systemic response (Kim et al., 2011).

Recently, many experimental studies have focused on examining the therapeutic effects of ADSCs in murine animal models of wounds healing; it was consistently evident that ADSCs-treated groups displayed improved wound healing due to multiple mechanisms. For instance, they deduced that ADSCs changed the expression profiles of some critical growth factors and cytokines, which lead to improved cellular proliferation, accumulation of collagen fibers, and enhanced migration of keratinocytes and endothelial cells (Ghaneialvar et al., 2017; Irons et al., 2017; Kuo et al., 2016; Maharlooei et al., 2011; Nie et al., 2011; Shi et al., 2016).

We can hence speculate that the fat tissue that was introduced in our study underneath the wounds might have dissolved and differentiated to MSCs, which then on served according to the needs of the local surrounding structures.

To further investigate the histological changes introduced by the fat treatment in this study, mast cells were counted in the wound area at different points, taking multiple fields into considerations. We looked at the burn area and the area that surrounds it, and in both mentioned fields mast cells were counted at the dermal area and the subcutaneous area. At day 3 post-op, a statistically significant increase in the number of mast cells of the D B group was seen when compared to the control group, at the dermis level ($p < 0.01$) and the subcutaneous level ($p < 0.001$), similarly the D B+F group showed a significant ($p< 0.05$) increase at the dermis level. These findings suggest an inflammation build-up around the wound, with more inflammation noted for the diabetic groups. This inflammatory response in the early phases of wound healing is essential for the production of important proinflammatory cytokines, recruitment of macrophages, and the stimulation of cell proliferation of angiogenesis (Paulino Do Nascimento & Monte-Alto-Costa, 2011). In addition, this response stimulates debridement and bacterial cell death in the early phases (Diegelmann & Evans, 2004; J. Li et al., 2007).

On day 7 post-op, both groups with fat transfer had a significant decrease in mast cell count in the subcutaneous non-burn area, with the D B+F group p-value <0.05 and the ND B+F group p-value < 0.01. We can deduce that, at this time point inflammation is decreasing with the presence of fat. As for day 21 post-op in the non-burn area, the D B group displayed a considerable increase in the dermis level, while all three experimental groups showed an increase at the subcutaneous level with the ND

B+F group having a significant increase with a p-value < 0.05 and the D B group having a higher significance with a p-value < 0.01. In addition, all groups at this point had considerable increases in mast cell counts in all fields at the burn area, however, the D B +F group had specifically a significant ($p<0.05$) increase at its dermal level, and the ND B+F group had a significant ($p<0.05$) increase at the subcutaneous level. Whereas the D B group showed significance at both its dermal and subcutaneous levels, close to the transplanted fat. On day 28, group D B continued to have a rise in its mast cell counts in all its fields. Also, the group ND B+F had a significant augmentation in the count at the burn area at both the dermal and subcutaneous levels.

Mast cells have been established as important participators in all phases of the healing process with a heightened role in the inflammatory phase (Barroso et al., 2012). They were mainly found under pathological conditions. They had a critical role in the formation of granulation tissue (Bolton & Montagna, 1993) as well as being chemoattractants to neutrophils (Kawamoto et al., 1995). It can well be established that their stimulation of the neuropeptide VIP induces the mitosis of keratinocytes (Wilkinson et al., 1994). Mast cells also improve angiogenesis by releasing bFGF, VEGF and TGF-ß (Grützkau et al., 1998; NILSSON et al., 1995; Qu et al., 1995). However, the prolonged deployment of mast cells until 21 days after the formation of the wound has been found to cause impairment in the formation of granulation tissue (Souza et al., 2006), as well as the excess mediators of mast cells specifically kinases was found to have a negative role, delaying epithelial cells migration (Cardoso et al., 2007; Ebihara et al., 2005).

However, the continued elevated levels of mast cells beyond day 21, in almost all the groups, could be explained by the mast cells' role in the remodeling phase.

Moreover mast cells have been found to be associated with fibroblast mitosis which leads to empowerment of collagen synthesis and secretion, as well as modification of ECM components (F. Levi-Schaffer & Rubinchik, 1995; Francesca Levi-Schaffer & Kupietzky, 1990; Ruoss et al., 1991; Schwartz, 1990), which are important landmarks that take place in the maturation phase.

As for the effect of fat cells at the molecular level, the expression of several interleukins was assessed at the wound site. To start, IL-6 gene expression levels were calculated at the 5 time points mentioned above for all the involved groups. On day 3 post-op, IL-6 gene expression was similar for all groups. Starting day 7, differences in the gene expression levels were noted: the groups with fat transferred showed significantly ($p<0.01$) increased levels of the gene compared to the control. The D B group had at this time point similar levels to the control. On day 14, both non-diabetic groups had higher levels of IL-6 expressed than the diabetic groups. Whereas at day 21, the D B group showed a considerable increase in its IL-6 levels. Day 28 was marked by all groups displaying similar levels of IL-6, except the ND B group which displayed slightly higher levels.

IL-6 is a pro-inflammatory cytokine that has been closely linked to wound healing through several mechanisms. It is a chemoattractant to neutrophils and plays a role in leukocyte recruitment to the wound area in the early phase of inflammation (Akira & Kishimoto, 1992; Werner & Grose, 2003a). IL-6 also has a powerful mitosis-inducing effect on keratinocytes and a crucial role in angiogenesis. Besides, it has been proved to enhance collagen deposition at the wound site (Duncan & Berman, 1991; Greenwel et al., 1993; Lin et al., 2003; Werner & Grose, 2003a). Furthermore, the lack

of IL-6 has been associated with delay in reepithelialization, predominantly due to the subsequent decrease in TGF-ß (Lin et al., 2003).

Several studies have in fact proved that IL-6 promotes cell proliferation, angiogenesis, and ECM synthesis in the early proliferative phase of wound healing. On the other hand, it was evident that prolonged expression of IL-6 entails chronic inflammation and the development of hypertrophic scarring (O'Reilly et al., 2012; Zhu et al., 2016). In our study, the D B group at day 21 post-op had elevated levels of IL-6 expression, consistent with prolonged inflammation and delayed wound healing.

Moreover, IL-6 has been found highly expressed in the hypertrophic scarring associated fibroblasts (H. Xue et al., 2000). A study by Foubert et al. on autologous adipose-derived regenerative cell (ADRC) therapy and development of hypertrophic scarring, showed that ADRC treated groups were linked to upregulated expression of IL-6 in the proliferative phase and downregulated expression of the gene in the remodeling phase, which leads to enhanced early healing and reduced hypertrophic scar formation (Foubert et al., 2017). This is evident in our study; in fact, the D B+F group specifically followed the course of expression of IL-6 as mapped out by Foubert et al.

In this study, IL-1α gene expression levels were calculated at the 5 time points mentioned above for all the groups involved. On day 3 all groups displayed lower levels than the control. On day 7, all groups continued to show lower levels of this gene, with the group D B+F having significantly ($p<0.05$) lower expression of IL-1α. Day 14 was marked by a significant decrease in the gene expression for the group D B+F ($p<0.01$) and the group D B ($p<0.05$). On day 21, all groups had similarly comparable levels of IL-1α expression except for the ND B+F group which now demonstrated a significant

increase ($p<0.01$). On day 28, on the other hand, all the groups fell back to comparable levels, lower than the levels expressed on day 3.

IL-1α is a pro-inflammatory cytokine, that plays an important role in wound healing through instigating the migration and proliferation of keratinocytes, in addition to stimulating the production of FGF (Werner & Grose, 2003a). IL-1α also regulated the expression of VCAM-1 and ICMA-1, important regulators of leukocyte extravasation (Lin et al., 2003). In our study, IL-1α expression was mostly downregulated after day 3 post-op. These observation could be linked to the fact that IL-1α exerts most of its effects at early stages of wound healing.

IL-12 gene expression levels were calculated at the 5 time points mentioned above for all the groups involved. On day 3, all groups expressed similar levels of the gene. Day 7 is when differences start to appear: the ND B+F group had a significant ($p< 0.05$) rise in gene expression. On day 14, both groups with fat had noticeable changes in IL-12 gene expression, with ND B+F having significantly ($p<0.05$) elevated levels. Day 21 was also distinct with the fat-treated groups with now both displaying significant increases in IL-12 gene expression: ND B+F $p<0.01$ and D B+F $p<0.001$. On day 28, all groups were back to similar gene expression levels except for the D B group which showed a slight increase at this time.

IL-12 is a pro-inflammatory cytokine that recruits neutrophils into the wound bed and enhances the differentiation of naïve T cells into T effector cells (Gillitzer, 2001; Matias et al., 2011; Trinchieri et al., 2003). Cytotoxic and helper T-cells have been found to have an essential role in the wound healing process, they both produce a plethora of lymphokines that are associated with the healing process, particularly effective on cellular subsets that are responsible for fibroblast replication and collagen

synthesis (Agren, 2016; Fishel et al., 1987). In addition, cytotoxic T cells have been associated with macrophage differentiation, as well as their activation and migration. T-cell-derived cytokines, for example, interferon γ which promotes the recruitment and activation of M1 macrophages, that in turn amplify adipose tissue inflammation and insulin resistance (Harford et al., 2011). However, IL-12 has anti-angiogenic activity (Voest et al., 1995), thus its overexpression might contribute negatively to the healing process. Moreover, a study established that the absence of IL-12 had accelerated epithelialization and promoted early inflammation, as well as the elimination of angiogenesis regulation (Matias et al., 2011).

The above manifestations demonstrate that fat was capable of elevating significantly the levels of IL-12 even at early stages, which could be responsible of the accelerated healing process.

CHAPTER VI

CONCLUSION

In this study, data are in line with results reported in the literature. However, it could demonstrate that autologous fat cells transported or transferred from the abdominal wall without any further treatment could also improve second degree burn wound healing by having: less scarring, smaller surface areas, faster regeneration of skin appendages (hair, sweat glands), and faster epithelialization. The fat tissue could have maintained a sustained low grade inflammatory reaction, as noted by histological studies, attracting more neutrophiles for quick clearing of necrotic tissue and thus reduction in ROS formation and enhancement of the proliferative phase. As a result of the secretory products from the fat cells, remodeling will be induced earlier with resolution of vascular bed, fibroblast proliferation and differentiation in the deep layers with early migration and myofibroblast activations. Consequently, the extracellular matrix becomes more organized with more collagen deposition and gaining of cumulative tensile strength and appropriate contraction, thus leading to a smaller area of the wound with less scarring.

A. Limitations of the study

Multiple limitations were faced in this study. First, we were aiming to study the effect of fat treatment in both diabetic and nondiabetic models through calculating the pAMPK/tAMPK ratio. However, since our quantification procedures depended on the Western Blot technique, we were unable to continue working on this aspect as this technique needed further optimization for our tissue type. Moreover, as indicated in the

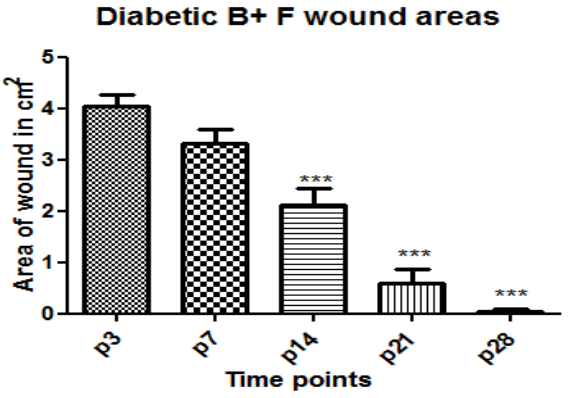
literature, several other interleukins and cytokines are equally important in wound healing, however, we did not have the tools to examine the progress of their expression under the different experimental conditions. Furthermore, we planned to perform a quantitative ROS production assay, but we were unable to do it due to technical difficulties, and lack of kits.

B. Future perspectives

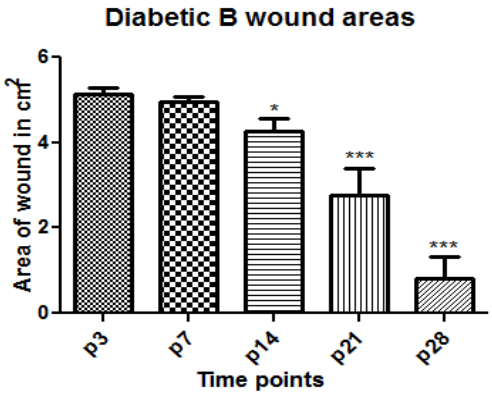
As for future perspectives, multiple areas can be further explored in this study, such as the fat cells migration and differentiation, as well fibroblasts formation and progression throughout the time points. While also, the observation of the migration patterns of different types of leukocytes could be assessed. In addition to the ROS technique that could be done on the remaining tissue samples, as well as heat shock protein 70 (HSP-70) expression.

APPENDIX

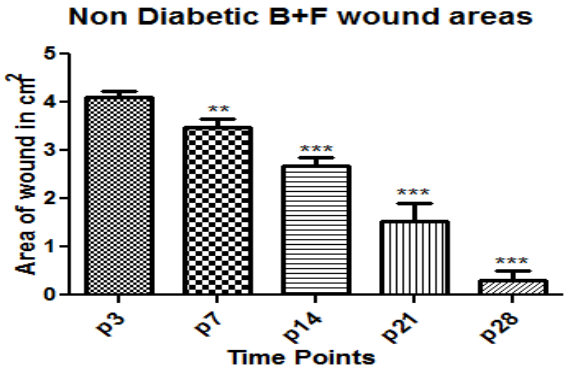
SUPPLEMENTARY DATA



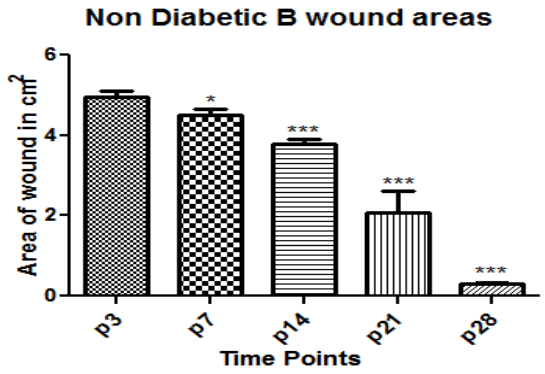
S1 – The D B+F group wound area progress over the 5 time-points. Data are expressed as averages of calculated wound areas obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (P3) via t-tests. *** for $P < 0.001$.



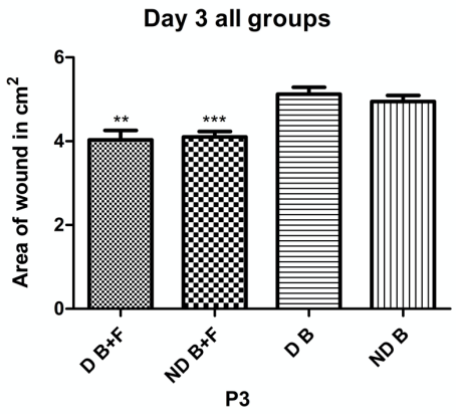
S2 – The D B group wound area progress over the 5 time-points. Data are expressed as averages of calculated wound areas obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (P3) via t-tests. *** for $P < 0.001$. * for $P < 0.05$.



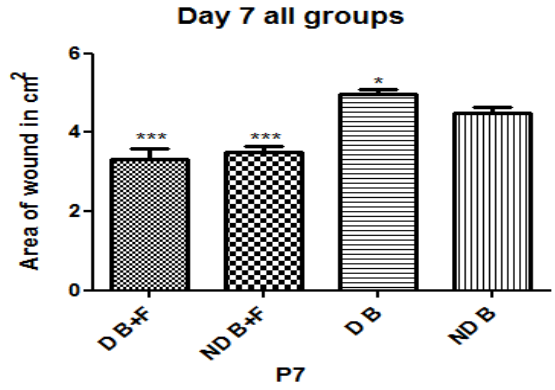
S3 – The ND B+F group wound area progress over the 5 time-points. Data are expressed as averages of calculated wound areas obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (P3) via t-tests. *** for $P < 0.001$. ** for $P < 0.01$.



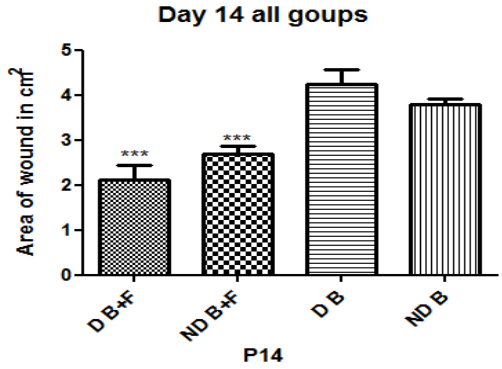
S4 – The ND B group wound area progress over the 5 time-points. Data are expressed as averages of calculated wound areas obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (P3) via t-tests. *** for $P < 0.001$. * for $P < 0.05$.



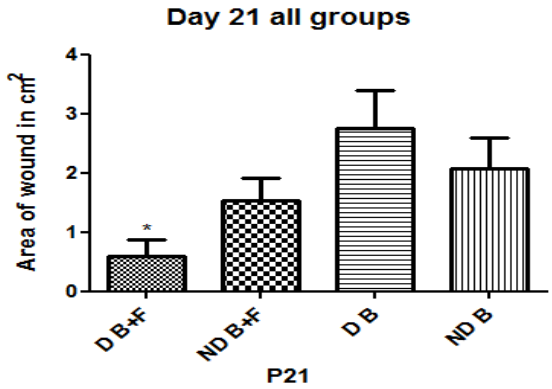
S5 – All groups compared to each other at Day 3. Data are expressed as averages of calculated wound areas obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (ND B) via t-tests. *** for P < 0.001. ** for P < 0.01.



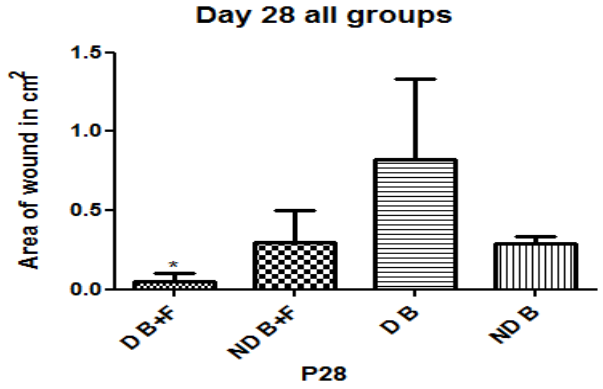
S6 – All groups compared to each other at Day 7. Data are expressed as averages of calculated wound areas obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (ND B) via t-tests. *** for P < 0.001. * for P < 0.05.



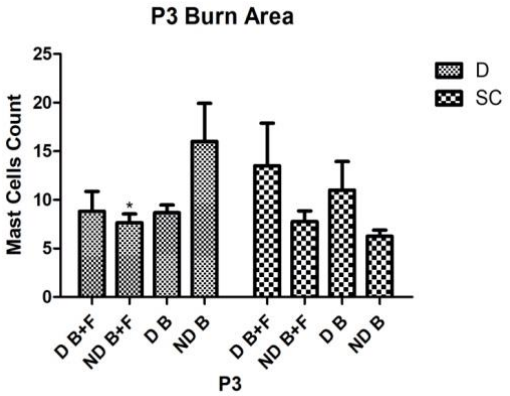
S7 – All groups compared to each other at Day 14. Data are expressed as averages of calculated wound areas obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (ND B) via t-tests. *** for P < 0.001.



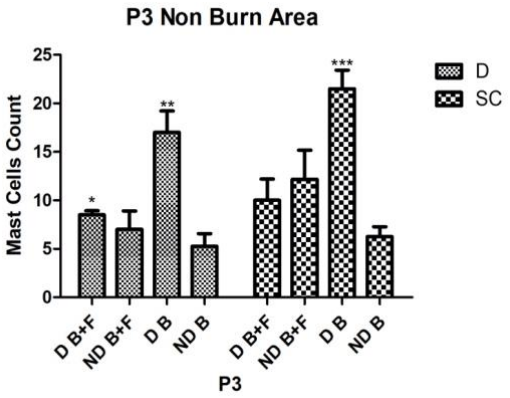
S8 – All groups compared to each other at Day 21. Data are expressed as averages of calculated wound areas obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (ND B) via t-tests. * for P < 0.05.



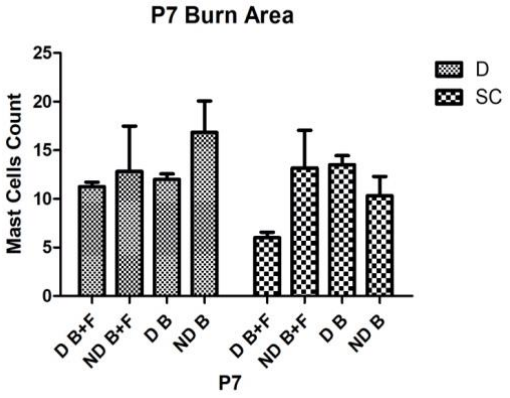
S9 – All groups compared to each other at Day 28. Data are expressed as averages of calculated wound areas obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (ND B) via t-tests. * for P < 0.05.



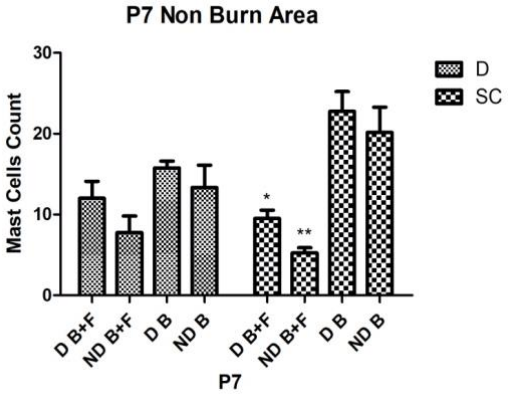
S10 - All groups compared to each other at Day 3. Data are expressed as averages of calculated mast cells in the burn area of the wound, obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (ND B) via t-tests. * for P < 0.05.



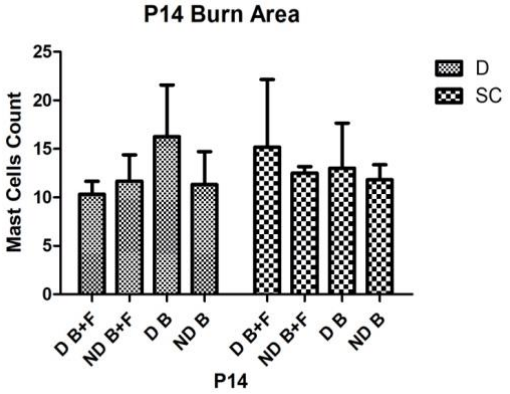
S11 - All groups compared to each other at Day 3. Data are expressed as averages of calculated mast cells in the non burn area of the wound, obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (ND B) via t-tests. *** for $P < 0.001$. ** for $P < 0.01$. * for $P < 0.05$.



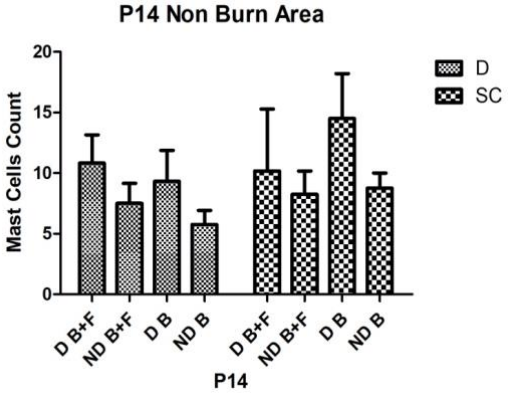
S12 - All groups compared to each other at Day 7. Data are expressed as averages of calculated mast cells in the burn area of the wound, obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (ND B) via t-tests.



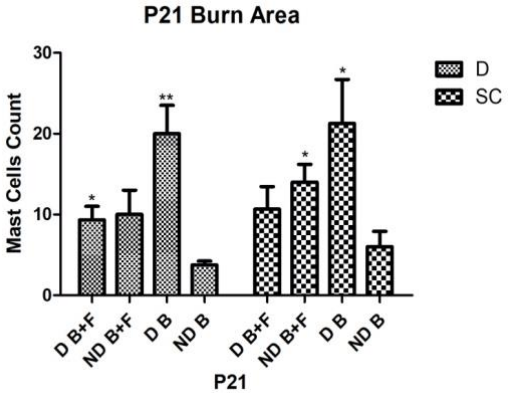
S13 - All groups compared to each other at Day 7. Data are expressed as averages of calculated mast cells in the non burn area of the wound, obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (ND B) via t-tests. ** for $P < 0.01$. * for $P < 0.05$.



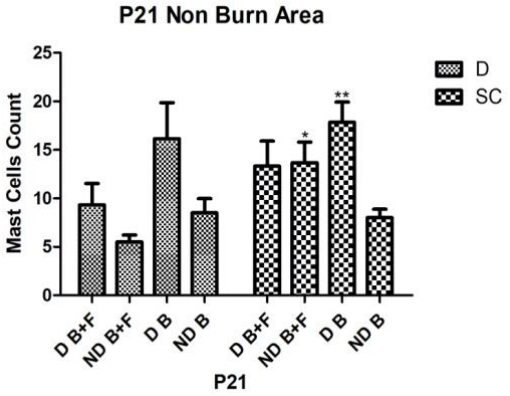
S14 - All groups compared to each other at Day 14. Data are expressed as averages of calculated mast cells in the burn area of the wound, obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (ND B) via t-tests.



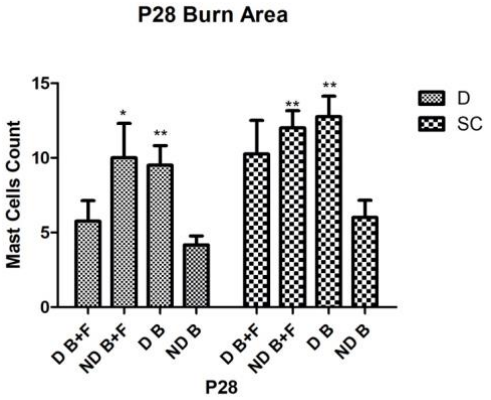
S15 - All groups compared to each other at Day 14. Data are expressed as averages of calculated mast cells in the non burn area of the wound, obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (ND B) via t-tests.



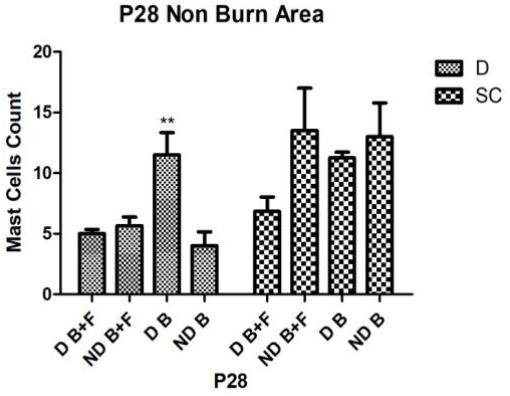
S16 - All groups compared to each other at Day 21. Data are expressed as averages of calculated mast cells in the burn area of the wound, obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (ND B) via t-tests. ** for $P < 0.01$. * for $P < 0.05$.



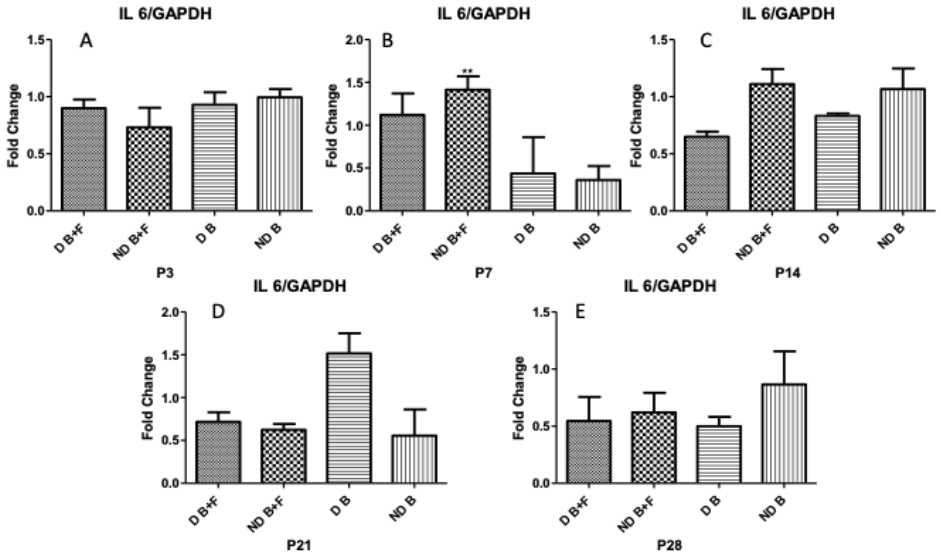
S17 - All groups compared to each other at Day 21. Data are expressed as averages of calculated mast cells in the non burn area of the wound, obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (ND B) via t-tests. ** for $P < 0.01$. * for $P < 0.05$.



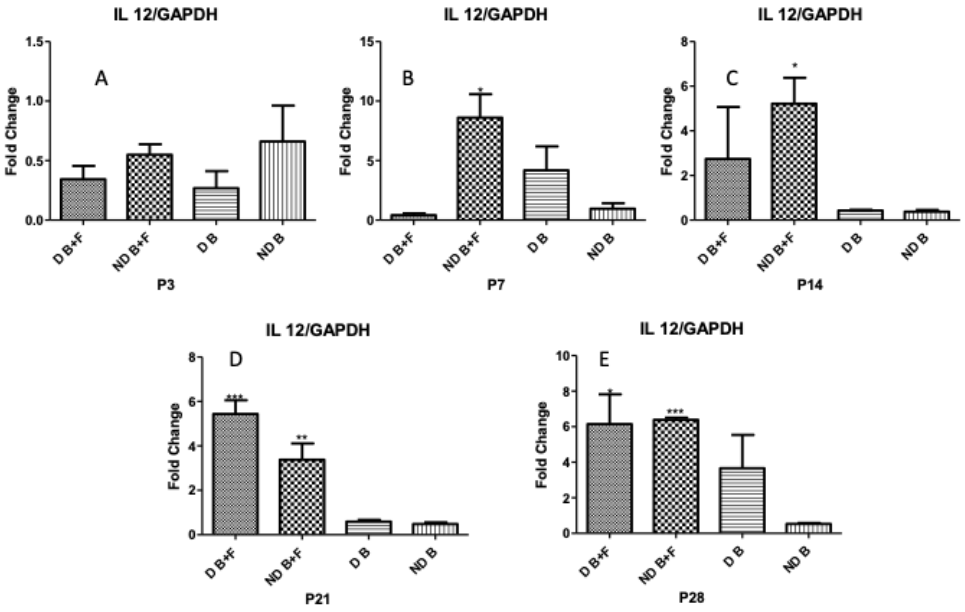
S18 - All groups compared to each other at Day 28. Data are expressed as averages of calculated mast cells in the burn area of the wound, obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (ND B) via t-tests. ** for $P < 0.01$. * for $P < 0.05$.



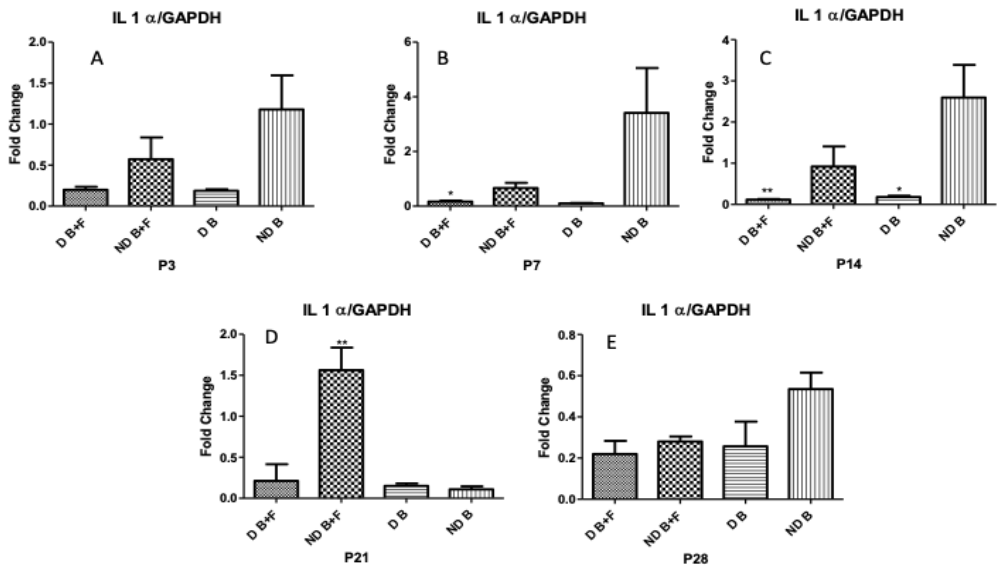
S19 - All groups compared to each other at Day 28. Data are expressed as averages of calculated mast cells in the non burn area of the wound, obtained from the 4 different rats sacrificed at each time point. The data from each time point are compared to the control (ND B) via t-tests. ** for $P < 0.01$.



S20 - Data are expressed as fold change relative to the control ND B P3. Data are compared to the ND B group at each time point via t-test. ** for $P < 0.01$.



S21 - Data are expressed as fold change relative to the control ND D P3. Data are compared to the ND B group at each time point via t-test. *** for $P < 0.001$. ** for $P < 0.01$. * for $P < 0.05$



S22 - Data are expressed as fold change relative to the control ND D P3. Data are compared to the ND B group at each time point via t-test. ** for $P < 0.01$. * for $P < 0.05$.

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