AMERICAN UNIVERSITY OF BEIRUT

EFFECT OF DENERVATION ON BURN WOUND HEALING

by
HISHAM SAADEDDINE DAOUK

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Anatomy, Cell Biology and Physiological Sciences
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
September 2016

AMERICAN UNIVERSITY OF BEIRUT

EFFECT OF DENERVATION ON BURN WOUND HEALING

by

Hisham Saadeddine Daouk

Approved by:

Dr. Abdo Jurjus, Professor — Advisor
Department of Anatomy, Cell Biology and
Physiological Sciences

Nada Lawand, Assistant Professor — Member of Committee
Department of Neurology

Dr. Georges Daoud, Assistant Professor — Member of Committee
Department of Anatomy, Cell Biology and
Physiological Sciences

Dr. Bisharah Atiyeh, Clinical Professor — Member of Committee
Department of Surgery

Date of thesis defense: September 8, 2016

AMERICAN UNIVERSITY OF BEIRUT

THESIS, DISSERTATION, PROJECT RELEASE FORM

AMERICAN UNIVERSITY OF BEIRUT

THESIS, DISSERTATION, PROJECT RELEASE FORM

Student Name: Daouk (Last) Hisham (First) Saadeddine (Middle)

● Master's Thesis ○ Master's Project ○ Doctoral Dissertation

☒ I authorize the American University of Beirut to: (a) reproduce hard or electronic copies of my thesis, dissertation, or project; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes.

☐ I authorize the American University of Beirut, to: (a) reproduce hard or electronic copies of it; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes after :

One ---- year from the date of submission of my thesis, dissertation, or project.
Two ---- years from the date of submission of my thesis, dissertation, or project.
Three ---- years from the date of submission of my thesis, dissertation, or project.

Daouk 16/09/2016

Signature Date

This form is signed when submitting the thesis, dissertation, or project to the University Libraries

ACKNOWLEDGMENTS

First, I would like to express my sincere gratitude to my advisor Professor Dr. Abdo Jurjus for the continuous support of my Masters and related research, for his patience, motivation and immense knowledge. His guidance helped me in all the time of research and writing of this thesis and also for his guidance throughout my career.

Besides my advisor, I would like to thank the members of the committee: Drs. Nada Lawand, Georges Daoud, and Bishara Atiyeh for their insightful comments and encouragement.

My sincere thanks also goes to Professor Dr. Nayef Saade for his continuous support and guidance throughout the experimental process and for guidance throughout my career. Another big “thank you” goes to Dr. Eid for his help and support. Dr. Faysal Saadeh, Dr. Elie Al-Chaer and Dr. Marwan El Sabban: thank you for your moral help and support.

I would like to thank the Department of Anatomy personnel: Sawsan Zaidan, Elia Maamari (a friend indeed), Bassem Najm and Talar Terzian for the help and support.

Farah Shamaa, thank you for your help and “pushing me” forward.

Another thank you goes to Hanine Haidar, Rateb Zeineddine and the staff at the Animal Care Facility.

Last but not the least, I would like to thank my family: my brother, my sisters, Ali, Abed El Rahman, Mohammad Nadim, and Samar for supporting me spiritually throughout writing this thesis and my life in general.

Thank you very much, everyone!

I dedicate this thesis to my: Father, Uncle Mustafa, Mom, wife Mirna and my precious little kids.

AN ABSTRACT OF THE THESIS OF

Hisham Saadeddine Daouk for Master of Science
Major: Anatomy, Human Morphology

Title: Effect of Denervation on Burn Wound Healing

Background and Aims: The skin forms a barrier between the interior milieu of the organism and its environment. This barrier has multiple physiological functions and is subject to an array of pathologies including wounds and burns. The present study aims to determine the effect of the nervous system on wound healing. Specifically, this study tested the effect of denervation by chemical ablation on the burn wound healing process using guanethidine for the sympathetic postganglionic neurons and resiniferatoxin for the Capsaicin-sensitive fibers.

Methods: Animals were divided into different groups of controls, sensory denervated and burned, sensory denervated non-burned, sympathetic denervated and burned, sympathetic denervated non-burned, vehicle sensory burned, vehicle sympathetic burned and non-denervated burned. We measured different morphologic and biochemical parameters such as wound surface area, histological alterations and mast cells. In addition, NGF, IL-1β, IL-6 and IL-8 were measured using the ELISA technique.

Results and Conclusion: The gross observations, the histological data including mast cells modulation as well as the molecular data speak in favor of a significant delay of burn wound healing caused by the sensory denervation and worse scar. On the other hand, results support the positive role of sympathetic denervation in reducing scar formation and speeding up the healing process. The dual effect of the nervous system on burn wound healing is being documented in an animal model for the first time.

CONTENTS

ILLUSTRATIONS

TABLES

List of Abbreviations	
TNF-α	Tumor Necrosis Factor Alpha
IL-1	Interleukin 1
IL-6	Interleukin 6
VEGF	Vascular Endothelial Growth Factor
IL-8	Interleukin 8
TGF-β	Transforming Growth Factor Beta
TGF-α	Transforming Growth Factor Alpha
FGF	Fibroblast Growth Factor
PDGF	Platelet Derived Growth Factor
IFN-*y*	Interferon gamma
EGF	Epidermal growth factor
KGF	Keratinocyte Growth Factor
IGF-1	Insulin-like Growth Factor 1
NGF	Nerve Growth Factor
αvβ3	Alpha-v beta-3 Integrin
αvβ5	Alpha-v beta-5

α5β1	Alpha-5 beta-3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
EGFR	Epidermal Growth Factor Receptor
FGF	Fibroblast Growth Factor
HIF-1α	Hypoxia-Inducible Factor 1 Alpha
VEGF-A	Vascular Endothelial Growth Factor A
FDA	Food and Drug Administration
PDGF-AA	Platelet Derived Growth Factor A
PDGF-BB	Platelet Derived Growth Factor B
PDGF-AB	Platelet Derived Growth Factor AB hetero dimer
VEGF-A-E	Vascular Endothelial Growth Factor A enzyme
BoNT	Botulinum Neuro Toxin
VAMP	Vesicle associated membrane proteins
CGRP	Calcitonin Gene Related Peptide
6-OHDA	6-hydroxydopamine
RTX	Resiniferatoxin

RTXB	RTX Burn
RTXnB	RTX non Burn
VRTX	Vehicle RTX
GB	Guanethidine Burn
VG	Vehicle Guanethidine
TEWL	Trans Epidermal Water loss
nDB	non-Denervated Burn
CTRL	Control
GnB	Guanethidine non Burn
CRH	Corticotropin-Releasing Hormone
ACTH	Adrenocorticotropic Hormone
SP	Substance P
SCF	Stem Cell Factor
IgE	Immuno globulin E

Effect of Denervation on Burn Wound Healing

1 INTRODUCTION

1.1 Preamble

The integumentary system is the largest system of the human body, covering almost its entire surface area. It forms a barrier between the interior milieu of the organism and its environment. What was merely thought of as a simple coat covering the human body, the skin has numerous other physiological functions, ranging from mechanic, metabolic, and regenerative, to energetic and immunological (Günter & Machens, 2012). It is also subject to disease and abnormal alterations including lacerations, wounds and burns.

A burn is defined as a damage inflicted to a body's tissue caused by heat, electricity, sunlight or radiation. Scalds from hot liquids and steam, building fires, and flammable liquids and gases are the most common causes of burns. Another kind is an inhalation injury, caused by inhaling smoke or chemical fumes (National Institute of Health, 2014). In general, a wound encompasses "tissue disruption of normal anatomic structure with consecutive loss of function" (Lazarus, et al., 1994). The disruption of anatomic, histologic, and molecular structures caused by wounds results in pathologic processes (Lazarus, et al., 1994). In this context, wounds and wound burns are breaks in the epithelial integrity of the skin (Enoch & Price, 2004). Wounds can be classified in many ways, primarily depending on their duration and their depth. They range from acute wounds which usually heal within 3 weeks to chronic wounds which may last for more than 3 months (Korting, Schollmann, & White, 2011).

1.2 Classification of Burns/Wounds

Burns have been classified into four types depending on the severity of burn: superficial, superficial partial-thickness, deep partial-thickness and full thickness.

1.2.1 In superficial wounds, damage may extend over the epidermis, like in superficial thermal wounds. An isolated loss of the epidermis, leaving the dermis intact, heals completely and without any scar formation within utmost of 8 days. An example is sunburns. In such a situation, there is no need for a specialized therapy (Günter & Machens , 2012).

1.2.2 Superficial Partial-thickness wounds extend over epidermis and superficial parts of the dermis mainly the papillary layer of the dermis (Gomez & Canico, 2007). Superficial partial-thickness burns form blisters, the base of which may be extremely painful if exposed to air (Günter & Machens , 2012).

1.2.3 Deep partial-thickness wounds covers the injury to the epidermis and deep dermis layers i.e. the papillary and the reticular layers of the dermis (Gomez & Canico, 2007). In such an injury, the necrotic layers of the skin should be removed by surgical debridement most of the times (Günter & Machens , 2012).

1.2.4 In full-thickness wounds the subcutaneous tissue layer is damaged (Korting, Schollmann, & White, 2011). If an acute full-thickness skin injury has occurred, any underlying tissue will be exposed to infection and trauma because the protecting barriers are being lost, namely the epidermis and dermis (Günter & Machens , 2012). Therefore, wound closure with sterility in such a case would be the best treatment approach (Günter & Machens , 2012).

1.3 Wound Healing

Wound healing is recognized as an important process for the survival of all living organisms. It is regulated by a complex interplay between cells, signaling pathways, and extracellular matrix.

An effective restoration of barrier function and its normal anatomic structure are associated with healing of any type of wound (Lazarus, et al., 1994), although scar formation may be possible (Korting, Schollmann, & White, 2011). This process of restoring skin to its original structure after any injury is called wound healing. However, wound healing is a complicated process formed of overlapping stages: Inflammation or Homeostasis, Proliferation, and Remodeling or Maturation. Therefore, skin healing of a wound "displays an extraordinary mechanism of cascading cellular functions which is unique in nature" (Reinke & Sorg, 2012).

1.4 Physiology of Adult Skin Wound Healing

Skin wound healing begins directly after wounding and might last for years. It is a dynamic process that is highly regulated by cellular, humoral and molecular mechanisms. Regeneration is a specific substitution of a tissue, i.e. the superficial epidermis, mucosa or fetal skin. However, skin repair is an unspecific form of healing

whereby fibrosis and scar formation are the result (Reinke & Sorg, 2012). The latter, unfortunately, takes place mainly in adult skin wound healing. Wound healing resembles an orchestra or acts of a drama (Krafts, 2010; Enoch & Leaper, Basic science of wound healing, 2005). A deficiency of a cell type or the absence of a mediator could be compensated for by others that are involved in wound healing; hence, the repair can still occur (Eming, Krieg, & Davidson, 2007). The acute wound healing process can be divided into 3 overlapping stages defined below.

1.4.1 Stage one: Hemostasis

This stage takes place immediately after injury and lasts for some hours. In this phase, the inflammatory process is initiated. The inflammatory phase of the wound healing cascade gets activated during the coagulation phase and can roughly be divided into an early phase with neutrophil recruitment and a late phase with the appearance and transformation of monocytes (Reinke & Sorg, 2012; DiPietro & Polverine, 1993).

This phase is also called the ‘lag-phase’, in which the organism manages the enrolment of many cells and factors for the healing process with no mechanical strength in the wound yet (Robson, Steed, & Franz, Wound healing: biological features and approaches to maximize healing trajectories, 2001). The different clotting cascades are then initiated by extrinsic clotting factors from the injured skin in addition to the intrinsic thrombocytes that get activated by exposed collagen for aggregation.

At the same time, the injured vessels go through a 5 to 10-min vasoconstriction caused by the platelets in order to reduce blood loss and fill the tissue gap by a blood clot composed of cytokines and growth factors (Martin, 1997). The blood clot contains fibrin molecules, fibronectin, vitronectin and thrombospondins, thus, forming a

provisional matrix as a scaffold for many cells to migrate; of these cells are leukocytes, keratinocytes, fibroblasts and endothelial cells. This matrix also acts as a reservoir of growth factors and the vasoconstriction is life-saving whereby clot formation causes a local perfusion failure. This lack of perfusion will result in a consecutive lack of oxygen, increased glycolysis and changes in pH (Woo, Park, Subeita, & Brennan, 2004). A vasodilation, in which thrombocytes invade the provisional wound matrix, will then follow the vasoconstriction (Robson, Steed, & Franz, 2001). In addition, the infiltration of leukocytes is influenced by chemotactic factors secreted by platelets, mast cells, and leukocytes would release cytokines and growth factors to: (1) initiate the inflammatory process (IL-1α, IL-1β, IL-6 and TNF-α), (2) stimulate the collagen synthesis (FGF-2, IGF-1, TGF- β), (3) activate the transformation of fibroblasts to myofibroblasts (TGF-β), (4) start the capillary sprouting (FGF-2, VEGF-A, HIF-1 α, TGF-β) (Werner & Grose, 2003). Vasodilation can be recognized by an edema (hyperemia) and by a local redness of the wound (Reinke & Sorg, 2012; Bauer, 2005).

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

About three days after injury, macrophages move to the injury zone to support the ongoing process. They perform phagocytosis of pathogens and cell debris

(Tziotzios, Profyris, & Sterling, 2012; Profyris, Tziotzios, & Do Vale, 2012), in addition to the secretion of growth factors, chemokines and cytokines. Furthermore, these secretions keep the healing process intact, because some of them are able to initiate the next phase of wound healing: the proliferative phase (Gurtner, Werner, Barrandon, & Longaker, 2008). The inflammatory response is very essential as it supplies growth factors and cytokine signals that regulate cellular and tissue movements, which are very crucial for the wound repair mechanisms in adult mammalians (Eming, Krieg, & Davidson, 2007; Leibovich & Ross, 1975). Evidence suggests that the severity of inflammation is very important in the extent of scar formation.

Macrophages have many functions in wound healing. Some of these are host defense, promotion and resolution of inflammation. In addition, they remove apoptotic cells, support cell proliferation, and restore tissue after an injury (Koh & DiPietro, 2011). They also function as antigen-presenting cells. Moreover, phagocytes during wound repair play an integral role in a successful healing response via the synthesis and secretion of numerous potent growth factors such as TGF- β, TGF- α, basic FGF, PDGF and VEGF. These growth factors promote cell proliferation and synthesis of extracellular matrix (ECM) molecules by cells present in skin (DiPietro & Polverine, 1993).

1.4.2 Stage two: Proliferation

The second stage is the phase of proliferation. It lasts for 3–10 days after wound infliction. In this stage, the main aim of the healing process is to cover the wound surface, to form a granulation tissue, and to restore the vascularization. Therefore, besides migration “of local fibroblasts along the fibrin network and the

beginning of reepithelialization from the wound edges" (Robson, Steed, & Franz, 2001), neovascularization and angiogenesis are triggered by capillary sprouting (Bauer, 2005; Arnold & West, 1991; Endrich & Menger, 2000). Granulated tissue formation stops via apoptosis, thus forming a mature wound being avascular and acellular (Reinke & Sorg, 2012; Endrich & Menger, 2000). Besides, the basis of a new matrix of connective is regulated by cytokines like IFN-γ and TGF-β, whereby, collagen synthesis, fibronectin, and other basic substances, needed for wound healing by fibroblasts, are used for the closure of tissue gaps and the restoration of the mechanical strength of the wound. "Subsequently, the synthesis of collagen increases throughout the wound, while the proliferation of fibroblasts declines successively, adjusting a balance between synthesis and degradation of the ECM" (Maden & Peacock, 1971).

Reepithelialization is ensured by local keratinocytes at the wound edges in addition to epithelial stem cells located at hair follicles or sweat glands (Martin, 1997; Lau, Paus, Tiede, Day, & Bayat, 2009; Miller, Bruke, Rader, Coulombe, & Lavker, 1998; Roh & lyle, 2006). This process of covering the wound by epithelial lining is activated by signaling pathways of epithelial and nonepithelial cells from nearby intact skin, thus releasing a myriad of different cytokines and growth factors: EGF, KGF, IGF-1 and NGF (Werner & Grose, 2003) among others. "Furthermore, the abolition of the contact inhibition and physical tension at desmosomes and hemidesmosomes produces lipid mediators and activates membrane-associated kinases (SRC kinases) resulting in an increased permeability of the membranes for ions, e.g. calcium" (Reinke & Sorg, 2012). This signaling to the cells at wound edges causes retraction and reorganization of their intracellular tonofilaments to adjust direction of the cellular migration. Intercellular desmosomes' loosening via collagenase and elastase enzymatic activity

causes keratinocytes to migrate along the maintained fibrin blood clot in the higher layers of the granulation tissue (Reinke & Sorg, 2012). This process is called the 'shuffling' of keratinocytes (Jacinto, Martinez-Arias, & Martin, 2001) and is characterized by the ability of such cells to migrate along a chemotactic gradient formed by mediators e.g. IL-1, and over a fibronectin-rich matrix to the wound's center (Clark, et al., 1982; Clark, Fibronectin in the skin, 1981).

The migration itself is accomplished via lamellipodial crawling and is directed into the defective site due to the polymerization of cytoskeletal actin fibers. The formation of a new focal adhesion at the ECM, which is regulated by integrins (Korting, Schollmann, & White, 2011), proceeds until the migrating cells touch each other which then leads to a reorganization of the cytoskeleton (Jacinto, Martinez-Arias, & Martin, 2001).

Binding of growth factors to their receptors on the endothelial cells of existing vessels is the first step in neovascularization, thereby activating intracellular signaling cascades. Endothelial cells that are activated will secrete proteolytic enzymes to dissolve the basal lamina and thus be able to proliferate and migrate into the wound area. This process is called 'sprouting'. The endothelial cells orient themselves at superficial adhesion molecules, e.g. integrins ($\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha 5\beta 1$). Then, they will exocytose matrix metalloproteinases at the front of proliferation to lyse surrounding tissue and hence support endothelial proliferation. Small tubular canals form the newly built sprouts. In turn, the sprouts will interconnect to others forming a vessel loop. The latter new vessels will differentiate into arteries and venules. Stabilization of these mature vessels is established via pericytes and smooth muscle cells' recruitment (Reinke & Sorg, 2012).

The last step in the proliferation phase is the formation of an acute granulation tissue. As the acute granulation tissue is a transitional tissue, it replaces the fibrin/fibronectin-based provisional wound matrix. Hence, it might produce a scar by maturation (Krafts, 2010) (Enoch & Leaper, Basic science of wound healing, 2005; Robson, Steed, & Franz, 2001; Martin, 1997; Gurtner, Werner, Barrandon, & Longaker, 2008; Nauta, Gurtner, & Longaker, 2011). Furthermore, it is composed of highly dense fibroblasts, granulocytes, macrophages, capillaries and loosely organized collagen bundles. This high amount of cellular compounds gives the granulation tissue its name. As angiogenesis is not yet completed, the tissue is highly vascular (Reinke & Sorg, 2012) making it appear with a classic redness that might be traumatized easily (Korting, Schollmann, & White, 2011). However, fibroblasts dominate in this phase, fulfilling different functions such as collagen bundles formation and ECM substances production (i.e. fibronectin, glycosaminoglycans, proteoglycans and hyaluronic acid) (Reinke & Sorg, 2012). ECM at this stage presents a scaffold for cellular adhesion in addition to regulation and organization of growth, movement, and cells' differentiation within it (Eckes, Nischt, & Krieg, 2010; Barker T. H., 2011). Fibroblasts are precursors of the provisional wound matrix on and in which cell migration and organization occurs (Jacinto, Martinez-Arias, & Martin, 2001). By the end of this phase, the numbers of mature fibroblasts will be reduced by myofibroblast differentiation and consecutively terminated by apoptosis (Hinz, 2007).

1.4.3 Stage three: Remodeling

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

A mature wound is, therefore, characterized as being avascular as well as acellular (Greenhalgh, 1998). During the maturation of the wound, the components of the ECM undergo changes. Collagen III, which was produced in the proliferative phase, is now replaced by the stronger collagen I (Reinke & Sorg, 2012; Guadry, et al., 1997). This type of collagen is oriented in small parallel bundles and is, therefore, different from the basket-woven collagen in healthy dermis (Gurtner & Evand, 2000). Later on, the myofibroblasts cause wound contraction by their multiple attachments to collagen and help decrease the surface of the developing scar (Tziotzios, Profyris, & Sterling, 2012; Profyris, Tziotzios, & Do Vale, 2012; Gurtner & Evand, 2000). Furthermore, the angiogenic processes diminishes, the wound blood flow declines, and the acute wound metabolic activity slows down and finally stops (Reinke & Sorg, 2012; Arnold & West, 1991).

1.4.4 Scarring

The physiological endpoint of mammalian wound repair displays the formation of a scar, which is directly linked to the extent of the inflammatory process throughout wound healing (Reinke & Sorg, 2012; Akaishi, Ogawa, & Hyakusoku, 2008). There are different situations which provide evidence that inflammation during the process of wound healing is directly linked to the extent of scar formation (Eming, Krieg, & Davidson, 2007). First, there is the fact that fetal wound healing, which shows a lack of the typical inflammatory response, is scarless up to a certain age (Bullard, Longaker, &

Lorenz, 2003; Redd, Cooper, Wood, Stramer, & Martin, 2004). In addition, scar formation seems to be extended when inflammation in fetal wounds is induced (Whitby & Ferguson, 1991). A second example indicating the role of inflammation on the formation of scars is the influence of reproductive hormones on this process (Reinke & Sorg, 2012). Studies showed that low estrogen levels in mice resulted in an impaired rate of healing, thus inducing excessive inflammation and scarring (Eming, Krieg, & Davidson, 2007; Aschroft, et al., 2003).

Most non-healing wounds fail to progress through the normal phases of wound repair, and remain in a chronic inflammatory state (Loots, et al., 1998) which leads to abnormal wound repair, e.g. to hypertrophic or keloid scars. Keloids contain thick collagen fibers, whereas hypertrophic scars contain thin fibers which are organized into nodules (Ehrlich, et al., 1994; Verhaegen, et al., 2009). Changes in collagen maturation are fundamental changes in excessive scar formation. The granulation tissue continues to grow due to the excessive secretion of growth factors and the lack of molecules required for apoptosis or ECM remodeling (Reinke & Sorg, 2012; Darby, et al., 2002). Hypertrophic scars contain excessive microvessels, which are mostly occluded due to the over proliferation and functional regression of endothelial cells induced by myofibroblast hyperactivity and excessive collagen production (Xi-Qiao, Ying-Kai, Chun, & Shu-Liang, 2009). Focal upregulation of p53 expression, which seems to play an important role in the inhibition of apoptosis, has been reported in situations of excessive scarring (Reinke & Sorg, 2012; Darby, et al., 2002). Furthermore, mechanical irritation in the early proliferative phase leads to hypertrophic scars by inhibiting apoptosis (Arabi, et al., 2007). Changes in the ECM and epithelium seem to be involved in excessive scarring also (Darby, et al., 2002; Bellemare, et al., 2005; Dabiri,

Tumbarello, Turner, & Van de Water, 2008). A neurogenic inflammation hypothesis has been suggested whereby, mechanical stress stimulates mechanosensitive nociceptors in skin sensory fibers that release neuropeptides involved in vessel modifications and fibroblast activation (Korting, Schollmann, & White, 2011). In brief, excessive scarring is a fibrotic disorder resulting from the disruption of the normal wound healing process (Sarrazy, Billet, Micallef, Coulomb, & Desmoulière, 2011).

1.5 Growth Factors and Cytokines in Wound Healing

An increased understanding of the molecular mechanisms that regulate the various events of wound healing has laid the foundation for therapeutic interventions attempting to improve the healing outcome (Kiwanuka, Junker, & Eriksson, 2012). The body's response to skin injury is focused on rapid wound closure, restraining invasion of microorganisms and preventing excessive fluid loss (Gurtner, Werner, Barrandon, & Longaker, 2008; Singer & Clarl, 1999; Aarabi, Longaker, & Gurtner, 2007; Mustoe, 2004).

1.5.1 Cytokines in Wound Healing

Cytokines are peptides and glycoproteins with a molecular weight of 5 to 30 kDa and are primarily produced by inflammatory cells (Rumalla & Borah , 2001). Cytokines regulate inflammatory and immune responses during wound healing by activating various cells (Kiwanuka, Junker, & Eriksson, 2012). The cytokines include chemokines, lymphokines, monokines, interleukins, colony-stimulating factors, and interferons. They can be distinguished from growth factors by the type of cells they influence (Kiwanuka, Junker, & Eriksson, 2012). Inflammatory cytokines are believed to have roles in wound healing, including migration and proliferation of keratinocytes and fibroblasts (Holman & Kalaaji, 2006). Interleukins 1 and 6 (IL-1 and IL-6) and

tumor necrosis factor a (TNF-a) are upregulated during the inflammatory phase of wound healing and are important in modulating reepithelialization (Werner & Grose, 2003). The granulocyte-macrophage colony-stimulating factor (GM-CSF) is involved in the activation of neutrophils and macrophages, and alters the activity of keratinocytes and fibroblasts (Hu, Sun, Han, Wang, & Yu, 2011).

1.5.2 Interleukins in Wound Healing

In an acute wound, IL-1β is produced by monocytes, macrophages, fibroblasts, and keratinocytes (Sauder, et al., 1990; Goretsky, Harriger, Greenhalgh, & Boyce, 1996; Finnerty, et al., 2006). Besides serving as a paracrine factor, IL-1 acts as an autocrine signal that induces the migration and proliferation of keratinocytes (Freedberg, Tomic-Canic, Komine, & Blumenberg, 2001; Chen, Lapiere, Sauder, Peavey, & Woodley, 1995; Gyulai, Hunyadi, Kenderessy-Szabo, Kemeny, & Dobozy, 1994). Exogenously administered IL-1 has been shown to promote healing of partial-thickness wounds in swine (Singer, McClain, Hacht, Batchkina, & Simon, 2006).

1.5.3 TNF-α in Wound Healing

Similar to IL-1, TNF-α is a proinflammatory cytokine that is produced by many cell types during wound healing (Brauchle, Angermeyer, Hubner, & Werner, 1994). The TNF-α level is elevated in chronic wounds, and its expression diminishes as healing progresses (Kiwanuka, Junker, & Eriksson, 2012).

1.5.4 GM-CSF in Wound Healing

GM-CSF is one of the most widely studied cytokines in wound healing (Hu, Sun, Han, Wang, & Yu, 2011; Malik, et al., 1998; Jaschke, Zabernigg, & Gattringer, 1999). Besides being a potent activator of neutrophils and macrophages, GM-CSF

influences the activity of keratinocytes and fibroblasts and increases the production of vascular endothelial growth factor (VEGF) (Cruciani, Lipsky, Mengoli, & de Lalla, 2005).

1.5.5 Growth Factors in Wound Healing

Growth factors are regulatory peptides that are synthesized and secreted by many of the cell types involved in wound healing, including inflammatory cells, platelets, fibroblasts, epithelial cells, and endothelial cells (Bennet & Schultz, 1993). Growth factors cause cells to migrate by chemotaxis, proliferate, differentiate, and synthesize extracellular matrix components (Robson, Robson MC. 1991). Growth factors may act by autocrine, paracrine, juxtacrine, or endocrine signaling (Kiwanuka, Junker, & Eriksson, 2012). On binding to the cell receptors, the growth factors trigger a cascade of intracellular events, leading to the activation of transcription factors, which results in gene expression (Kiwanuka, Junker, & Eriksson, 2012).

Growth factors are classified into several families based on their characteristics (Kiwanuka, Junker, & Eriksson, 2012). The most relevant growth factor families for wound healing are the epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF) and VEGF (Kiwanuka, Junker, & Eriksson, 2012).

1.5.5.1 EGF Family

The EGF family is composed of: EGF, TGF-α, Amphiregulin and Heparin-binding EGF (Kiwanuka, Junker, & Eriksson, 2012). EGF was first isolated from the submandibular glands of rats but is primarily produced by platelets and macrophages

(Cohen, 1962). The roles of the EGFs and growth factors that signal through the EGF receptor (EGFR) are well characterized.

1.5.5.2 FGF Family

The FGF family includes more than 20 growth factors (Kiwanuka, Junker, & Eriksson, 2012). However the most relevant to wound healing are: FGF-2, FGF-7, FGF-10, and FGF-22 (Kiwanuka, Junker, & Eriksson, 2012). FGF-7 is also known as the keratinocyte growth factor 1 (KGF-1) and is secreted by dermal fibroblasts and T cells (Jameson, et al., 2002; auf demKeller, Krampet, Kumin, Braun, & Werner, 2004; Steiling & Werner, 2003). Moreover, FGF-2, also known as basic FGF, is released from damaged endothelial cells and is one of the most potent isoforms (Kiwanuka, Junker, & Eriksson, 2012). FGF-2 is a mitogenic and chemotactic factor for fibroblasts and endothelial cells, and stimulates angiogenesis (Kiwanuka, Junker, & Eriksson, 2012). Its concentration is upregulated during wound healing, and exogenous administration of FGF-2 has been shown to increase wound reepithelialization (Sanz Gracia, et al., 2000; Hebda, Klingbeil, Abraham, & Fiddes, 1990).

1.5.5.3 TGF-β Family

The TGF-β family consists of: TGF-β1-3, Bone Morphogenetic Proteins (BMPs), Activins, Nodals, Growth and differentiation factors and Mullerian-inhibiting substances (Shi & Massague, 2003). The TGF-β superfamily is so named because it was believed to induce normal cells to become malignant (Broughton, Janis, & Attinger, 2006). At present, TGF-β cells are seen as important regulators of wound healing.

1.5.5.4 PDGF Family

The PDGF is the first and only recombinant growth factor to be approved by the FDA for topical administration to wounds. The PDGF family consists of several homodimeric or heterodimeric growth factors including: PDGF-AA, PDGF-BB, and PDGF-AB. PDGF is produced by platelets, macrophages, endothelial cells, fibroblasts and keratinocytes (Kiwanuka, Junker, & Eriksson, 2012). Each of these cells plays a role in each phase of wound healing (Niessen, Andriessen, Schalkwijk, Visser, & Timens, 2001; Uutela, et al., 2004).

1.5.5.5 VEGF Family

The members of the VEGF family are VEGF-A-E in addition to the placental growth factor (Kiwanuka, Junker, & Eriksson, 2012). The VEGF is produced by a variety of cell types during wound healing, and is a potent stimulator of proliferation and migration in endothelial cells (Nissen, et al., 1998; Guadry, et al., 1997).

1.6 The Nervous System and Wound Healing

The nervous system is present in the skin, especially in the dermis, as they share similar embryonic origin. It forms a complex 3-dimensional network, modulating immune and inflammatory responses, controlling thermoregulation and homeostasis, and is being involved in wound healing and skin appendages regulations (Besne, Descombes, & Breton, 2002; Esteves Junior, et al., 2009; Ferreira, Gragnani, Furtado, & Hochman, 2009; Liang, et al., 2004). Neurogenic inflammatory disorders have been linked to various skin diseases including psoriasis (Saraceno, Kleyn, Terenghi, & Griffiths, 2006), atopic dermatitis (Misery, 2011), and contact dermatitis (Pavlovic, et al., 2011). The neurogenic inflammation concept was conceived after discovering that primary afferents stimulation mediates inflammatory effects (Jancso, 1960).

“Neurogenic inflammation is caused by antidromic activation of nerve endings by exogenous stimuli” (Besne, Descombes, & Breton, 2002; Pavlovic, et al., 2011; Roosterman, Goerge, Schneider, Bunnett, & Steinhoff, 2006; Caviedes-Bucheli, Munoz, Azuero-Holguin, & Ulate, 2008).

In neurogenic inflammation, dermal and epidermal nerve endings secrete proinflammatory cutaneous neuropeptides, such as substance P (SP) and calcitonin gene-related peptide (CGRP), in the proximal direction (Lapin, et al., 2015). These neuropeptides affect and modulate the inflammatory and immune responses in the skin via different pathways such as paracrine, juxtacrine, and endocrine pathways (Peters, et al., 2006).

“Substance P is an 11-amino-acid peptide of the tachykinin family that is released from nerve endings and acts through neurokinin 1 receptors causing degranulation of mast cells and activation of keratinocytes, fibroblasts, macrophages, and B and T lymphocytes” (Lapin, et al., 2015), including vasodilation (flare), plasma extravasation (Chahl, 1988; Maggi, 1991), changes in smooth muscle contractility, an increase in fibroblast activity, and effects on leukocytes” and vice versa (Arvier, Chahl, & Ladd, 1977; Levine, Dardick, Roizen, Helms, & Basbaum, 1986). The latter cells, then, synthesize and secrete immunomodulators and proinflammatory agents like histamine, tumor necrosis factor α, prostaglandin D2, leukotriene B4, neurokinin 1, and interleukins IL-1 α, IL-1β, and IL-8 that initiate chemotaxis of neutrophils and eosinophils (Misery, 2011; Pavlovic, et al., 2011), in addition to activation of the immune system, and wound healing. Substance P also shows involvement in inflammatory dermatoses such as atopic dermatitis, where its release, triggered by stress, greatly enhances the inflammation of skin lesions (Lapin, et al., 2015). It can be

summed up that SP is one of the endocrine mediators of the immune and nervous system (Saraceno, Kleyn, Terenghi, & Griffiths, 2006; Misery, 2011).

On the other hand, "calcitonin gene-related peptide is a 37-amino-acid neuropeptide that has 2 forms in humans, β- and α-CGRP, which differ by 3 amino acids" (Schmelz & Petersen, 2001). β-CGRP is mainly released in skin by nerve endings, on the other hand, α-CGRP is present in the epidermis especially on enteric nerve endings (Schmelz & Petersen, 2001). CGRP is commonly found in the same nerve fibers as SP in skin (Steinhoff, et al., 2003). Upon its secretion, "CGRP activates β-CGRP and α-CGRP receptors on mast cells, macrophages, fibroblasts, melanocytes, dendritic cells, keratinocytes, and B and T lymphocytes". CGRP is involved in complex processes, especially those related to proinflammatory activities in the acute phase of inflammation. CGRP has many important "local effects including vasodilation, T-lymphocyte suppression, modulation of wound healing, chemotaxis of inflammatory cells, and consequent pain modulation" (Moore & Salvatore, 2012; Brain & Grant, 2004; Birklein & Schmelz, 2008).

Other evidence indicates another major role for sympathetic postganglionic neurons (SPGNs) in neurogenic inflammation, where they also secrete SP and CGRP. It has also been reported that in wound healing is delayed in denervated cutaneous wounds (Barker, Rosson, & Dellon, 2006; Fukai, Takeda, & Uchinuma, 2005; Younan, et al., 2010).

1.6.1 Resiniferatoxin mode of action

TRP channels are involved in cellular homeostasis and growth control, regulation of cell fate and survival, immune and inflammatory mechanisms, and endocrine and exocrine secretory processes (Nilius & Owslanik, 2010; Boesmans,

Owslanik, Tack, Voets, & Vanden Berghe, 2011; Denda & Tsutsumi, 2011; Moran, McAlexander, Bíró, & Szallasi, 2011; Fernandes, Fernandes, & Keeble, 2012). Resiniferatoxin (RTX), an excitotoxic agonist for transient receptor potential vanilloid receptor 1 (TPRV1) (Tender, Li, & Cui, 2013). TPRV1 provides responses to temperature, pH and endogenous lipids (Alawi & Keeble, 2010). When activated TPRV1 may open transiently and initiate depolarization mediated influx of sodium and calcium ions resulting in action potentials in nociceptive sensory nerves like capsaicin-sensitive fibers and some Aδ fibers. These signals may be interpreted by the brain and/or spinal cord as warming, burning, stinging or itching sensations. However, stimulation by agonists such as RTX and capsaicin can generate biochemical signals with persistent effects. High concentrations of RTX lead to impaired local nociceptor function to extended periods (Bley, 2010).

Chemical ablation of sympathetic fibers with either 6-hydroxy dopamine (6-OHDA) (Helme & Andrews, 1985; Lee, Coderre, Basbaum, & Levine, 1991), guanethidine (Green, 1974; Loh & Nathan, 1978), or reserpine (Gozsy & Kato, 1966) significantly decreases vascular permeability. On the other hand, chemical and electrical sympathetic stimulation lead to vasodilation and increased plasma extravasation in both rat and human skin (Gozsy & Kato, 1966).

1.6.2 Guanethidine Mode of Action

Guanethidine has been reported to deplete peripheral noradrenaline stores (Cass, Kuntzman, & Brodie, 1960; Devine, Robertson, & Simpson, 1967). Guanethidine has been shown to diminish noradrenaline accumulation and dense-cored vesicles of sympathetic nerves in vitro in a dose-dependent manner (Tomlinson, Mayor, Mitchel, &

Banks, 1971). Guanethidine, like reserpine, increases deaminated catechols in urine when administered parenterally to rats (Kopin & Gordon, 1963). Thus indicating that the catecholamines removed from their intravesicular storage sites by the action of guanethidine were broken down by monoamine oxidase. Guanethidine, at high doses in vivo and high concentrations in vitro, interferes with noradrenaline uptake and retention by the amine-storage granules (Tomlinson, Mayor, Mitchel, & Banks, 1971). Guanethidine reduces the release of norepinephrine, by displacing it from the presynaptic release vesicles, resulting in a chemical sympathectomy (Gil, et al., 2016).

1.6.3 Nerve Growth Factor (NGF)

NGF was the first to be isolated of the neurotrophin family and the best-characterized member. NGF has a critical role in survival, differentiation, and function of peripheral sensory and sympathetic nerves and brain neurons of mammals (Sofroniew, Howe, & Mobley, 2001). NGF has been assigned as a neurotrophic and immunomodulatory factor and in control of cutaneous morphogenesis, wound healing and inflammatory processes (Lambiase, Manni, Rama, & Bonini, 2003). “It was originally recognized for its effects in stimulating growth and differentiation in neurons” (Jurjus, et al., 2007). Evidence has shown NGF to play a renowned role in promoting healing processes (Matsuda, et al., 1998). “NGF levels are significantly higher at wounded sites after skin punching than at uninjured control skin sites” (Chen, et al., 2014). NGF is responsible for many activities in the immune system and beyond (Micera, Puxeddu, Aloe, & Levi-Schaffer, 2003). Although NGF is considered a pro-inflammatory factor, it is also pro-angiogenic (Graiani, et al., 2004). Vasoactive neuropeptides dilate blood vessels hence delivering more serum proteins at wound site (Graiani, et al., 2004). Neuropeptides modulate pain protecting from further injury

(Cruise, Xu, & Hall, 2004). “This may partly explain why sensory innervation is so critical for wound healing” (Jurjus, et al., 2007).

“NGF was demonstrated to be an important component of wound healing and tissue repair process both in vivo and in vitro” (Graiani, et al., 2004; Muangman, et al., 2004; Kawamoto & Matsuda, 2004; Shi, Qu, & Cheng, 2003). The removal of the tissues that store large amounts of NGF affects recovery in mouse wounded skin healing considerably, while exogenous NGF added at the site of injury markedly accelerated wound contraction rate (Li, Koroly, Schattenkerk, Malt, & Young, 1980). NGF stimulates proliferation and inhibits apoptosis of keratinocytes and thus wound healing kinetics are improved by promoting epithelialization (Kawamoto & Matsuda, 2004). It is reported that NGF has a robust effect on fibroblast migration, “a favorable factor for early re-epithelialization” (Jurjus, et al., 2007). Clinical experience clearly shows that NGF topical administration accelerates the healing of surgical corneal wounds effectively (Genirini, et al., 2004). “NGF promotes healing of diabetic foot ulcers” (Genirini, et al., 2004), and may be an effective therapy for severe pressure ulcers in patients (Landi, et al., 2003). “Experiments demonstrated that local NGF administration prevented diabetes-induced expressional alterations, enhanced reparative capillarization, and accelerated wound healing” (Jurjus, et al., 2007). Topical administration of NGF was associated with a three-fold increase in endothelial cell proliferation, and 50% reduction in apoptosis (Jurjus, et al., 2007). “NGF increased also the release of immunoreactive vascular endothelial growth factor-A (VEGF-A)” (Graiani, et al., 2004).

1.7 Aim

We hypothesize that sympathetic and capsaicin-sensitive fibers denervation affects burn wound healing. This study aimed to determine the effect of the nervous system on wound healing via its interactions with the inflammatory response initiated by burn wound inflection in a rat model.

Specifically, this research assessed the effect of denervation by chemical ablation on the burn wound healing process using guanethidine for the sympathetic postganglionic neurons and resiniferatoxin for the Capsaicin-sensitive fibers.

2 MATERIALS AND METHODS

The effect of skin denervation on the healing process of burn wound was assessed in the experimental rat model through a series of parameters:

(1) Clinical observation and gross inspection of wound healing

(2) Comparison of the histopathological changes over time

(3) Measurement of the changing rates of NGF, IL-1β, IL-6, and IL-8 at various time points.

2.1 Animals:

A total of 143 adult females Sprague-Dawley rats (250-300g) were used in this experiment after approval of the Institutional Animal Care and Use Committee at the American University of Beirut. The animals were randomly assigned to 2 major groups:

2.1.1 Group one included 54 rats which were devoted to assess the effect of resiniferatoxin (RTX), the chemical that can ablate capsaicin-sensitive primary afferent fibers. The 54 animals in this group were divided into 3 subgroups A, B and C:

2.1.1.1 Subgroup A (RTXB) consisted of 30 rats that were subjected to burning and treated with RTX

2.1.1.2 Subgroup B (RTXnB) consisted of 15 rats that were treated with RTX but were not subjected to burn

2.1.1.3 Subgroup C (VRTX) consisted of 9 animals that were subjected to burning and received only the vehicle for RTX, namely absolute alcohol.

2.1.2 Group two consisted of 54 rats and was subdivided into similar subgroups like in Group One with the only difference being the treatment by Guanethidine (G) instead of RTX. Similarly there were three other subgroups; D treated with Guanethidine and subjected to burn (GB), E treated with guanethidine only GnB, and F burned and treated with the vehicle (VG), namely physiological saline.

2.1.3 Group three included 30 rats that were all subjected to burning without any treatment.

2.1.4 Group four included 5 rats which were left untouched and were considered as normal healthy controls.

Rats that underwent a standardized burning procedure were left to heal without any dressing. They were housed throughout the experiment on 12 h light/dark cycles with temperature of about 22 to 24^0C. They had access to standard rodent chow and water ad libitum. Animals from each group were sacrificed at multiple time points on days 0, 3, 8, 14, 21 and 28 as these dates are standards in wound healing literature.As for the normal healthy controls, they served all time points.

2.2 Pre-operative preparation

The backs of all animals were shaved with a commercial electric shaving machine one day before the burning procedure.

2.2.1 Sensory denervation

Following Ossipov (1999) protocol, sensory ablation was achieved by intra-peritoneal injection of Resiniferatoxin, 0.1mg/kg dissolved in 100% pure ethanol (Ossipov, Bian, Malan Jr, Lai, & Porreca, 1999) . Desensitization was verified after 3 days by the indifference to a corneal application of capsaicin in the RTX group (Hammond & Ruda, 1991).

2.2.2 Peripheral block of sympathetic efferents

This block was performed according to Coderre et al.'s protocol (Coderre, Basbaum, & Levine, 1989), whereby, Guanethidine (1-[2-guanidinoethyl] octahydroazocine) monosulfate (1:1) (from Sigma) was used to block the sympathetic efferents. It was dissolved in sterile saline, 30 mg/ml, and injected subcutaneously in the area of the burn one hour before burn.

2.2.3 Anesthesia:

Burning was performed under deep anesthesia with a mixture of atropine (atropine sulfate, Laboratoire Aguettant, 0.05 mg/kg) and chlorpromazine (Largactil®, 8 mg/kg), injected intra-peritoneally (i.p.) as pre-anesthetics and followed 10 min later by an i.p. injection of ketamine (Ketalar®, 50 mg/kg).

2.3 Burn injury:

A modified version of the aluminum stamp described by Knabl et al. (Knabl, et al., 1999) has been used. The desired temperature was maintained and controlled via an electronic temperature controller with a thermo-coupling feedback sensor and a dimmer knob. A burn area of 4.9 cm^2 was produced by applying an ordinary soldering iron (20 W) retrofitted with a 2.5 cm diameter aluminum stamp. The desired temperature of 80˚ C was reached after preheating the device for 15 mins. The stamp was applied for 55 sec to produce a consistent deep partial thickness burn (Jurjus, et al., 2007). The iron was held vertically, applying its own weight with no additional pressure to ensure a reproducible experimental burn.

2.4 Treatment:

The experimental burns were left to heal with no dressing or any other interference.

2.5 Biopsy and observational phase:

2.5.1 Macroscopic examination:

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

2.5.2 Wound surface:

Wound surface was measured in square cm (area = πr^2) of the burn as it progressed since the aluminum stamp was circular.

2.5.3 Biopsies:

On days 0, 3, 8, 14, 21, and 28 punch biopsies of 3.5mm diameter were taken, under deep anesthesia, from the rostral (for light microscopy) and caudal parts (for ELISA) of the burn areas. Animals were then sacrificed.

2.6 Light microscopy:

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

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

2.7 Enzyme-Linked ImmunoSorbent Assay (ELISA):

Caudal biopsy samples were used for the quantitative assessment of IL-1β, IL-6, IL-8 and NGF using ELISA. Biopsies were snapped directly into liquid nitrogen then stored in deep freeze at -80^0C until processing.

2.7.1 Tissue Processing:

Collected tissues were homogenized for 45 sec on ice at 21000 rpm using a homogenization probe (Tissue Tearor, Polytron, Biospec Products, Inc.) along with freshly prepared ice-cold extraction buffer (Tris 100mM, NaCl 150mM, EGTA 1mM, EDTA 1mM, Triton X-100 1%, Sodium deoxycholate 0.5%; pH=7.4; 1000µl/tissue) and protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany; 2 tablets/100 ml). The homogenates were then centrifuged at 4°C for 1 hour at a speed of 11,000 rpm (15,000xg) and the supernatants were collected, in sterilized test tubes, and stored at -80°C.

2.7.2 ELISA

ELISA was used to evaluate IL-1β, IL-6, IL-8 and NGF in the supernatants of the homogenized tissues. The protein concentration of each sample was first determined using the BCA protein assay according to the manufacturer's guidelines (Bio-Rad Laboratories, Hercules, CA). The concentrations of NGF were detected using the four-day ELISA kit (R&D Systems, Minneapolis, MN) and following the protocol provided by the manufacturer. The concentrations of each cytokine; however, were determined using a modified two-site sandwich ELISA as previously described (Saadé, et al., 2002; Saab, et al., 2009). First, the ELISA plates were coated with immunoaffinity-purified polyclonal sheep anti-rat IL-1β, IL-6 or IL-8 antibodies diluted in coating buffer and incubated overnight at 4°C (100µl/well; NISBC, England). The plates were washed the second day with wash/dilution buffer, blocked for non-specific binding with a blocking buffer at 37°C (3% BSA, 0.1% Tween 20 in PBS) and then incubated with the samples overnight at 4°C. On the third day, the plates were washed and respective biotin-

conjugated immunoaffinity-purified polyclonal antibody diluted (1:4000) in 1% Normal Sheep Serum (NSS; abcam, Cambridge, UK) was added and incubated at 4°C overnight. On the last day, the plates were washed then loaded with the enzyme streptavidin horse-radish peroxidase (Amersham; diluted 1:8000 in wash buffer containing 1% BSA; 100µl/well) and a tetramethylbenzidine-containing substrate solution was added. Finally, the reaction was stopped by an acidic stop solution (1M H2SO4; 100µl/well) and the optical density was measured using a 450nm filter. Data were then analyzed using a four-parameter logistics curve-fit by Ascent Software for iEMS Reader. Cytokine levels were expressed as picograms per milligram protein.

The protein concentration in the supernatant was quantified using the DC Protein Assay following the manufacturer's instructions (DC Protein Assay Reagent Kit, Bio-Rad) with minor modifications. Samples were pipetted as duplicates (5 µl/well) in a 96 well microtiter plate (Nunc). Each plate was inserted into a plate reader (iEMS Reader MF, Labsystems, Finland) to read the optical density of each well at an absorbance of 750 nm. Data were analyzed using Ascent Software for iEMS Reader (Saadé, et al., 2002; Saab, et al., 2009; Safieh-Garabedian, Poole, Allchorne , Winter, & Woolf, 1995).

2.8 Statistical Analysis:

Microsoft Excel 2013 software was used to determine statistical significance using student T-test double sided with unequal variance when comparing one group to another.

3 RESULTS

3.1 Clinical Observations

Daily observation of the animals showed that at D0, in the non denervated burned group (nDB), the wounds were well delineated with an elevated rim and a high degree of redness (+3). On the other hand, the guanethidine treated animals (GB) presented with a well delineated softer burn and little redness (+1), while the resiniferatoxin treated animals (RTXB) reacted similarly with little redness (+1). The burned spots in the RTXB and GB groups were relatively softer than controls.

On the third day, (+3) redness continued in the nDB group with a relatively harder crest, than in GB (+2) and RTXB (+1), no other major changes were noted.

After the first week, on day 8, the wounds in the nDB animals showed redness of rims (+2) with dryness and hardening of the crusts as well as partial elevation of the rims. On the other hand, the GB animals exhibited less hardening with elevation of the rims and less necrosis and partial redness (+2). The RTXB depicted a clinical picture similar to GB with less redness (+1).

After two weeks, the crust was partially shed away in the nDB group leaving good vascularization behind (+2). In the GB the crust was completely shed away with excellent vascularization or redness (+3). On the other hand, the RTXB wounds showed hard elevated crusts but still in place with a rim of redness (+2).

After 3 weeks, the crust was completely sloughed in GB group but partially remaining in the nDB group. In the RTXB sloughing was partial. Vascularization in all

groups was good, however it was best in GB and least in RTXB treated rats compared to the nDB group.

The burn wounds were checked for the last time on day 28, they all healed and vascularized with hairs starting to grow, however, the RTXB still had a very loose curst and the healing was less advanced.

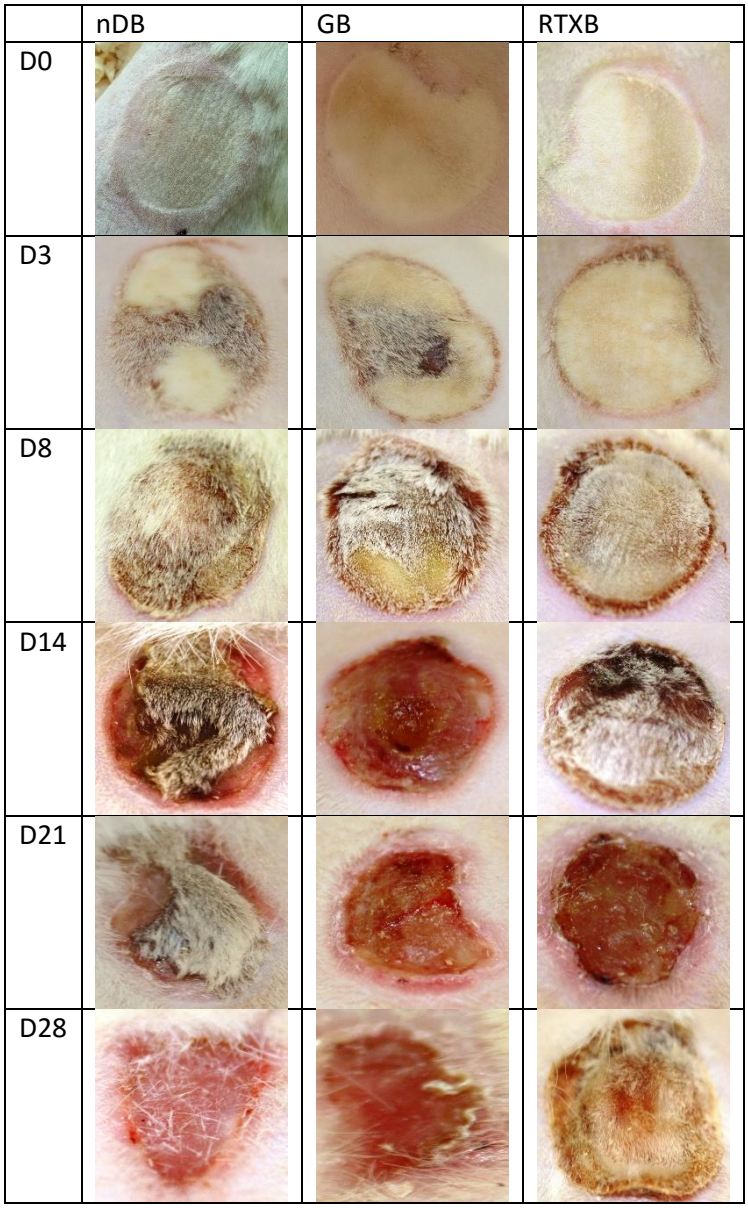

Figure 1. Gross wounds of the different experimental groups at all time points.

3.2 Area of Burn Wound:

The area of burn at the various time points was also considered as valid indicator for the progression of the healing process.

Table 1. Average wound area (in cm2) ± SEM of different experimental groups at all time points.

Time Points	nDB	GB	RTXB
D0	4.91 ± 0	4.91 ± 30	4.91 ± 0
D3	4.91 ± 0	4.91 ± 0	4.91 ± 0
D8	4.91 ± 0	4.91 ± 0	4.91 ± 0
D14	3.90± 0.03	4.18 ± 0.05	3.89 ± 0.07
D21	2.83 ± 0.05	2.60 ± 0.06	2.83 ± 0.07
D28	1.72 ± 0.06	1.08 ± 0.06	2.27 ± 0.06

Measurements of the surface area of the wounds, on a regular basis, showed that during the first week (D0 to D8), there were no changes in the areas or diameters of the burns. However, they significantly differed by the end of the fourth week, with the GB group healing significantly faster with much less surface area of 1.08 ± 0.06 compared to RTXB (2.27 ± 0.06) and nDB group (1.72 ± 0.06).

Such results lead us to conclude that the rate of healing is relatively faster in the GB and slower in the RTXB, compared to controls nDB. In brief, GB treated animals presented relatively faster closure velocity especially after day 21.

According to data in Table 2, which compared the surface area of the wounds inflected in the GB group against the nDB group showed no significant difference except at D28 where P is 0.002. Again comparing RTXB against nDB groups, a p-value of 0.003 indicates a significant difference at one time point (D28). In brief, the closure

of the burn wounds were more advanced and faster in the GB versus nDB versus RTXB. When comparing GB against RTXB there were marked differences at all time points (Table 2) in favor of a faster healing rate in the GB group.

Table 2. Same Day Comparison of Wound Area among experimental groups.

Time points	nDB Vs GB	nDB Vs RTXB	GB Vs RTXB
D0	-	-	-
D3	-	-	-
D8	-	-	-
D14	0.097	0.366	0.241
D21	0.223	0.449	0.76
D28	**0.002**	**0.003**	0.222

N.B. Student T-test analysis. Significance $p \leq 0.05$.

Table 3 shows a comparison of the wound areas within the same group. Accordingly, significant differences exist at three time points for each group. Group GB shows significant differences at three time points, days 14, 21 and 28 where their respective p-values are 0.016, 0.001 and 0.00009, respectively. In the RTXB group a similar result is detected at the same time points and the respective p-values are 0.017, 0.018, and 0.004. Furthermore, in the nDB a statistically significant difference exists at days 14, 21 and 28, very similar to the other groups with corresponding p-values of 0.002, 0.001 and 0.00004, respectively (Table 3).

In brief, the areas of the closing wound on day 28 are smallest for GB and largest for RTXB compared to nDB's.

Table 3. Comparison of Wound Area within each group.

	GB	RTXB	nDB
D3 Vs D0	-	-	-
D8 Vs D0	-	-	-
D14 Vs D0	**0.016**	**0.017**	**0.002**
D21 Vs D0	**0.001**	**0.018**	**0.001**
D28 Vs D0	**0.00009**	**0.004**	**0.00004**

N.B. Student T-test analysis. Significance $p \leq 0.05$.

3.3 Histological Inspection:

3.3.1 Microscopic changes of the skin in various groups across time

- The controls of various categories (CTRL, GnB, RTXnB) showed normal skin histology with distinct well layered epidermis, continuous ducts and glands well delineated along with a well-organized dermis.
- The non-denervated burn wounds (nDB) showed disrupted and sloughed epidermis with edema and disorganized dermis with glands and ducts destroyed. The remnants of such glands reached surface of burned skin. The collagen bundles in the upper dermis were also disorganized. The picture remained as such till day 8 where an epidermal layer or two were growing and bridging the gaps created by the burns.

  On day 14, the epidermal gaps were closed, however, the glands and ducts did not fully recover and collagen bundles were getting reorganized to a good extent.

  By D21 the wounds were fully recovered; the epidermis well layered and the dermis well organized, however, the glands and ducts were not fully organized.

By Day 28 the skin over the wound area was back to normal.

- The GB group showed a similar pattern to nDB except that the edema was less on day 3, by day 8 the epidermal and dermal layers showed less disorganization then the nDB group with the growing of distinct epithelial layers (one or two). On D14, more organized and thicker epidermis and well organized dermis were encountered but the adnexia were not fully recovered until day 21. By day 28, the skin was normal like controls except that the crust present in the RTB to a great extent.
- On the other hand, in the RTXB group the picture on days 0 and 3 was similar to nDB. On day 8, however, disorganization remained very distinct and epidermal growth was minimal; otherwise, the picture was pretty similar to day 8 of nDB and GB.

  Moreover, the picture on day 21 was pretty similar to D14 of GB and nDB and the picture on day 28 was similar to day 21in other groups. In brief, almost one week delay from nDB. With the vehicles, the picture was very much similar to nDB or a little better.

In brief, RTXB delayed the time of healing for at least a weak and the results of the scar was worse. The best healing occurred in the GB group, faster and better quality scar.

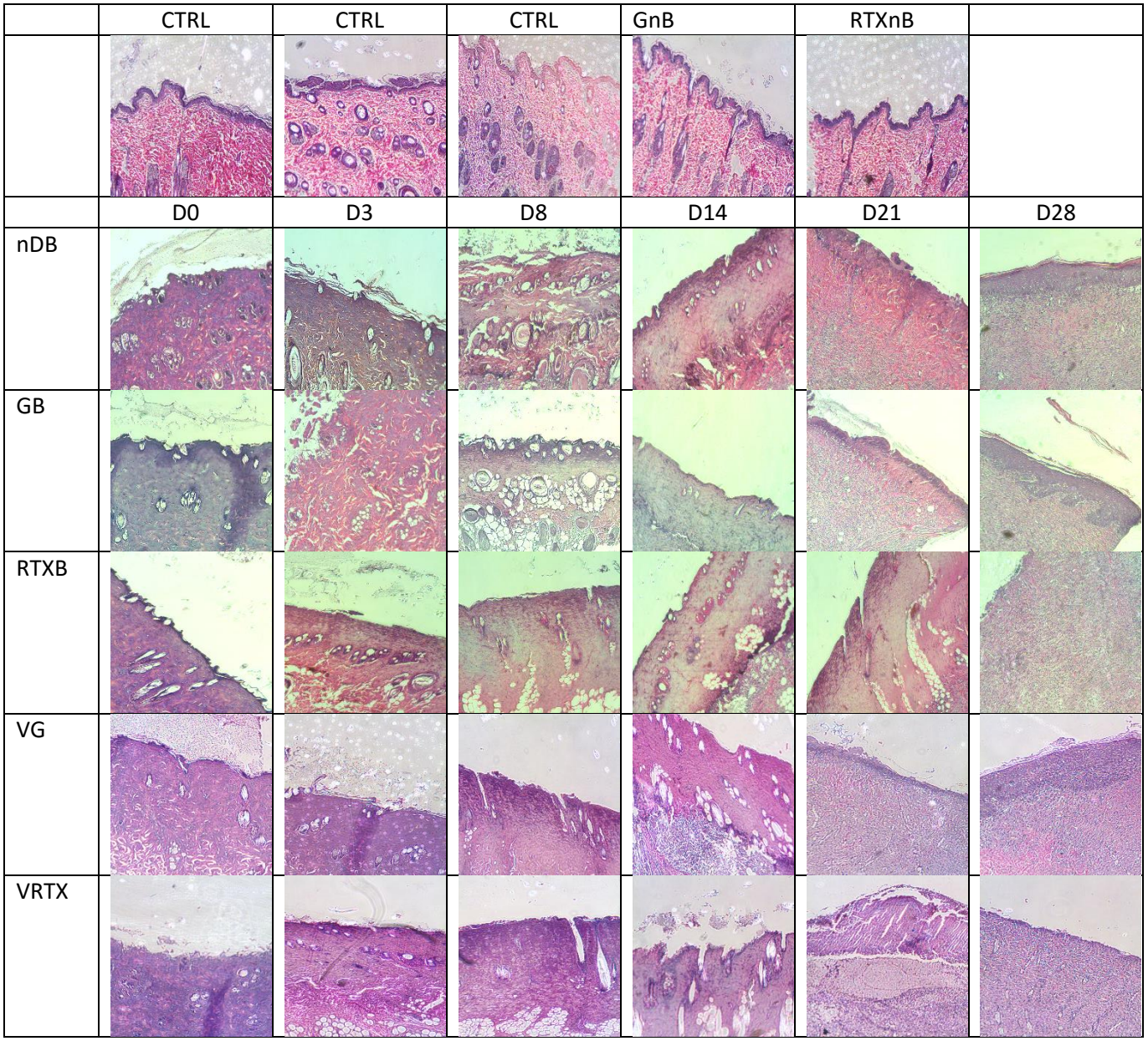

Figure 2. Histological sections of the various experimental groups stained with H&E (x100).

3.4 Mast Cells alterations in the various groups across time and treatment

Mast cells expressed alterations in their relative number in the various groups compared to the CTRL controls which showed 2-3 mast cells per field.

- In the nDB group, mast cells on day 0 were about 6 per field, almost twice the control CTRL group, and kept increasing on day 3 to a slightly higher number 7-8 mast cells per field on the average. On the other hand, they decreased on day 8 with less cells 4-6 cells per field. On D14 it went back to D0 levels 4-6 cells per field, then increased again on D21 to 7 or more, to settle on D28 to a count of 4-6 cells per field.
- In the GB group, the picture on D0 was similar to nDB, 4-6 cells per field, and they maintained a similar presence on D3 then to decrease on D8 to very low levels or even absence and later to increase tremendously on day 14 to 7 cells or more per field and then remained numerous (4-6) per field on D21, to increase tremendously on day 28 to 7 or more cells per field.
- In the RTXB group, on D0 the picture was similar to nDB or GB, however, a drop or even absence of mast cells was noted on days 3, 8 and 14. On the other hand, numerous mast cells, 7 cells or more per field, were encountered in the last week of the experiment during the remodeling and scarring phases.
- In the VG group the count started similar to nDB at D0 and began to rise on day 3 to 4-6 cells per field and continued to increase tremendously on day 14 to 7 cells or more per field. However, the number of mast cell became moderate for the last 2 weeks of the experiment with a cell count of 2-4 cells/field.
- VRTX presented a higher count than CTRL for D0, 4-6 cells/field. Later, on days 3 and 8 the count decreased to the level of controls (2-3 cells per field). It then rose back to a moderate count of 4-6 cells per field for the rest of the experiment

In brief, in the RTXB rats the mast cells decreased in numbers until almost day 21 then increased during the last week of the experiment but remained above CTRL levels. Where as in the GB the number of mast cells started moderate to decrease by the first week of the experiment then to rise tremendously on the second week, then to decrease on the third week to rise back again tremendously on the fourth week. In addition, the presence of G or RTX led to more degranulations of mast cells.

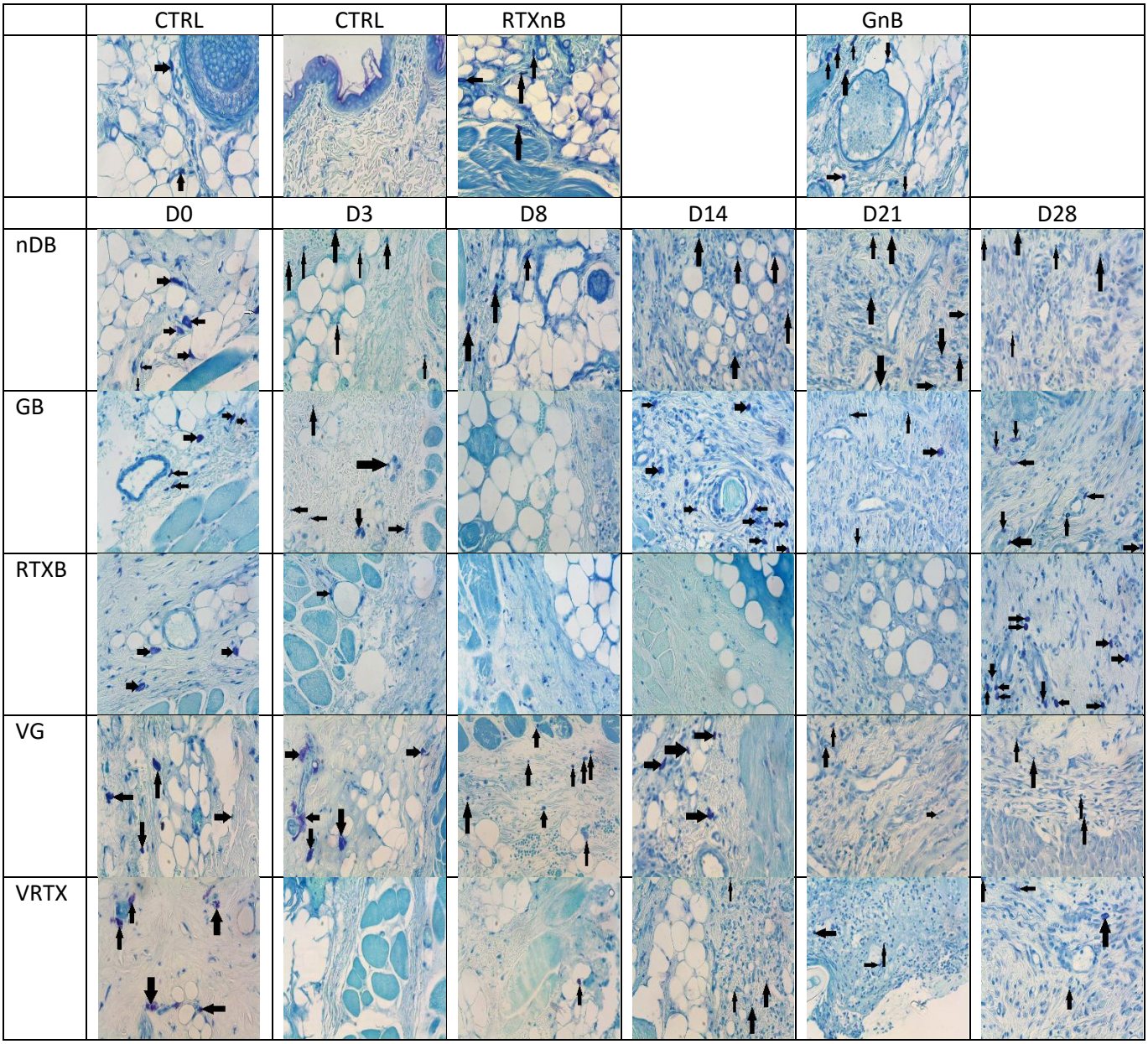

Figure 3. Histological sections stained Toluidine-blue for Mast Cell (x400).

3.5 Molecular Parameters:

Multiple molecular parameters were assessed in the various groups at all time points considered. Such parameters included the: NGF, the IL-1β, IL-6 and IL-8.

3.5.1 NGF modulation:

A thorough analysis of NGF data showed that:

- NGF concentration diminished slightly in the denervated non-burned (RTXnB and GnB) compared to normal control non-burned (CTRL) or to denervated and burned animals (RTXB and GB) (Table 7). The decrease was more obvious in the RTX treated groups (range 45%, 47%, and 41%) than in Guanethidine denervated non-burned rats (range 37%, 36%, 46%). These differences were significant at multiple time points (Figures 5-7).
- The burning increased the levels of NGF in the denervated animals (RTXB and GB) compared to non-burned denervated animals (RTXnB and GnB). However, in general the non-denervated burn had the highest concentrations of NGF at days 0, 14 and 28.
- As shown in figures 4 and 5, NGF levels were different in different groups and at different time points. The concentrations of NGF in RTXB group oscillated. Figure 5 shows the NGF level in the RTXB group at D0 to be 12 pg/mg. This level has risen up to 16 pg/mg at D3 presenting a statistical significance of 0.04 of t-test. Then it has fallen down to 11 pg/mg at D8 with p-value of 0.01. Then it decreased again to 10 pg/mg at D14. On the other hand, at D21, NGF concentration increased up of 13 pg/mg to fall again to a lowest recorded level of 8 pg/mg at D28, p=0.03, after healing (Figure 5).

- On the other hand, NGF levels in nDB group ranged from 8 pg/mg to 16 pg/mg. At D0 it showed a value of 14 pg/mg then decreased at D3 to 8 pg/mg. At D8, there was an increase to 11 pg/mg. This rise continued to D14 showing a value of 16 pg/mg, with a statistical difference to D0 ($p \leq 0.05$), then to fall again at D21 to 11 pg/mg, thus presenting a significant difference to D14 of 0.005. On the last day of the experiment, D28 showed a rise back to the same value at D0 (14 pg/mg) (Figure 6), thus showing a statistical significance against D21 of $p=0.04$ (Figure 6), slightly higher than normal healthy controls (12.32 pg/mg).
- On the other hand, NGF levels in the GB group ranged from 10.74 pg/mg to 12.87 mg/pg, values that are close to the normal control of 12.32 mg/pg. At D0 the level of NGF was 12.06 pg/mg. It rose a little at D3 to 12.14 pg/mg. At D8 the concentration fell to 10.74 pg/mg then rose again on D14 to 11.42 pg/mg. The rise continued to D21 which showed a concentration of 12.87 pg/mg. at D28 a fall was noted to 11.74 pg/mg. In this group no statistical significance existed at all time points compared to control (Figure 7).

In brief, the denervation decreased the levels of NGF but more so in the RTXB group, however, due probably to the burning effect, the levels rose at specific time points significantly beyond all controls as well as non-denervated with burns.

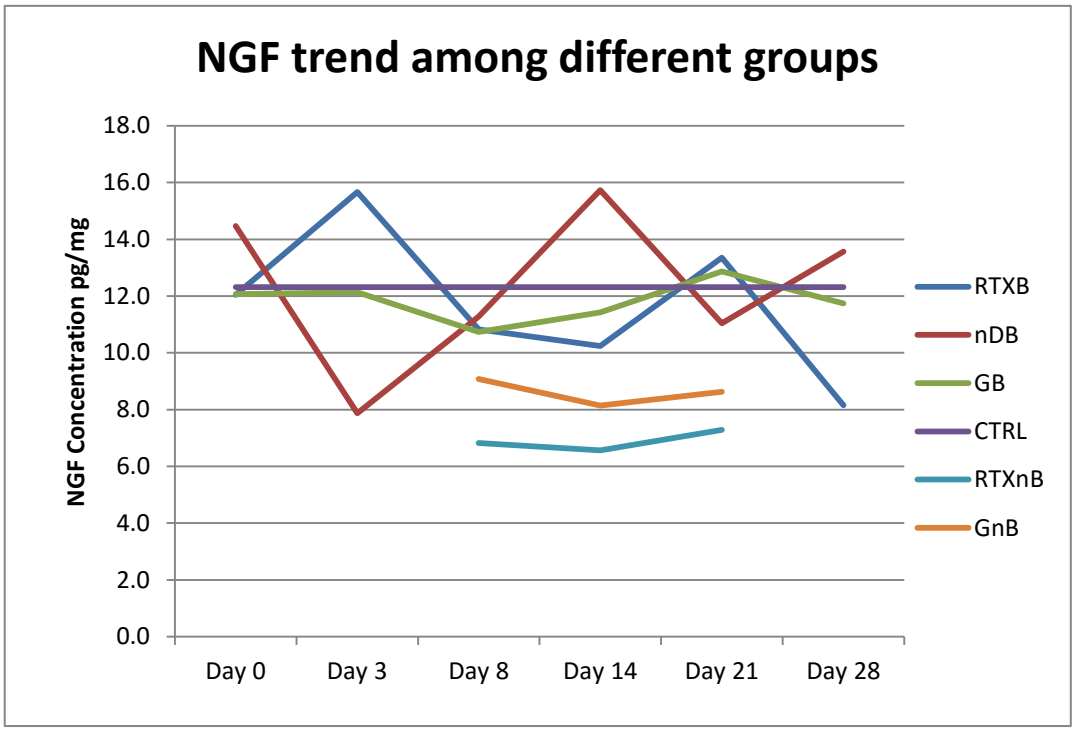

Figure 4. NGF Levels among different groups.

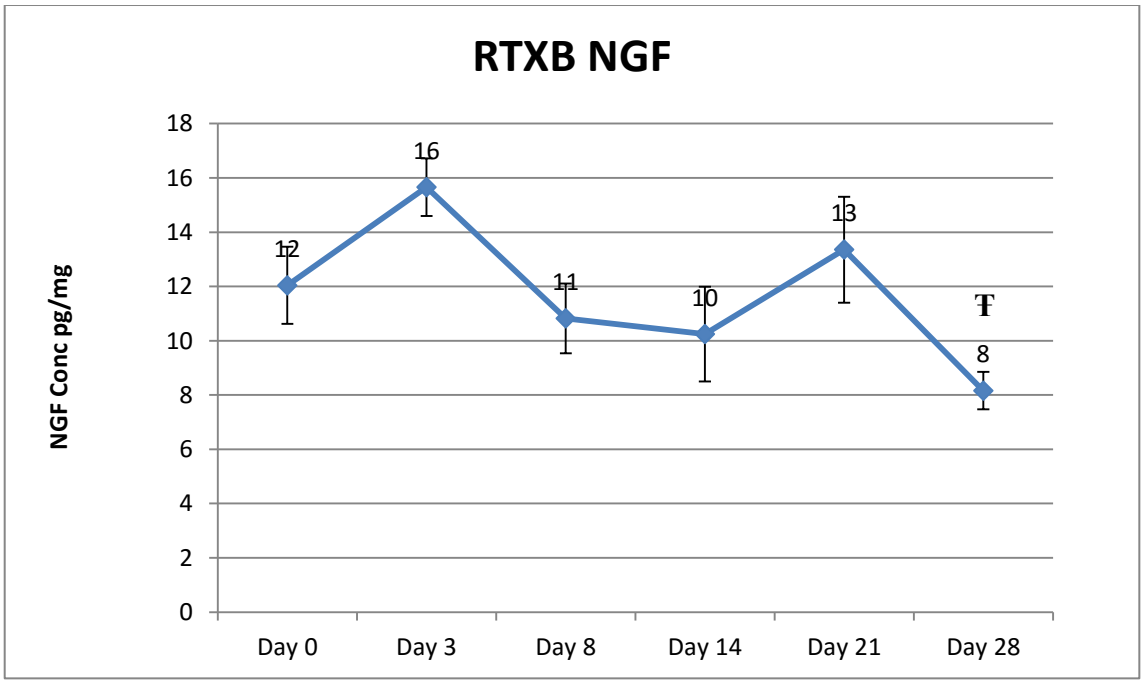

Figure 5. NGF levels in RTXB group.Ŧ shows statistical significance in comparison to CTRL (p-value ≤ 0.05)

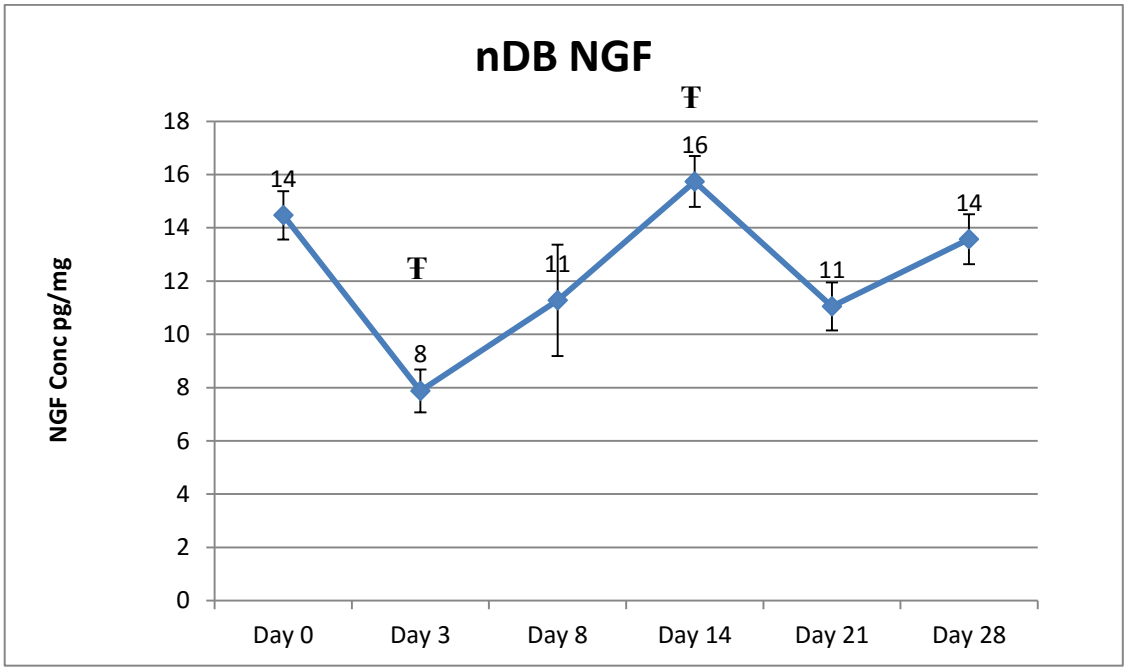

Figure 6. NGF levels in nDB group.Ŧ shows statistical significance in comparison to CTRL (p-value ≤ 0.05)

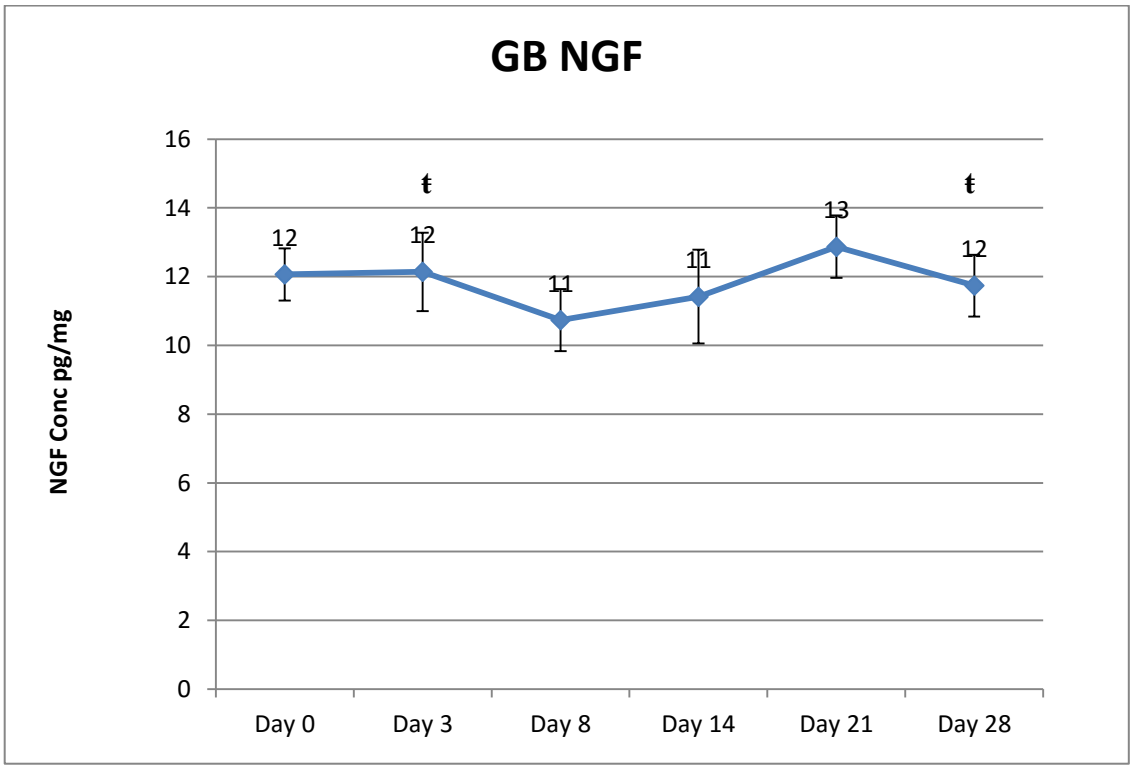

Figure 7. NGF levels in GB group. ŧ shows statistical significance in comparison to RTXB (p-value ≤ 0.05)

In brief, NGF concentrations were altered markedly and sometimes to relatively significant levels, thus oscillating around control levels.

3.5.2 IL-1β modulation

- The assessment of IL-1β exhibited undetectable levels in the non-burned normal healthy skin and in the RTX treated non-burned skin (RTXnB) as well as extremely low levels in the GnB group ranging between 2.81 ± 0.2, 3.68 ± 0.4, and 6.82 ± 0.4 (Figures 9 and 11).
- On the other hand, in the non denervated burns nDB, IL-1β increased slowly but steadily during the experimental period from 134 ± 15 on day 0 to 353 ± 36 on day 28 (Figures 10). This behavior was significantly different from the other burned groups RTXB and GB (Figures 9 and 11) ($P \leq 0.05$). In the denervated burned skins, IL-1β showed different patterns of expression among RTXB and GB on one hand and among RTXB and GB with nDB on the other hand.
- As shown in Figure 9 , IL-1β in the RTXB group picked up after two weeks (185 ±25) to reach its maximal level on the third week (534 ± 43) and to remain high (471 ± 39) by the end of the experiment.
- On the other hand, IL-1β in burned animals treated with guanethidine (GB) remained suppressed for 3 days then started to increase to reach the levels of denervated burned skin by day 8 and to keep up reaching its maximal level of 585 ± 43 and then to remain significantly high for the rest of the duration, 378 ± 24 week 3 and 388 ± 30 week 4 (Figures 8-11).

In brief, denervation of burns with RTXB and GB suppressed IL-1β initially in the acute inflammation phase. The concentrations of IL-1β went up to non-denervation levels after one week with guanethidine and after two weeks after RTXB treatment.

Figure 11 exhibits a comparison among the experimental groups, GB data against CTRL data show significant differences on days 3, 8, 14 and 28. The p-values are 0.05, 0.001, 0.009 and 0.04, respectively. Concentrations from RTXB against those of CTRL show also significant differences at 4 time points with p-values of 0.01, 0.05, 0.006, and 0.01 at time points D3, D14, D21 and D28, respectively (Figure 9). On the other hand, the nDB concentrations against those of the CTRL present significance at all time points with p-values of 0.01, 0.02, 0.02, 0.02, 0.008, and 0.002 for day 0, 3, 8, 14, 21 and 28, respectively (Figure 10). However, a comparison of values from GnB against CTRL shows no significance and so is the comparison of RTXnB against the CTRL group, since the concentrations are numerically equivalent. In addition, comparing IL-1β values of RTXnB against those of RTXB show significant differences at two time points, at days 14 and 21where their respective p-values are 0.05 an0.006 (data not shown). Furthermore, GnB against GB showed also significant differences also at two time points. However, these time points are on days 8 and 21 with p-values of 0.0007 and 0.009, respectively (data not shown). When comparing RTXnB against GnB no significance is presented (data not shown). Nevertheless, RTXB against GB also show significant differences at two time points, days 8 and 21 where the p-values are 0.03 and 0.029, respectively (Figure 11).

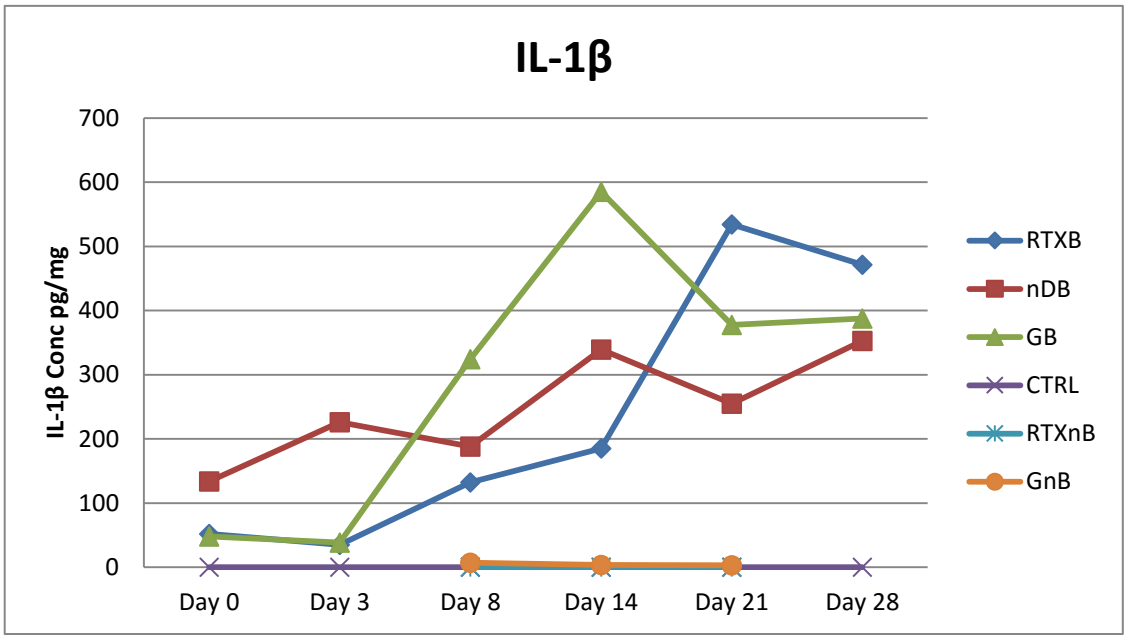

Figure 8. IL-1β levels among different groups.

Figure 9 shows the trend of increasing concentrations of the IL-1β in the RTXB group. The slight increase on the first four time points showed no significance when compared to its D0 and D3. However, significance was present on days 14, 21 and 28 compared to D0 and D3 when a rise of IL-1β concentration took place from 185 pg/mg to 534 pg/mg with a p-value of 0.02.

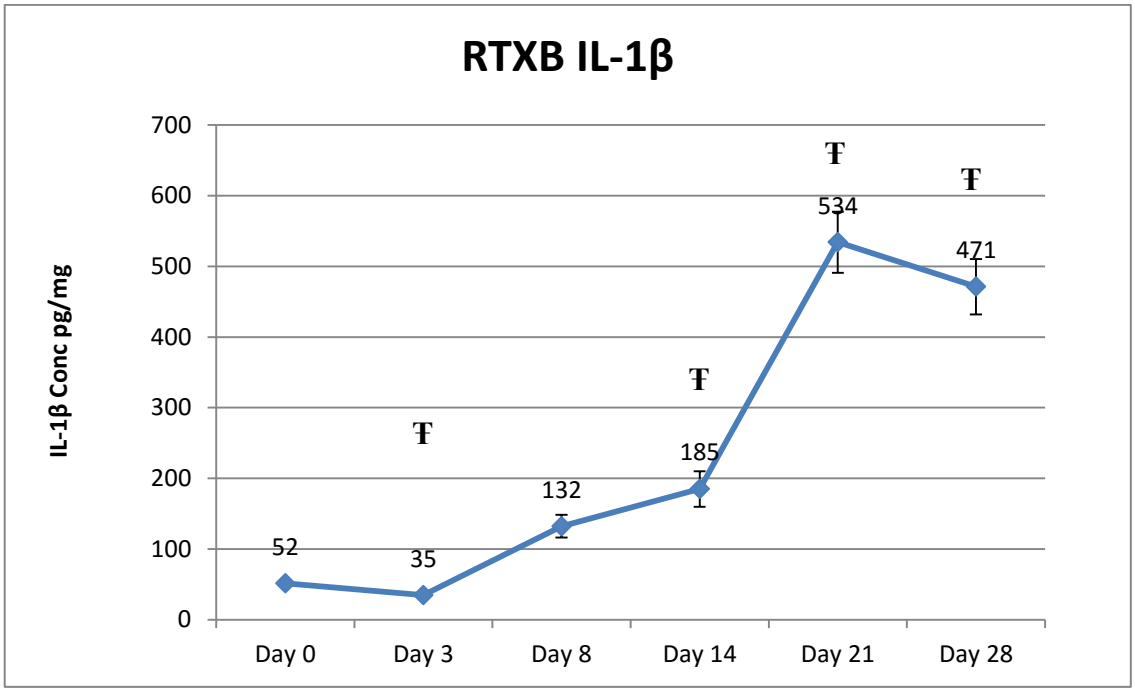

Figure 9. IL-1β Level in RTXB group. Ŧ shows statistical significance in comparison to CTRL (p-value ≤ 0.05)

Figure 10 shows also an increase in the concentrations of IL-1β in the nDB group. The graph shows a trend of a steady increase. When comparing one time point to its previous one, no statistical significance was present. However, comparing concentrations at D0 against D14, D21 and D28, a statistical significance of p-values $p < 0.05$ existed.

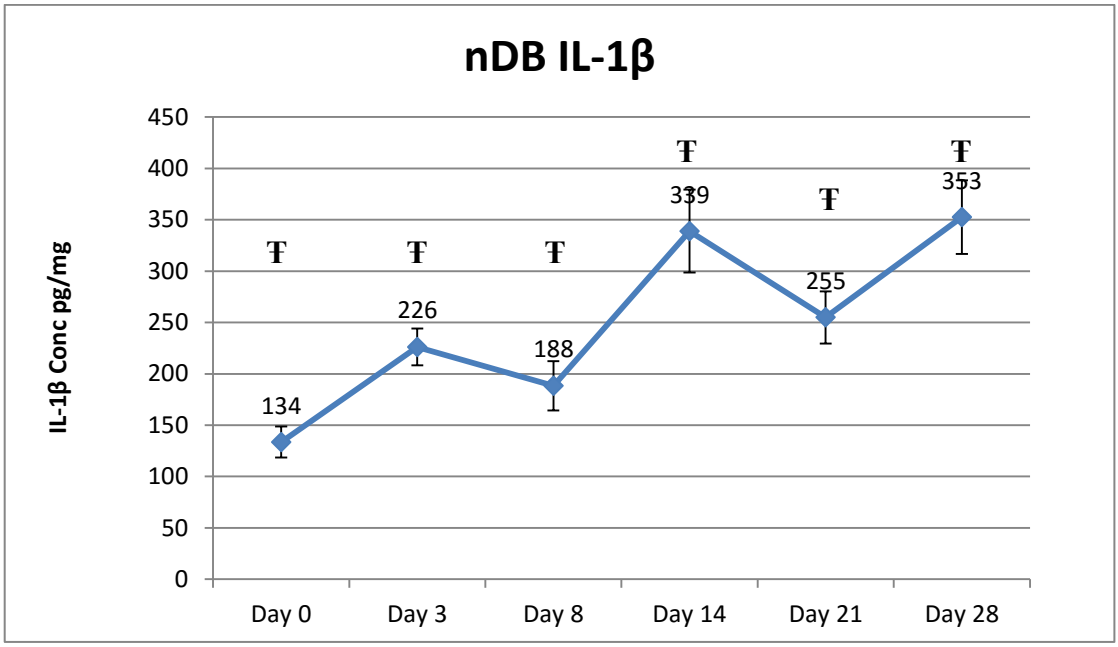

Figure 10. IL-1β in nDB group. Ŧ shows statistical significance in comparison to CTRL (p-value ≤ 0.05)

It is noteworthy that IL-1β had a different expression pattern in the GB group. Figure 11 shows a marked decrease in the concentration of IL-1β from 48 pg/mg to 38 pg/mg on day 0 to day 3. However, on day 8 the concentration increased significantly to 324 showing a p-value of 0.0003. The rise continued on D14 to mark 585 pg/mg D14 (p-value is 0.04). On day 21, the concentration dropped to 378 pg/mg with no significance against D14 but still significant compared to D0 of p-value equals to 0.04. On the other hand, on D28 the concentration increased to 388 pg/mg with no significance to D21 but was still significant to D0, p-value equals to 0.03.

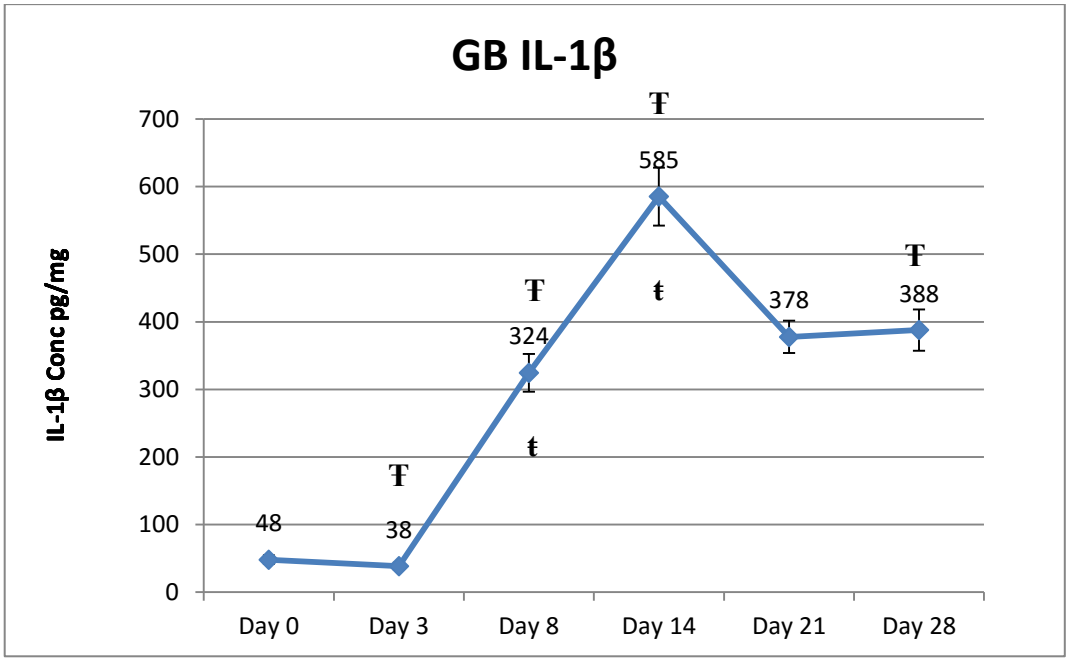

Figure 11. IL-1β in GB group. ŦŦ *shows statistical significance in comparison to CTRL.* ŧ *shows statistical significance in comparison to RTXB* (*p-value ≤ 0.05)*

3.5.3 IL-6 Quantification

IL-6 concentrations started high in all burned skin groups, however, in the non-denervated group (nDB), the levels of IL-6 were significantly higher 1523 ± 58 pg/mg compared to 1062 ± 101 pg/mg and 1068 ± 78 pg/mg in the RTXB and GB, respectively ($P < 0.05$) (Figures 13-15).

Comparing IL-6 levels in the GB against those of the CTRL groups, they exhibited significant differences at four time points where the p-values are 0.005, 0.008, 0.001, and 0.002 for days 0, 3, 8 and 14, respectively. RTXB against CTRL showed also significant differences at five time points D0, D3, D8, D21 and D28 with p-values of 0.02, 0.04, 0.047, 0.0002 and 0.0005, respectively. Furthermore, comparing nDB against CTRL data, they showed statistically significant differences at all time points with p-values of 0.01, 0.017, 0.013, 0.0001 and 0.02 for days 0, 3, 8, 21, and 25, respectively. On the other hand, when comparing values of GnB against those of CTRL and values of RTXnB against those of CTRL no significant difference was recorded. In addition, when comparing RTXnB against RTXB, the effect of burn in modulating IL-6 is significant at various time points, days 8, 14, and 21 for respective p-values of 0.05, 0.03 and 0.0004 (data not shown). GnB against GB shows a similar pattern, again with significant differences at three time points, days 8, 14, and 21 with p-values of 0.001, 0.04, and 0.007, respectively (data not shown). On the other hand, comparing RTXnB to GnB shows a significant difference at one time point at D8 where p-value is 0.03 (data not shown). Furthermore, RTXB against GB groups when compared present statistical significance at three time points, days 14, 21 and 28 with respective p-values of 0.03, 0.0004 and 0.01 (Figures 13-15).

In brief, burning has led to the production of high levels of IL-6 in the acute phase of healing process and more so in the denervated burns.

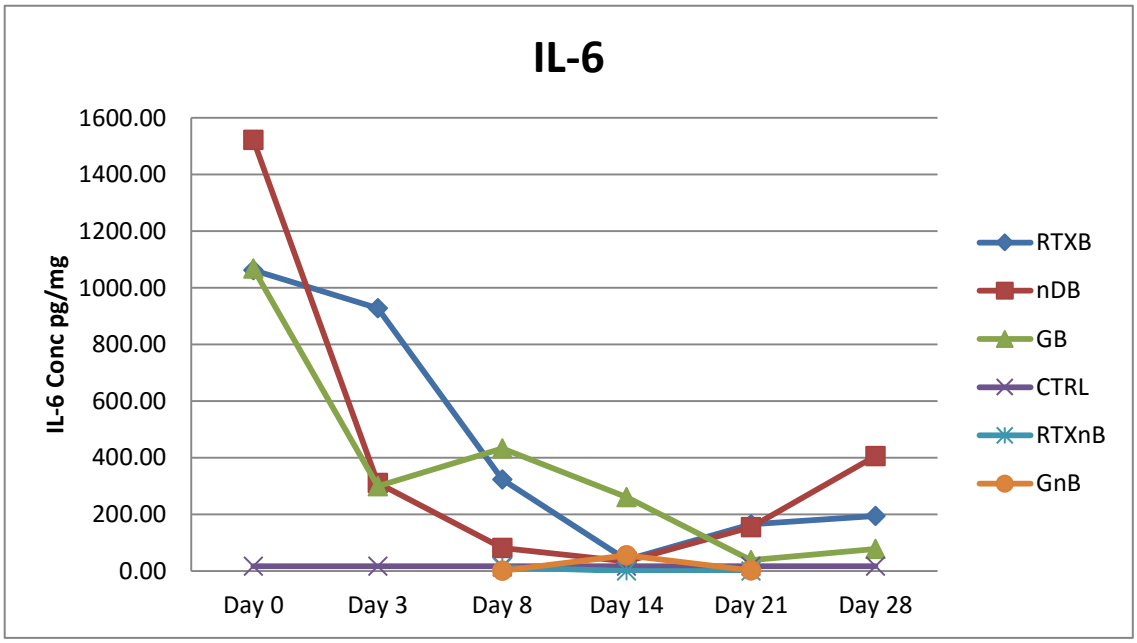

Figure 12. IL-6 levels among different groups.

On the other hand, Figure 13 exhibits the high concentrations of IL-6 early in the process within the RTXB group at early time points. At D0 the concentration scores a 1062 pg/mg to fall gradually on D3 to 928 pg/mg without showing a statistical significance against D0. The fall continues to D8 presenting a concentration of 285 pg/mg with a statistical significance against D3 of p-value of 0.05. On D14 the concentration was down significantly to 40 pg/mg ($p < 0.05$) to rise on D21 to 165 pg/mg with a statistical significance against D14 of 0.0001, and to 194 pg/mg on D28. In brief, IL-6 values on D8, D14, D21 and D28 show a statistical significance against D0 with p-value of 0.019, 0.011, 0.016 and 0.017, respectively. Consequently, there was significance drop in IL-6 with time. Such a drop was slower with RTXB compared to the nDB or GB groups.

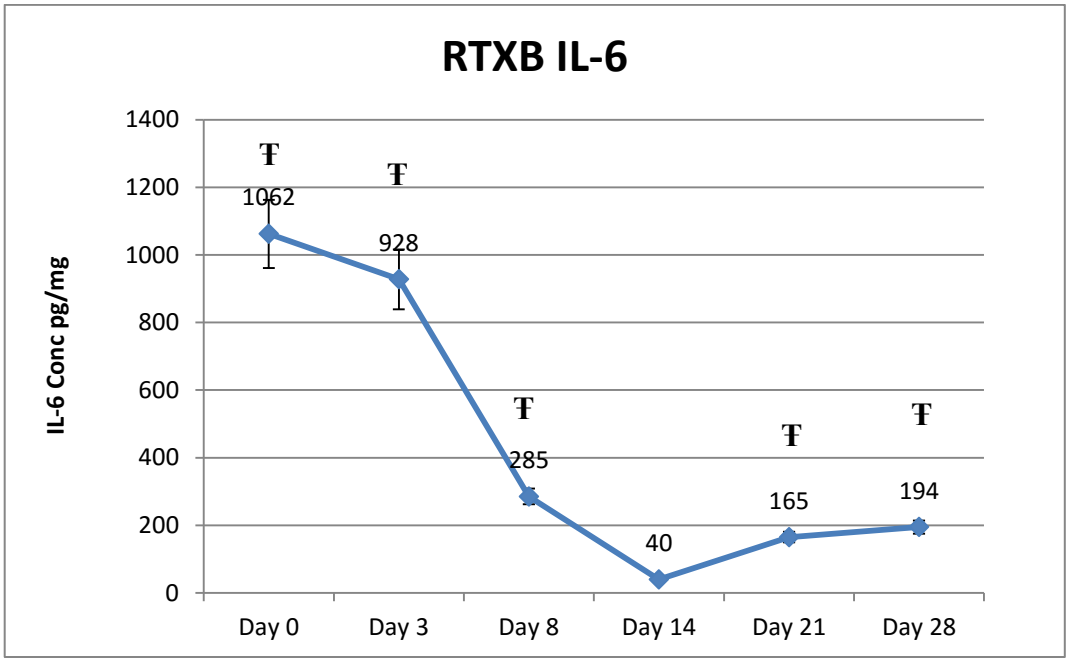

Figure 13. IL-6 Levels in RTXB group. ∓ shows statistical significance in comparison to CTRL (p-value ≤ 0.05)

On the other hand, in the non-denervated burned group (nDB), IL-6 has an initial concentration of 1523 pg/mg at D0, to fall to 311 pg/mg on D3, (p=0.018). The fall continues to 81 pg/mg on D8 with p-value of 0.018 against D3. On D14 the concentration decreased more to 34 pg/mg to score a statistical significance against D8 of p-value equals to 0.023. On D21 the concentration rises again significantly to 155 pg/mg (p=0.0001) against D14. The concentration then rises again on D28 to 406 pg/mg, showing a significance against D21 of p-value equals to 0.031. In brief, all time points show statistical significance against D0 with p-values of 0.018, 0.007, 0.006, 0.008, and 0.014 for day 3, 8, 14, 21 and 28, respectively (Figure 14).

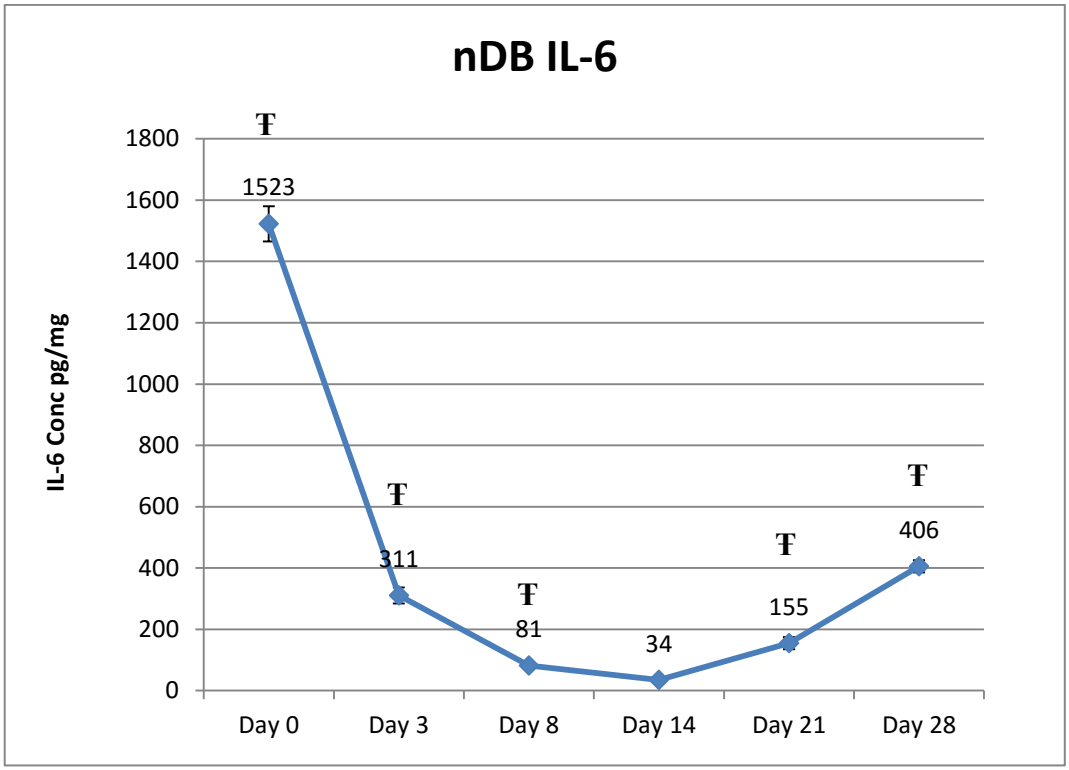

Figure 14. IL-6 levels in nDB group. ₸ shows statistical significance in comparison to CTRL (p-value ≤ 0.05)

Furthermore, in the GB group, IL-6 concentration scores 1068 pg/mg on D0, very close to RTXB, to fall to 300 pg/mg on D3 thus presenting a statistical significance of p-value equals to 0.004. On D8 the concentration rose again to 433 pg/mg then falls again on D14 to score 261 pg/mg showing also a statistical significance against D8 of p-value equals to 0.04. On D21st, however, a concentration of 38 pg/mg was detected with a statistical significance against D14 with a p-value of 0.015. On day 28 the concentration went up to 79 pg/mg with no statistical significance against D21. In brief, concentrations at all time points show statistical significance against D0 with p-values, of 0.004, 0.008, 0.003, 0.003, and 0.003, respectively (Figure 15).

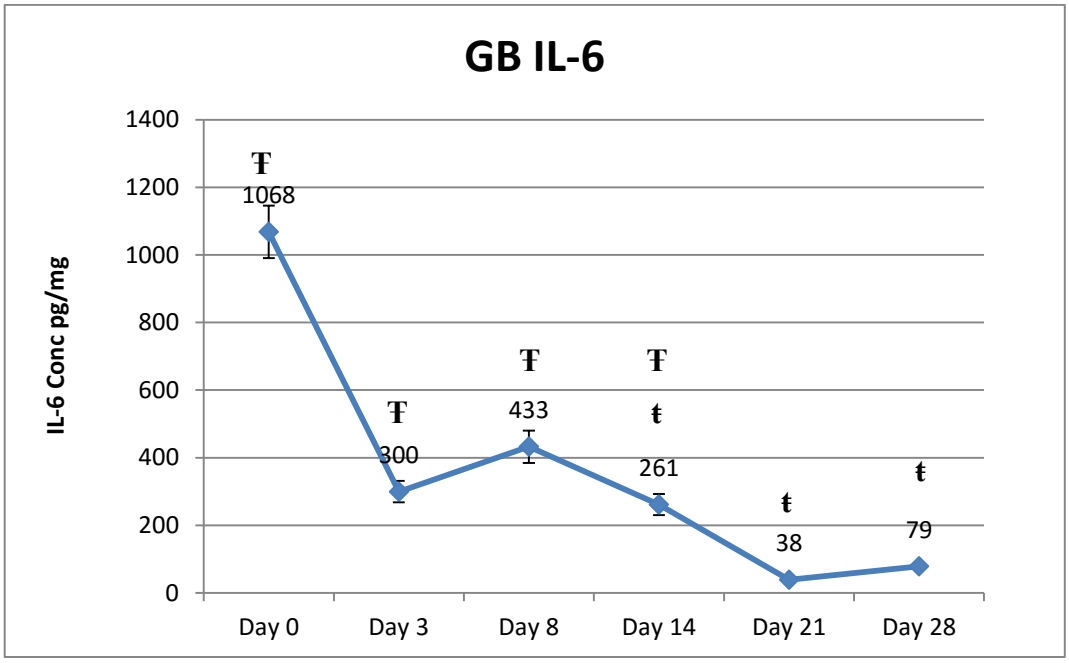

Figure 15. IL-6 levels in GB group. Ŧ shows statistical significance in comparison to CTRL. ŧ shows statistical significance in comparison to RTXB (p-value ≤ 0.05)

3.5.4 IL-8 Modulation

The data collected on IL-8 showed that denervation of burned skin decreased significantly its levels during the first week with RTXB and the first 3 days in the GB groups compared to the non denervated burned group (nDB). The concentration picked up later on to closer levels in the 3 burned groups. However, the concentrations of IL-8 in the non-burned and control groups were undetectable (Figures 17-19).

A comparison of the GB group IL-8 values against those of the CTRL group values shows significant differences at four time points days 8, 14, 21, and 28 with respective p-values of 0.000001, 0.002, 0.049 and 0.004. However, when comparing the

RTXB against the CTRL groups, they show also significance at four time points D8, D14, D21 and D28 with p-values of 0.002, 0.028, 0.002 and 0.010 respectively (Figure 17). Furthermore, the comparison of nDB vs CTRL presents significance at all time points days, 3, 8, 14, 21 and 28 with respective p-values of 0.006, 0.002, 0.008, 0.004, and 0.004 (Figure 18). Again, comparing RTXB against RTXnB shows three significant time points at D8, D14 and D21 with p-values of 0.002, 0.003 and 0.002 respectively (data not shown). Furthermore, comparing GnB against GB shows significance at two time points days 8 and 14. Their respective p-values are 0.000001 and 0.002 (data not shown). In brief, when comparing RTXB against GB there was difference only on D8 with p-value of 0.000001 (Figure 19).

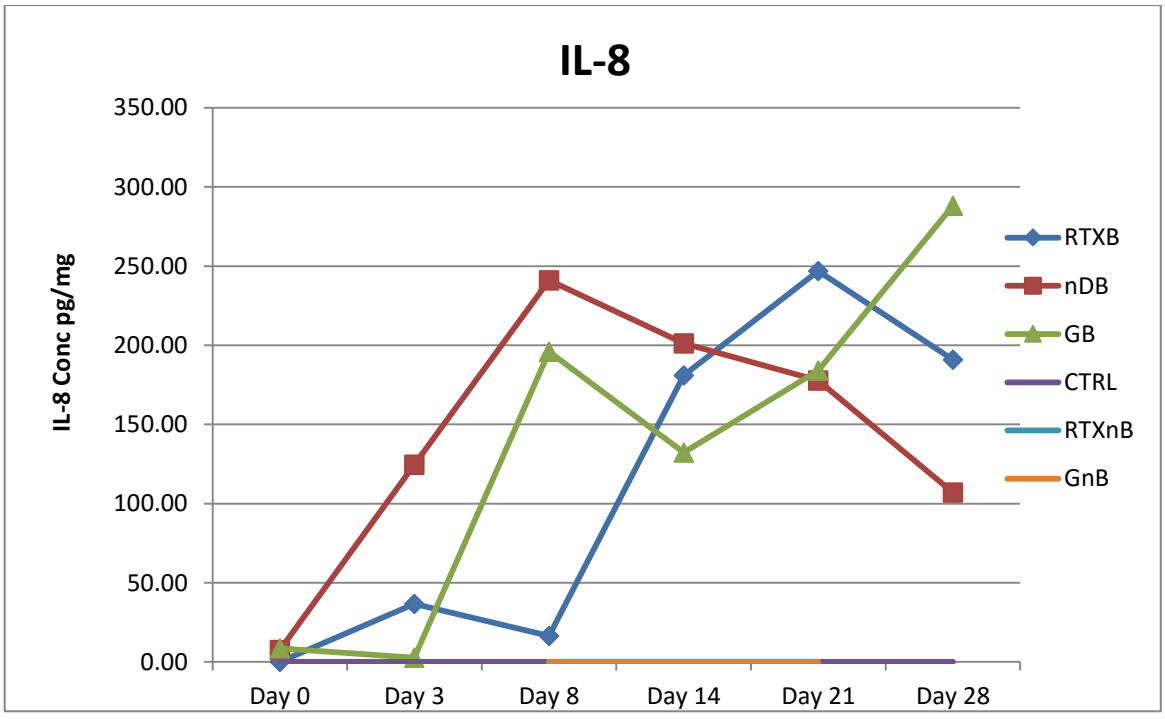

Figure 16. IL-8 levels among different groups

However, when looking into the expressions of IL-8 in the RTXB group, Figure 16 shows that the IL-8 concentration in the RTXB group at D0 was undetectable

then rose to 37 pg/mg at D3 to fall again on D8 to 16 pg/mg. On D14 there was an abrupt significant increase of the concentration to 181 pg/mg (p=0.02). The increase continued on D21 and presented 247 pg/mg then to fall to 191 pg/mg on D28. In brief, values of D8, D14, D21 and D28 time points show statistical significance against D0 with p-values of 0.001, 0.014, 0.0008 and 0.005, respectively (Figure 17).

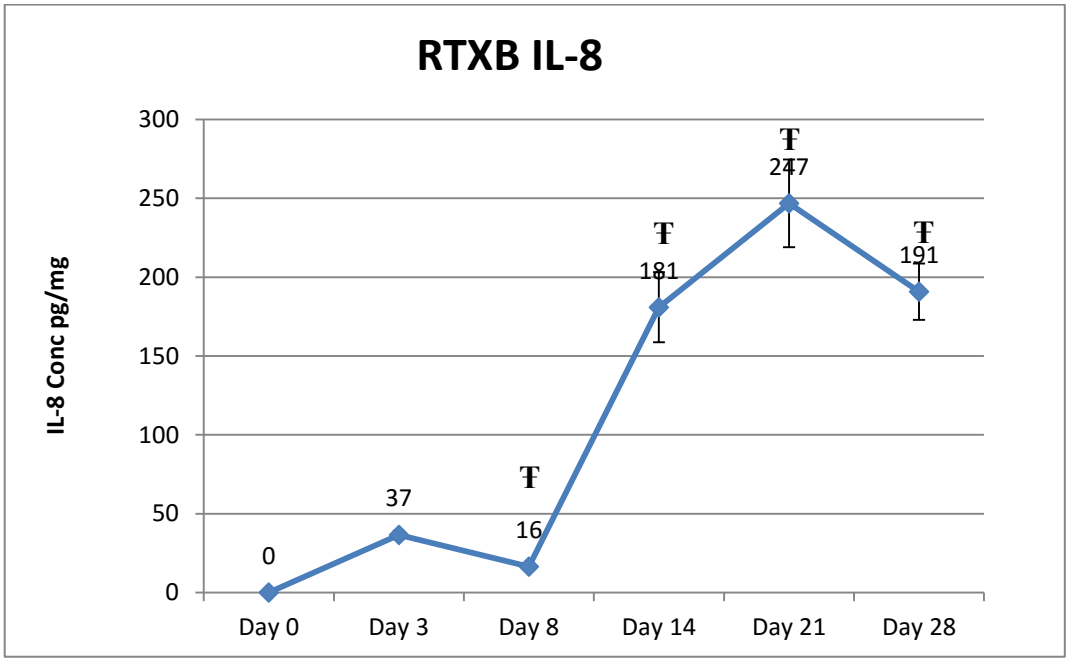

Figure 17. IL-8 levels in RTXB group. ŦŦ shows statistical significance in comparison to CTRL (p-value ≤ 0.05)

Concerning the nDB group, figure 20 shows the average concentrations of IL-8 at various time points. At D0 the concentration scores 7 pg/mg to rise significantly on day 3 to 125 pg/mg (p=0.002). The concentration continues to rise on day 8 to 241 pg/mg, thus showing a statistical significance against D3 with a p-value of 0.004. This concentration falls on day 14 to 201 pg/mg, then to 178 pg/mg on day 21. This decrease continues at time point D28 to score 107 pg/mg, again showing a statistical significance

against D21 (p=0.020). On the other hand, D3, D8, D14, D21 and D28 show statistical significance against D0 with p-values of 0.002, 0.0005, 0.0027, 0.0012 and 0.0007, respectively (Figure 18).

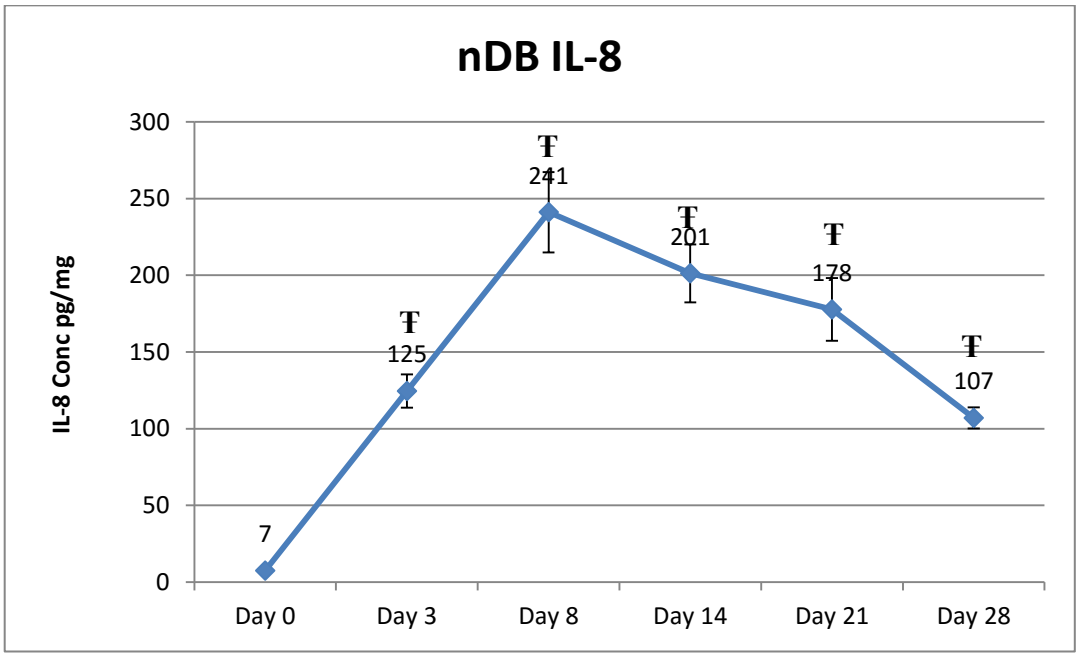

Figure 18. IL-8 levels in nDB group. ₸ shows statistical significance in comparison to CTRL (p-value ≤ 0.05)

Lastly, concerning IL-8 values in the GB group, figure 23 shows the average IL-8 concentrations at the different time points. At D0, the concentration is 9 pg/mg to fall at D3 to 3 pg/mg. On day 8, the concentrations rises to 196 pg/mg to show a statistical significance against D3 of p-value equals to 0.0000001. On day 14, the concentration falls significantly to 132 pg/mg, thus presenting a statistical significance against D8 of p-value equals to 0.017. It then rises on D21 to 163 pg/mg and on day 28 the concentration scores a 288 pg/mg with a statistical significance against D21 (p=0.050). In brief, the time points D8, D14, D21 and D28 show statistical significance against D0 ($p<0.05$) (Figure 19).

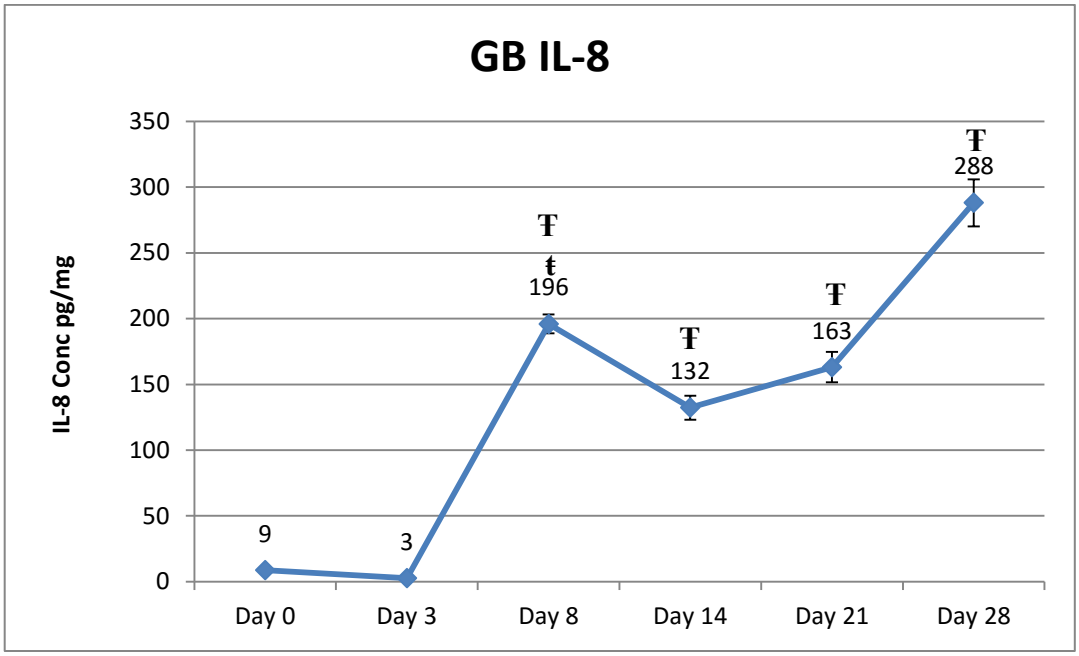

Figure 19. IL-8 levels in GB group. ₸ shows statistical significance in comparison to CTRL. ŧ shows statistical significance in comparison to RTXB (p-value ≤ 0.05)

3.6 Summary of the changes of molecular parameters per treatment

The results when grouped by treatment type exhibited multiple alterations in the concentrations of NGF, IL-1β, IL-6 and IL-8 (Tables 4-8 and Figures 20-24).

3.6.1 In the regular non-denervated burns (nDB) the followings were recorded:

- NGF

In the non denervated burned group (nDB), NGF decreased from D0 t0 D3 (14.5 pg/mg to 7.9 pg/mg) then went up steadily after day 3 to regain the D0 level and beyond after 2 weeks (15.7 pg/mg) and then back within the range of D0, 11.0 pg/mg on D21 and 13.6 pg/mg on D28 (Figure 20a).

- IL-1β

The concentration of IL-1β increased slowly but steadily all the duration of the experiment; 134 pg/mg on D0 to 353 pg/mg on D28 (Figure 20b).

- IL-6

The burn affected significantly the concentration of IL-6, it decreased sharply from D0 to D3, D8, D14 and remained very low for the rest of experiment; from 1523 pg/mg at D0 to 311 pg/mg on D3, then to 81 pg/mg on D8 to a minimum of 34 pg/mg was on D14 (Figure 20c).

- IL-8

The concentration of IL-8 increased steadily from D0 (9 pg/mg) to D3 (3 pg/mg) and D8 (196 pg/mg) where it reached its maximal concentration. It plateaued between D14 (132 pg/mg) and D21 (184 pg/mg) around the control levels to reincrease markedly on D28 (288 pg/mg) (Figure 20d).

Table 4. nDB Group Parameters.

nDB				
	NGF	IL-1β	IL-6	IL-8
D0	14.5	134	1523	9
D3	7.9	226	311	3
D8	11.3	188	81	196
D14	15.7	399	34	132
D21	11	255	155	184
D28	13.6	353	406	288

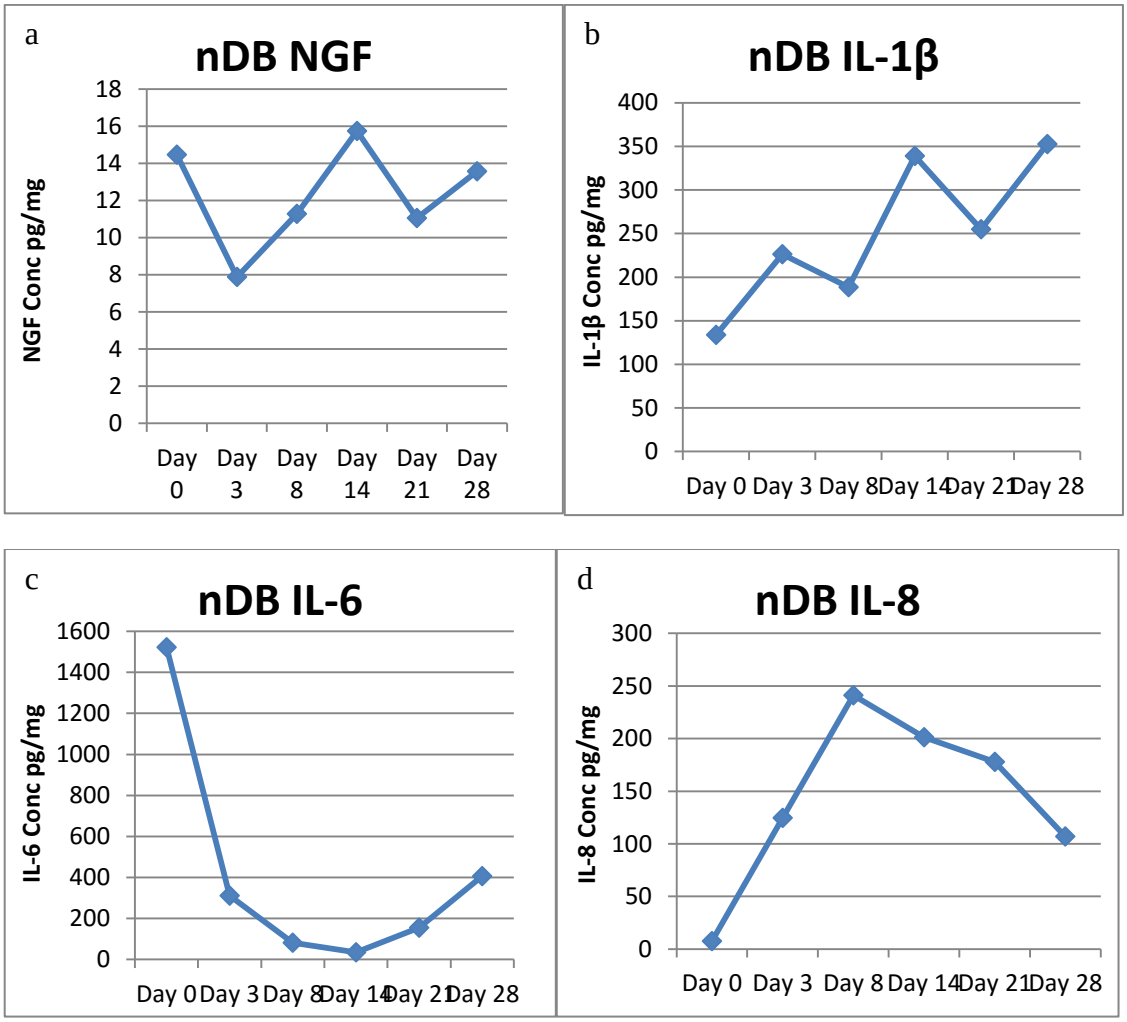

Figure 20. nDB Group Parameters

3.6.2 RTXB treatment

Denervation by RTX of burned wounds has altered the concentrations of various parameters at the various time points (Figure 21).

- NGF

In the RTXB, NGF concentration increased from D0 (12.0 pg/mg) to D3 (15.7) pg/mg steadily, then decreased till days 8 (10.8 pg/mg) and 14 (10.2 pg/mg) to go up to almost control levels and beyond by day 21 (13.4 pg/mg) and then to decrease a little by

day 28 (8.2 pg/mg). That is, after an initial acute rise, the concentration of NGF went down althrough the duration of the experiment (Figure 21a).

- IL-1β

The secretion of IL-1β was suppressed for almost a week to pick up later. It was suppressed from D0 (52 pg/mg) to D3 (35 pg/mg) and started to increase by days 8 (132 pg/mg), 14 (185 pg/mg) to reach a maximum on D21 (534 pg/mg) and remained high by day 28 (471 pg/mg). Consequently, there was a delay in its production in the acute phase (Figure 21b).

- IL-6

In the presence of RTX, IL-6 behaved a little different by slowing down its decrease. IL-6 started to decrease slowly from D0 (1062 pg/mg) to D3 (928 pg/mg) and D8 (323 pg/mg) to reach complete suppression afterwards on D14 (40 pg/mg) and to remain at very low levels afterwards; 165 pg/mg and 194 pg/mg by D21 and D28, respectively (Figure 21c).

- IL-8

It was suppressed significantly from D0 (0 pg/mg) to D3 (37 pg/mg) and D8 (16 pg/mg) then increased steadily by D14 (184) to reach a maximum by day 21 (247 pg/mg) and remained high on D28 (191 pg/mg) (Figure 21d).

Table 5. RTXB Group Parameters.

RTXB				
	NGF	IL-1β	IL-6	IL-8
D0	12	52	1062	0
D3	15.7	35	928	37
D8	10.8	132	323	16
D14	10.2	185	40	181
D21	13.4	534	165	247
D28	8.2	471	194	191

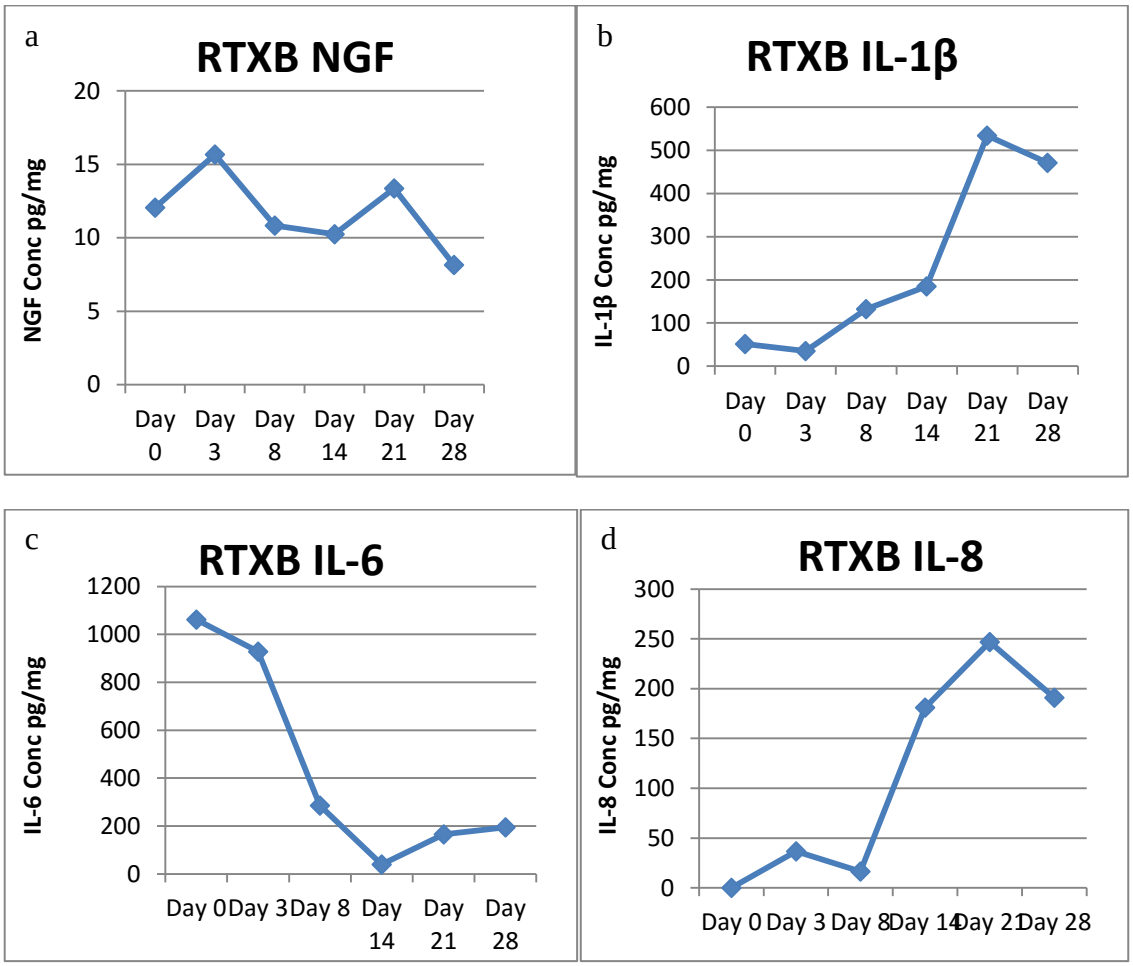

Figure 21. RTXB Group Parameters.

3.6.3 GB Treatment

The changes in the various parameters in the guanethidine treatment were close to those of RTX.

- NGF

In the GB group, NGF oscillated around the controls CTRL's level with the lowest concentrations level reached on D8 (10.74 pg/mg) and the highest on day 21 (12.87 pg/mg) (Figure 22a).

- IL-1β

Results showed that IL-1β was actually suppressed till D3 (38 pg/mg) then increased sharply afterwards D8 (324 pg/mg), to reach its maximal concentration on D14 (585 pg/mg) and then to decrease a little and remain high by D21 (378 pg/mg) and D28 (388 pg/mg) (Figure 22b).

- IL-6

The changes in IL-6 concentrations were similar to nDB and slightly different from RTXB. IL-6 concentrations decreased sharply from D0 (1068 pg/mg) to D3 (38 pg/mg) and remained very low throughout the duration of the experiment (Figure 22c).

- IL-8

IL-8 concentrations did not seem to be affected by guanethidine treatment. It remained suppressed from D0 (9 pg/mg) to D3 (3 pg/mg) then picked up on D8 (196 pg/mg) and remained high on days 14 (132 pg/mg) and 21 (184 pg/mg) to reach a maximum on day 28 (288 pg/mg) (Figure 22d).

Table 6. GB Group Parameters.

GB				
	NGF	IL-1β	IL-6	IL-8
D0	12.1	48	1068	9
D3	12.1	38	300	3
D8	10.7	324	433	196
D14	11.4	585	261	132
D21	12.9	378	38	184
D28	11.7	388	79	288

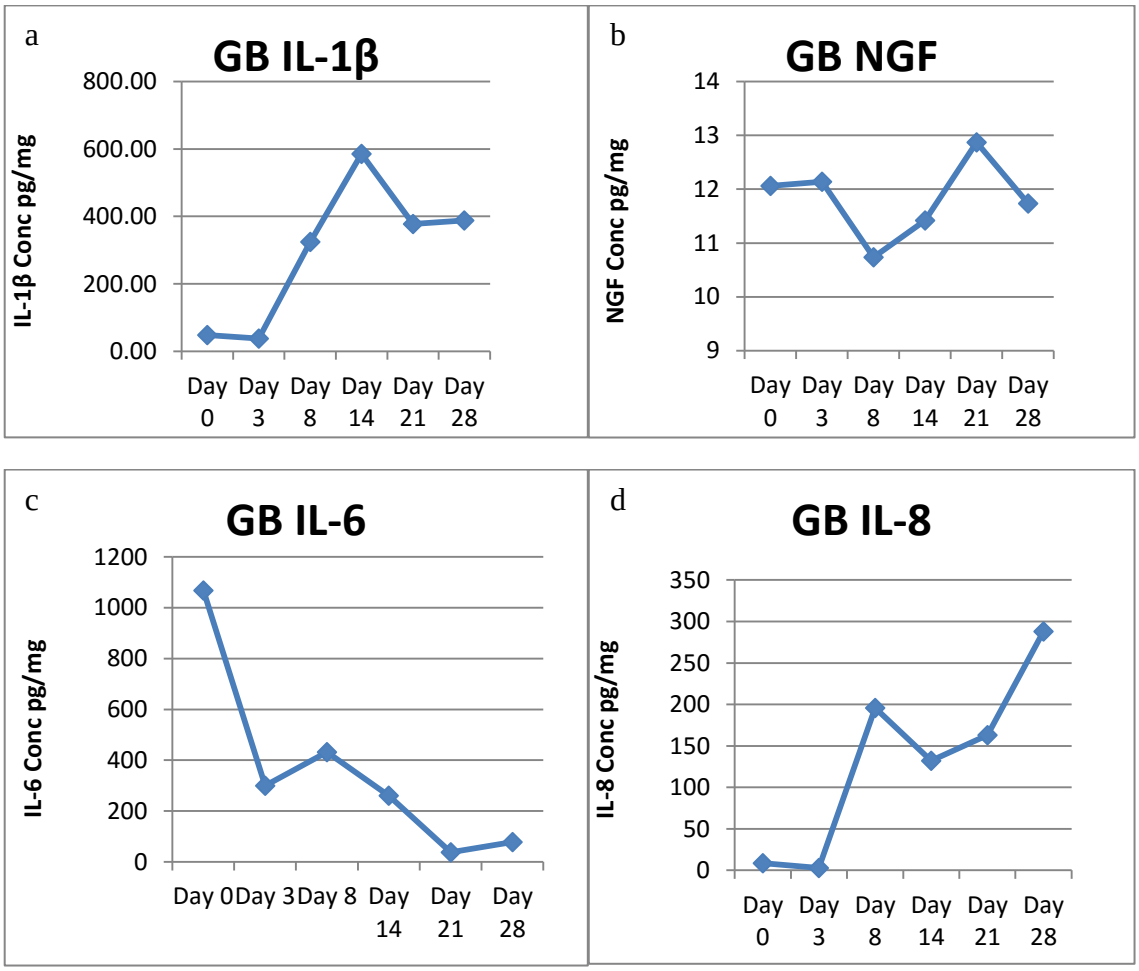

Figure 22. GB Group Parameters.

3.6.4 RTXnB and GnB

In the absence of burn RTX by itself lead to a decrease in NGF and IL-6 for 2 weeks. For the rest of the duration, NGF increased while IL-6 remained low (Figure 23 a,c). On the other hand, RTX suppressed IL-1β and IL-8 althrough the experiment (Figure 23 b,d). As for guanethidine, in the absence of burn, it also suppressed NGF for two weeks and IL-8 althrough the experiment (Figure 24 a,d). on the other hand, IL-1β decreased slowly from 8 to 21 days while IL-6 increased sharply during the second week (8-14 days) then decreased sharply (Figure 24 b,c).

Table 7. RTXnB Group Parameters.

RTXnB				
	NGF	IL-1β	IL-6	IL-8
D8	6.82	0	14	0
D14	6.57	0	0	0
D21	7.29	0	0	0

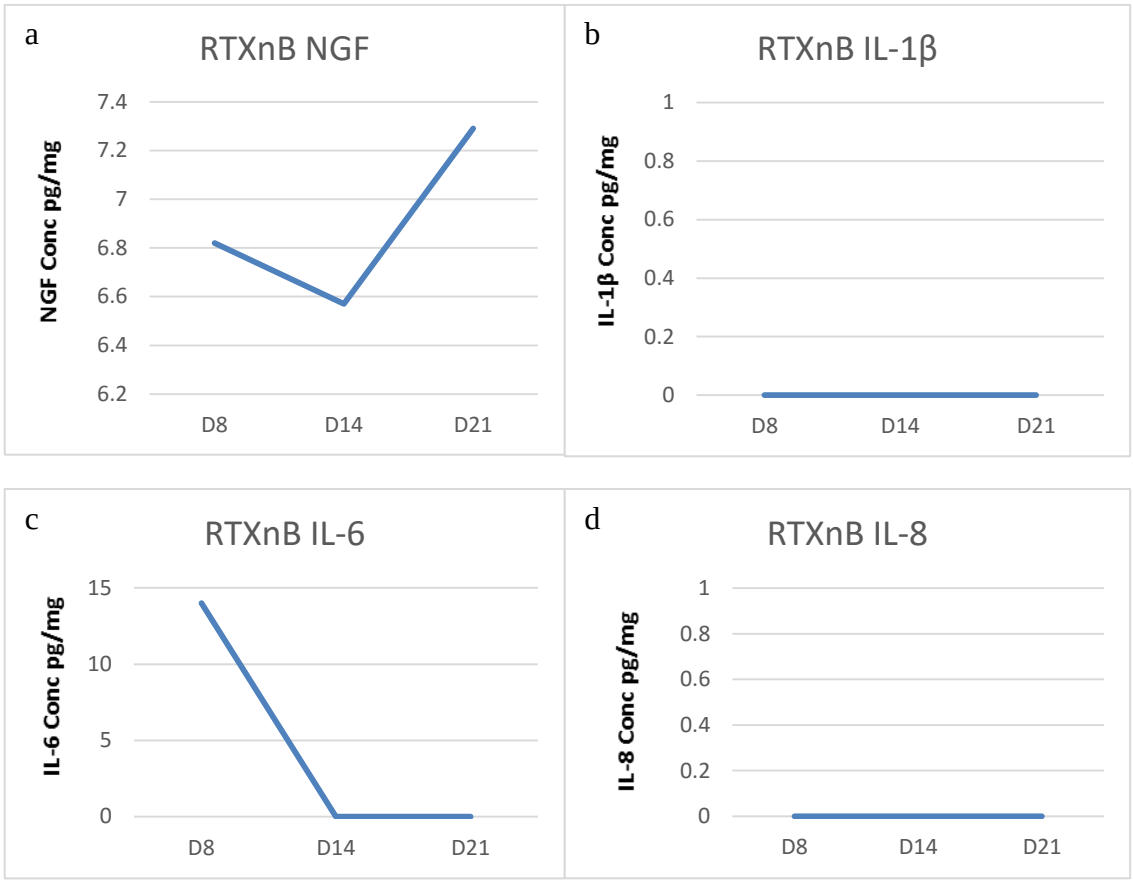

Figure 23. RTXnB Group Parameters.

Table 8. GnB Group Parameters.

GnB				
	NGF	IL-1β	IL-6	IL-8
D8	9.08	6.82	1	0
D14	8.14	3.68	55	0
D21	8.63	2.81	3	0

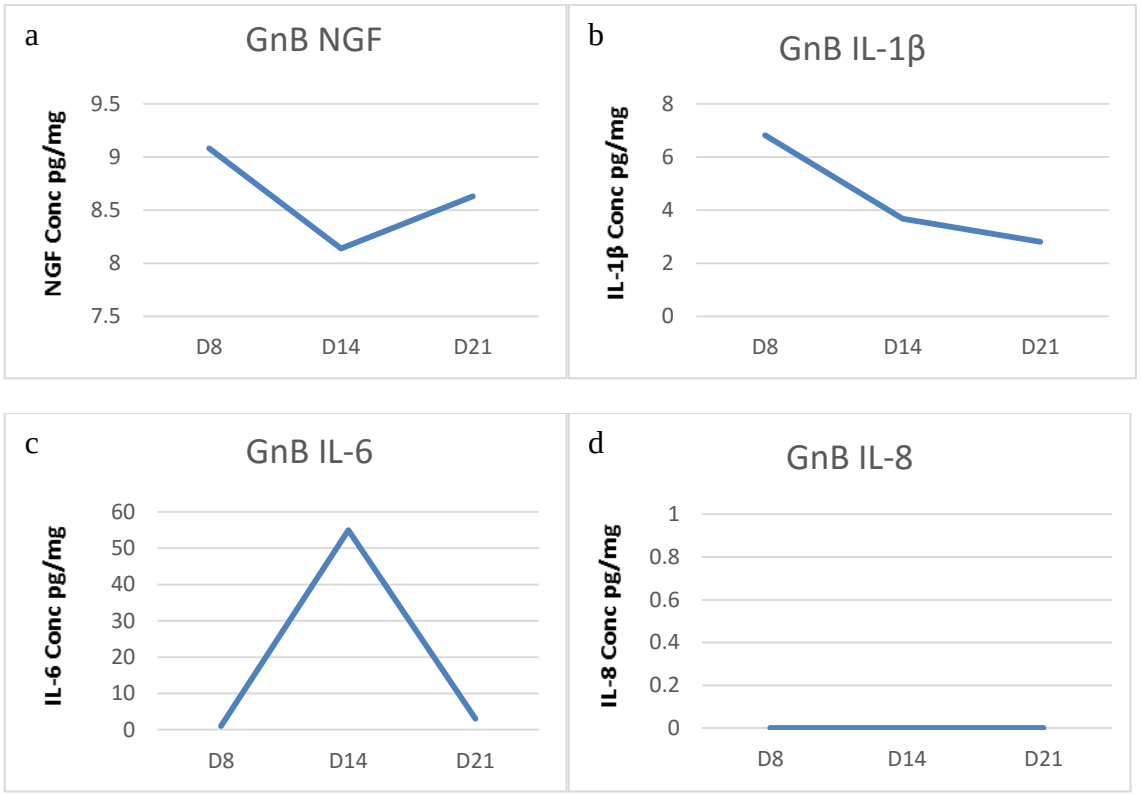

Figure 24. GnB Group Parameters.

In brief, burning the skin has stimulated significant alterations in all parameters tested; it increased pro-inflammatory cytokines (IL-1β) and downregulated to variable extents various anti-inflammatory cytokine (IL-8). IL-6 was maintained in the early phase and its decrease was delayed mostly in the RTX group. However, in the GB as well as in the nDB the decrease was sharp and acute.

The denervation has delayed for one (GB) or two weeks (RTXB) the up regulation of pro inflammatory cytokines and inhibited the production of IL-6 and IL-8 in the early phases.

4 DISCUSSION

The results of this study demonstrated an important role of sensory and postganglionic sympathetic fibers in the healing process of skin burn injury. The denervation of the sympathetic postganglionic neurons seemed to hasten the process with an earlier shedding of the crust and better vascularization compared to the non-denervated group. On the other hand, the denervation of the capsaicin-sensitive fibers by resiniferatoxin delayed the inflammation process, vascularization and sloughing of the crust for almost a week compared to the nDB group. Towards the end of the experiment (D28), the burn surface was reduced significantly in the GB and nDB groups, while the crust was widely maintained in the RTXB group. Such results are in line with reported data indicating that the wound surface area was larger by almost 36% in the final stage compared to the controls (Smith & Liu, 2002). It has also been reported that capsaicin denervation impairs migration of stem cells progeny present in hair follicles to participate in the reepithelialization process, thus resulting in longer times needed by capsaicin denervated wounds to heal (Martinez-Martinez, Galvan-Hernandez, Toscano-Marquez, & Gutierrez-Ospina, 2012; Barker, Rosson, & Dellon, 2006; Younan, et al., 2010; Fukai, Takeda, & Uchinuma, 2005). On the other hand, Souza et al. concluded that in full thickness wounds sympathetic denervation accelerated wound healing, although it partially impaired reepithelization (Souza, Cardoso, Amadeu, Desmouliere, & Costa, 2005). Their findings are in agreement with our data by showing that reepithelialization was relatively slow in the first two weeks then it picked up faster than non-denervated group by day 21 and definitely much faster than in the RTXB group which was well delayed. Consequently, by day 28, the

guanethidine treated rats had the best results of least surface area and scar with better vascularization. Sympathetic innervation is very important in regulating vascularity, in that chronic loss of sympathetic activity may contribute to abnormal vascular proliferation. Effects originate in the vascular tone from disruption of sympathetic fibers, causing the dilation and reorientation of choke vessels within the tissue of interest (Steinle, Pierce, Clancy, & Smith, 2002). “The term “choke vessel” refers to the portions of the vasculature that are located on the periphery of one vascular territory, or angiosome” (Glotzbach, Levi, Wong, Longaker, & Gurtner, 2010).

In this study, guanethidine helped in maintaining a more favorable microenvironment for the healing process while the resiniferatoxin did not. However, it is important to note that the dermis has a very important effect on the epidermal layer and both layers “cross talk” (Muller, 1971). In our study, the dermis was not fully destroyed, suggesting that in a partial-thickness burn, sympathetic inhibition may not be complete and might recover in 2-3 weeks, thus leading to a rebounce and to a modulation of alterations in the levels of cytokines and growth factors involved or even affecting cell populations, like mast cells, myofibroblasts or even stem cells and others.

4.1 Mast Cells

Skin mast cells constitute the peripheral response to stress, a major key player in neurogenic inflammation’ (Bienenstock et al., 1987), and as a major stress guard at the interface of the neuroimmunoendocrine environment (Maurer, et al., 2003). They secrete many proinflammatory cytokines and their cell life cycle has been regulated by stress mediators such as CRH, ACTH, SP, NGF and SCF among other factors, thus promoting activation and proliferation, however, inhibition is mainly mediated by catecholamines or corticosteroids (Theoharides et al., 1998). In addition, mast cells have

been documented to play a role in wound healing mostly in the homeostasis phase, and in particular in the inflammatory cascade.

Many studies have indicated that mast cells degranulate when subjected stress (Theoharides et al., 1998; Singh, Pang, Alexacos, Letourneau, & Theoharides 1999; Peters et al., 2005; Kiernan J. A., 1974). The authors described different stress modules of hormone, physiologic and mechanical stimuli. Kiernan JA and Siebenhaar F. also reported degranulation of mast cells by antidromic effect by sensory fibers (Kiernan J. A., 1975; Siebenhaar et al., 2008). Siebenhaar also concluded that sensory denervation impairs skin inflammation induced by mast cells (Siebenhaar et al., 2008). On the other hand, Bunker et al. reported decreased number of mast cells after capsaicin topical application in human subjects with compromised stratum corneum (Bunker et al., 1991). Our data are in line with the reported literature, they showed that the mast cells increased during the inflammatory phase in the regular nDB to go back to normal control levels later. However, in the RTXB group there was a drop and even absence of mast cells during the first two weeks of the healing process to reappear later during the last healing phase of remodeling and scarring whereby the scar was maintained for longer periods. On the other hand, in the GB group the effect on mast cells was similar to RTXB, but less drastic. Mast cells disappeared only for a short time during the first week to regain their presence later during the remodeling and scarring phases. Moreover, IgE activation of mast cells is reduced markedly in the absence of sensory innervation (Siebenhaar, et al., 2008). Siebenhaar et al. also reported that sensory innervation contribute to allergic skin responses suggesting that they augment inflammatory response driven by mast cells. Bunker et al. suggested that capsaicin induced the release of peptides from neurons could lead to mast cell degranulation

(Bunker, et al., 1991). On the other hand, it has been reported that SP immunoreactive nerve fibers were present near degranulated mast cells, thus indicating plasticity in the peptidergic innervation and skin neuro-immune communication (Peters, et al., 2005) via NK-1 (Paus, Theoharides, & Arck, 2006).

4.2 Changes of molecular parameters per treatment:

Our data showed that burning the skin has stimulated significant alterations in tested parameters; it increased the pro-inflammatory cytokine (IL-1β) and downregulated to variable extents the anti-inflammatory cytokine (IL-8). The denervation has delayed for one week in GB or two weeks in RTXB the up regulation of pro inflammatory cytokines and inhibited the production of IL-8 in the early phases. On the other hand, NGF had its non-significant ups and downs alterations.

The Nerve growth factor (NGF), a member of the neurotrophin family of polypeptides, is crucial for the development and survival of certain sympathetic and sensory neurons in the nervous systems: central and peripheral (Lewin & Mendell, 1993). “It plays a key role in the initiation and maintenance of inflammation in various organs” (Werner & Grose 2003). Accordingly it is documented that NGF is also involved in skin wound healing (Werner & Grose 2003). Applying exogenous NGF accelerates wound healing in normal and diabetic mice (Matsuda, et al., 1998; Li, Koroly, Schattenkerk, Malt, & Young, 1980) and promotes pressure ulcers healing in humans (Bernabei, et al., 1999).

Constantinou et al. found a marked increase in NGF levels of neonatal but not of adult rats after wounding (Constantinou, Reynolds, Woolf, Safieh-Garabedian, & Fitzgerald, 1994). NGF levels also increased at the wound site and so did serum levels

of NGF in the same wound model, and NGF mRNA was detected at the wound edge in new epithelial cells and in fibroblasts granulated tissue (Matsuda, et al., 1998). In addition, in myofibroblasts granulation tissue of rat wounds, high expression of NGF was found. On the other hand, much lower levels of NGF being found in adult animals' myofibroblasts compared with neonatals (Hasan, Zhang, Liu, Warn, & Smith, 2000). However, NGF presence in higher levels in neonatals compared with adult wounds indicates it is partially "responsible for the faster healing observed in neonatal animals" (Werner & Grose, 2003), independent of its mechanism of action. RTX is an agonist of TPRV1 and has blocked the sensory neurons from secreting NGF and other peptides hence the low levels of NFG in the RTXnB groups. After burning NGF was back to around CTRL levels. This is may be due to that NGF is usually secreted by different cell types present at wound site including fibroblasts, mast cells, T-cells, and keratinocytes (Radtke, Rennekampff, Reimers, Vogt, & Kocsis, 2013). Keratinocytes produce and secrete active NGF, stimulating neuritic outgrowth and also acting on keratinocytes to migrate and proliferate promoting repithelialization (Di Marco, et al., 1993; Pincelli & Marconi, 2000) hence, contributing to wound healing (Di Marco, et al., 1993). In addition, Ratdke et al., in their study, concluded that "keratinocytes up-regulate NGF production and proliferation in the presence of sensory neurons" (Radtke, Rennekampff, Reimers, Vogt, & Kocsis, 2013). In our study, NGF levels decreased more in denervated non-burned rats (RTXnB) than GnB, nDB or in controls indicating a successful denervation by RTX with maximal effect compared to guanethidine or controls.

In the RTXB, after an initial non-significant increase, NGF decreased all through the experiment and so did IL-6 which also decreased slowly, the first three days

then sharply to zero levels by day 8 to remain low later on. In addition IL-1β presence was delayed and started to increase significantly after the first week, therefore, delaying the inflammatory homeostasis phase. Such a response corresponded to a similar increase of IL-8.

During the experiment, NGF did not seem to vary significantly among the various groups and so was the case of IL-6, which dropped significantly to zero levels in nDB and later in RTXB groups, but remained present for more time in the GB group, till end of the second week. In conclusion, sensory neurons are needed for the upregulation of NGF production by keratinocytes and mast cells. The sensory nerve fibers entering wounds may contribute to keratinocyte stimulation and epithelial proliferation at wound edges and hence more NGF and IL-1β secretion (Radtke, Rennekampff, Reimers, Vogt, & Kocsis, 2013).

Our results come mostly in agreement with the reported literature concerning skin wounds and the nervous system. Actually, the inflammatory cytokines that are secreted with barrier disruption by wounds or burns can recruit and trap inflammatory cells in the dermis and epidermis and store an array of cytokines and growth factors (Nickoloff & Naidu, 1994). These preformed cytokines: interleukin-1α and β, NGF and tumor necrosis factor-α (TNF-α), among others, are released from the keratinocytes, mast cells and granular cells in response to minimal external perturbations (Nickoloff & Naidu, 1994; Wood, Jackson, Elias, Grunfeld, & Feingold, 1992; Wood, et al., 1996; Elias, Wood, & Feingold, 1999). The levels of IL-1β, IL-6 and IL-8 released at the site of injury, are indicative of progress in wound closure and of surface area of the burn wound. In brief, IL-1β levels increase has been linked to myofibroblasts apoptosis

increase (Ziche, et al., 1994) and IL-6 normally present in small amounts in cells of the epidermis, quickly increase in levels after barrier disruption (Wang, et al., 2004). The presence of burn and denervation have decreased or inhibited IL-6 in our case a possible indication of delayed inflammatory response and reepithelialization. Simultaneously, changes leading to repair are aided by cytokines like IL-8, triggering proliferation and rebuilding of the epidermis (Elias & Feingold, 2001). However, it is proven that the mitogenic effect of IL-6 can inflict a dramatic delay of reepithelialization for keratinocytes in IL-6 Knock out mice, not to mention that granulation tissue formation could be impaired too (Werner & Grose, 2003). Thus, IL-6 appears to be crucial for the initiation of the healing process via its mitogenic effect on keratinocytes and neutrophils recruitment (Werner & Grose, 2003) and it decreased dramatically in the denervated rats, in particular, in the RTXB group may be due to the delayed inflammatory process.

On the other hand, IL-8 being an anti-inflammatory cytokine is also expressed in healing skin wounds. It has been suggested that it reduces inflammation in fetal wound healing (Liechty, Crombleholme, Cass, Martin, & Adzick, 1998) due to their higher presence in fetal wounds (Werner & Grose, 2003) and hence may be contributing to scarless wound repair [224]. In vivo application of IL-8 stimulated reepithelialization on human skin grafts in a chimeric mouse model (Werner & Grose, 2003). Data in our study showed that the increase of IL-8 appearance coincided with the progress of the healing process.

The inflammatory process can sustain the abnormal skin condition initiated by the primary barrier disruption (Liechty, Crombleholme, Cass, Martin, & Adzick, 1998). Acute and chronic disruption of the cutaneous permeability barrier increase messenger ribonucleic acid (mRNA) levels for TNF, granulocyte-macrophage colony-stimulating

factor (GMCSF), IL-1α, IL-1β, and IL-6 in the epidermis (Fore-Pfliger, 2004). Treating the abnormality in the skin barrier is a key factor in reducing the inflammatory process (Ganz, 2002). It could also be the opposite, decreasing inflammation severity by guanethidine could enhance the healing process for a shorter time and leading to a better scarring condition like in our experiment.

Tian et al. reported that in their controls which are equivalent to the nDB group in this study, inflammatory cells appeared at site of injury after six hours of superficial second degree burn infliction (Tian, et al., 2016). Hence the inflammation phase of wound healing has started almost instantly. As reported in this study, IL-1β concentration at D0 after injury inflection was134 pg/mg more than double those of the RTXB (52 pg/mg) and the GB (48 pg/mg). Moreover, IL-6 concentration on D0 was 1523 pg/mg which was higher also than RTXB (1062 pg/mg) and GB (1068 pg/mg). However, it took more time for IL-8 to pick up. This difference might be explained by the suppression of the inflammatory process in the first week (GB group) and second weeks (RTXB group). Richards A. M. et al. found a significant reduced cell count of monocytes and macrophages in the granulation tissue of the sensory denervated flap compared with his controls (Richards, Floyd, Terenghi, & McGrouther, 1999).

As the inflammatory phase picked up pace, IL-1β concentrations started to rise and IL-6 concentrations continued to decrease in the homeostasis and proliferation phases. The pace was, however, slower for RTXB compared to GB or nDB group. IL-1β levels were almost significant at all time points in RTXB, GB and nDB groups compared with the control group. However, NGF did not seem have much influence in this case or not much affected, since close concentration levels, not significantly

different, were detected in all groups. On the other hand, IL-8 increase corresponded very well to the progress in the healing process.

It is important to note that estrogen has an inhibitory role on the inflammatory process after burn injury in female rodents (Deitch, et al., 2006; Gregory, Duffner, Faunce, & Kovacs, 2000). Ovariectomy was shown to result in increased neutrophil activity in the periphery (Deitch, et al., 2006). Moreover, the percentage of reepithelialized biopsy surface was significantly larger in male patients (Wehrens, Arnoldussen, Booi, & van der Hulst, 2016). In brief, "Estrogen modulates all phases of wound healing by reducing inflammation, accelerating re-epithelialization, stimulating the formation of granulation tissue, and regulating proteolysis" (Emmerson & Hardman, 2012). Based on this information, cytokines levels in this study may have been affected by the estrogen cycle. Hence, in burn studies done on females, time points should take into consideration the estrogen cycle which is about 5 days in female rats.

In conclusion, the denervation by RTX of sensory fibers delayed the healing time and resulted in a hard and worse scar. On the other hand, denervation by guanethidine of the sympathetic nerves created a better environment for healing of the burned wounds leading to a faster and better scar.

5 REFERENCES

(2014, April 21). Retrieved from National Institute of Health: http://www.nlm.nih.gov/medlineplus/burns.html

Aarabi, S., Longaker, M. T., & Gurtner, G. C. (2007). Hypertrophic scar formation following burns and trauma: new approaches to treatment. *PLoS Medicine*, 4:e234.

Abdallah Hajj Hussein, I., Dali Balta, N., Jurjus, R. A., Watfa, W., Gerges, A., Atiyeh, B., . . . Jurjus, A. R. (2012). Rat model of burn wound healing: effect of Botox. *Journal of Biological Regulators and Homeostatic Agents*, 289-400.

Akaishi, S., Ogawa, R., & Hyakusoku, H. (2008). Keloid and hypertrophic scar: neurogenic inflammation hypotheses. *Medical Hypotheses*, 32-38.

Alawi, K., & Keeble, J. (2010). The paradoxical role of transient receptor vanilloid 1 receptor in inflammation. *Pharmacology and Therapeutics*, 181-195.

Apfel, S. C., Arezzo, J. C., Brownlee, M., Federoff, H., & Kessler, J. A. (1994). Nerve growth factor administration protects against experimental diabetic sensory neuropathy. *Brain Research*, 7-12.

Arabi, S., Bhatt, K. A., Paterno, J., Chang, E. L., Loh, S. A., Holmes, J. W., . . . Gurtern, G. C. (2007). Mechanical load initiates hypertrophic scar formation through decreased cellular apoptosis. *FASEB*, 3250-3261.

Arnold, F., & West, D. C. (1991). Angiogenesis in wound healing. *Pharmacology and therapeutics*, 407-422.

Arvier, P. T., Chahl, L. A., & Ladd, R. J. (1977). Modification by capsaicin and compound 48/80 of dye leakage induced by irritants in the rat. *British Journal of Pharmacology*, 61-68.

Aschroft, G., Mills, S., Lei, K., Jeong, M. J., Taniguchi, M., Burrow, M., . . . Nakayama, T. (2003). Estrogen modulates cutaneous wound healing by downregulating macrophage migration inhibitory factor. *The Journal of Clinical Investigation*, 1309-1318.

Atiyeh, B. S., & Hayek, S. N. (2005). Moisture and Wound Healing. *Journal des Plaies et Cicatrisation*, 7-11.

Atiyeh, B. S., Ioannovich, J., Al-Amm, C. A., & El-Musa, K. A. (2002). Management of acute and chronic open wounds: the importance of moist environment on optimal wound healing. *Current Pharmaceutical Biotechnology*, 179-195.

auf demKeller, U., Krampet, M., Kumin, A., Braun, S., & Werner, S. (2004). Keratinocyte growth factor: effects on keratinocytes and mechanisms of action. *European Journal of Cell Biology*, 607-612.

Bansal, C., Omlin, K. J., Hayes, C. M., & Rohrer, T. E. (2006). Novel cutaneous uses for botulinum toxin type A. *Journal of Cometic Dermatology*, 268-272.

Barker, A. R., Rosson, G. D., & Dellon, A. L. (2006). Wound healing in denervated tissue. *Annals of Plastic Surgery*, 339-342.

Barker, T. H. (2011). The role of ECM proteins and protein fragments in guiding cell behavior in regenerative medicine. *Biomaterials*, 4211-4214.

Bauer, S. M. (2005). Angiogenesis, vasculogenesis, and induction of healing in chronic wounds. *Vascular & Endovascular Surgery*, 293-306.

Baum, J., & Duffy, H. S. (2011). Fibroblasts and myofibroblasts: what are we talking about? . *Journal of Cardiovascular Pharmacology*, 376-379.

Beam, J. W. (2008). Occlusive dressings and the healing of standardized abrasions. *Journal of Athletic Training*, 600-607.

Bellemare, J., Roberge, C., Bergeron, D., Lopez-Valle, C. A., Roy, M., & Moulin, V. J. (2005). Epidermis promotes dermal fibrosis: role in the pathogenesis of hypertrophic scars. *The Journal of Pathology*, 1-8.

Bennet, N. T., & Schultz, G. S. (1993). Growth factors and wound healing: biochemical properties of growth factors and their receptors. *American Journal of Surgery*, 728-737.

Bennett, M. K., Calakos, N., & Scheller, R. H. (1992). Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science*, 255-259.

Bennett, S. P., Griffiths, G. D., Schor, A. M., Leese, G. P., & Schor, S. L. (2003). Growth factors in the treatment of diabetic foot ulcers. *British Journal of Surgery*, 133-146.

Bernabei, R., Landi, F., Bonini, S., Onder, G., Lambiase, A., Pola, R., & Aloe, L. (1999). Effect of topical application of nerve-growth factor on pressure ulcers. *Lancet*, 307.

Besne, I., Descombes, C., & Breton, L. (2002). Effect of age and anatomical site on density of sensory innervation in human epidermis. *Archives of Dermatology*, 1445-1450.

Bienenstock, J., Tomioka, M., Stead, R., Ernst, P., Jordana, M., Gauldie, J., . . . Denburg, J. (1987). Mast cell involvement in various inflammatory processes. *American Review of Respiratory Disease*, S5–S8.

Birklein, F., & Schmelz, M. (2008). Neuropeptides, neurogenic inflammation and complex regional pain syndrome (CRPS). *Neuroscience Letters*, 199-202.

Blasi, J., Chapman, E. R., Link, E., Binz, T., Yamasaki, S., De Camilli, P., . . . Jahn, R. (1993). Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature*, 160-163.

Bley, K. R. (2010). *TRPV1 agonist approach for pain management. In: Gomatsyan A, Flatynek CR eds. Vanilloid TRPV1 in Durg Discovery: Targetting Pain an other Phathological Disorders.* New York: Wiley.

Boesmans, W., Owslanik, G., Tack, J., Voets, T., & Vanden Berghe, P. (2011). TRP Channels in neurogastroenterology: opportunities for therapeutic intervention. *British Journal of Pharmacology*, 18-37.

Bolton, L. (2007). Operational definition of moist wound healing. *Journal of Wound, Ostomy, and Continence*, 23-29.

Brain, S. D., & Grant, A. D. (2004). Vascular actions of calcitonin gene-related peptide and adrenomedullin. *Pysiological Reviews*, 903-934.

Brauchle, M., Angermeyer, K., Hubner, G., & Werner, S. (1994). Large induction of keratinocyte growth factor expression by serum growth factors and pro-inflammatory cytokines in cultured fibroblasts. *Oncogene*, 3199-3204.

Breuing, K., Eriksson, E., Liu, P., & Miller, D. R. (1992). Healing of partial thickness porcine skin wounds in a liquid environment. *The journal of Surgical Research*, 50-58.

Broughton, G., Janis, J. E., & Attinger, C. E. (2006). The basic science of wound healing. *Plastic and Reconstructive Surgery*, 12S-34S.

Bullard, K. M., Longaker, M. T., & Lorenz, H. P. (2003). Fetal wound healing: current biology. *World Journal of Surgery*, 54-61.

Bunker, C. B., Cerio, R., Bull, H. A., Evans, J., Dowd, P. M., & Foreman, J. C. (1991). The effect of capsaicin application on mast cells in normal human skin. *Agents & Actions*, 195-196.

Cass, R., Kuntzman, R., & Brodie, B. B. (1960). Norepinephrine depletion as a possible mechanism of action of guanethidine (SU5364), a new hypotensive agent. *Proceedings of the Society for Experimental Biology and Medicine*, 871-872.

Caviedes-Bucheli, J., Munoz, H. R., Azuero-Holguin, M. M., & Ulate, E. (2008). Neuropeptides in dental pulp: the silent protagonists. *Journal of Endodontics*, 773-788.

Cha, D., O'Brien, P., O'Toole, E. A., Woodley, D. T., & Hudson, L. G. (1996). Enhanced modulation of keratinocyte motility by transforming growth factor-alpha (TGF-alpha) relative to epidermal growth factor (EGF). *Journal of investigative Dermatology*, 590-597.

Chahl, L. A. (1988). Antidromic vasodilatation and neurogenic inflammation. . *Pharmacology and Therapeutics*, 275-300.

Chen, J. C., Lin, B. B., Hu, H. W., Lin, C., Jin, W. Y., Zhang, F. B., . . . Chen, R. J. (2014). NGF Accelerates Cutaneous Wound Healing by Promoting the Migration of Dermal Fibroblasts via the PI3K/Akt-Rac1-JNK and ERK Pathways. *BioMed Research International*, 1-13.

Chen, J. D., Lapiere, J. C., Sauder, D. N., Peavey, C., & Woodley, D. T. (1995). Interleukin-1 alpha stimulates keratinocyte migration through an epidermal growth factor/transforming growth factor-alpha-independent pathway. *The Journal of Investigative Dermatology*, 729-733.

Cherington, M. (2004). Botulism: update and review. *Seminars in Neurology*, 155-163.

Choi, J. C., Lucarelli, M. J., & Shore, J. W. (1997). Use of botulinum A toxin in patients at risk of wound complications following eyelid reconstruction. *Opthalmic Plastic and Reconstructive Surgery*, 259-264.

Clark, R. A. (1981). Fibronectin in the skin. *The Journal of Investigative Dermatology*, 475-479.

Clark, R. A., Lanigan, J. M., DellaPelle, P., Manseau, E., Dvorak, H. F., & Colvin, R. B. (1982). Fibronectin and fibrin provide a provisional matrix for epdiermal cell migration during wound reepithelialization. *The Journal of Investigative Dermatology*, 264-269.

Claus, E. E., Fusco, C. F., Ingram , T., Ingersoll, C. D., Edwards, J. E., & Melham, T. J. (1998). Compasrison of the effects of selected dressings on the healing of standardized abrasions. *Journal of Athletic Training*, 145-149.

Coderre, T. J., Basbaum, A. I., & Levine, J. D. (1989). Neural control of vascular permeability: interactions between primary afferents, mast cells, and sympathetic efferents. *Jrnal of Neurophysiology*, 48-58.

Cohen, S. (1962). Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *The Journal of Biological Chemistry*, 1555-1562.

Constantinou, J., Reynolds, M. L., Woolf, C. J., Safieh-Garabedian, B., & Fitzgerald, M. (1994). Nerve growth factor levels in developing rat skin: upregulation following skin wounding. *Neuroreport*, 2281–2284.

Cruciani, M., Lipsky, B. A., Mengoli, C., & de Lalla, F. (2005). Are granulocyte colony-stimulating factors beneficial in treating diabetic foot infections?: a meta-analysis. *Diabetes Care*, 454-460.

Cruise, B. A., Xu, P., & Hall, A. K. (2004). Wounds increase activin in skin and a vasoactive neuropeptide in sensory ganglia. *Developmental Biology*, 1-10.

Dabiri, G., Tumbarello, D. A., Turner, C. E., & Van de Water, L. (2008). Hic-5 promotes the hypertrophic scar myofibroblast phenotype by regulating the TGF-beta1 autocrine loop. *The Journal of Investigative Dermatology*, 2518-2525.

Darby, I. A., Bisucci, T., Pittet, B., Garbin, S., Gabbiani, G., & Desmoulière, A. (2002). Skin flap-induced regression of granulation tissue correlates with reduced growth factor and increase metalloproteinase expression. *The Journal of Pathology*, 117-127.

Daveltov, B., Bajohrs, M., & Binz T. (2005). Beyond BOTOX: advantages and limitations of individual botulinum neurotoxins . *Trends in Neurosciences*, 446-452.

Deitch, E. A., Ananthakrishnan, P., Cohen, D. B., Xu, D. Z., Feketeova, E., & Hauser, C. J. (2006). Neutrophil activation is modulated by sex hormones after trauma-hemorrhagic shock and burn injuries. *American Journal of Physiology. Heart and Circulatory Physiology*, 456-465.

Denda, M., & Tsutsumi, M. (2011). Role of transient recepotr potential proteins (TRPs) in epidermal ketatinocytes. *Advances in Experimental Medicine and Biology*, 847-860.

Devine, C. E., Robertson, A. A., & Simpson, F. O. (1967). Effect of symphtolytic drugs on sympathetic axonal fine structure and tissue catecholamine levels. *New Zealand Medical Journal*, 390.

Di Marco, E., Mathor, M., Bondanza, S., Cutuli, N., Marchisio, P. C., Cancedda, R., & De Luca, M. (1993). Nerve growth factor binds to normal human keratinocytes through high and low affinity receptors and stimulates their growth by a novel autocrine loop. *The Journal of Biological Chemistry*, 22838-22846.

DiPietro, L. A., & Polverine, P. J. (1993). Role of the macrophage in the positive and negative regulation of wound neovascularization. *Behring Institute Mitteilungen*, 238-247.

Dolly, O. (2003). Synaptic transmission: inhibition of neurotransmitter release by botulinum toxins. *Headache*, S16-S24.

Douglas, H. E. (2010). TGF-ß in wound healing: a review. *Journal of Wound Care*, 403-406.

Dyson, M., Young, S., Pendle , C. L., Webster, D. F., & Lang, S. M. (1988). Comparison of the effects of moist and dry conditions on dermal repair. *The Journal of Investigative Dermatology*, 434-439.

Eaglstein, W. H. (1985). Experiences with biosynthetic dressings. *Journal of the American Academy of Dermatology*, 434-440.

Eckes, B., Nischt, R., & Krieg, T. (2010). Cell-matrix interactions in dermal repair and scarring. *Fibrogensis and Tissue Repair*, 3:4.

Ehrlich, H. P., Desmoulière, A., Diegelmann, R. F., Cohen, I. K., Compton, C. C., Garner, W. L., . . . Gabbiani, G. (1994). Morphological and immunochemical differences between keloid and hypertrophic scar. *The American Journal of Pathology*, 105-113.

Elias, P. M., & Feingold, K. R. (2001). Does the tail wag the dog? Role of the barrier in the pathogenesis of inflammatory dermatoses and therapeutic implications. *Archives of Dermatology*, 1079-1081.

Elias, P. M., Wood, L. C., & Feingold, K. R. (1999). Epidermal pathogenesis of inflammatory dermatoses. *American Journal of Contact Dermatisis*, 119-126.

Eming, S. A., Krieg, T., & Davidson, J. M. (2007). Inflammation in wound repair: molecular and cellular mechanisms. *Journa of Investigative Dermatology*, 514-525.

Emmerson, E., & Hardman, M. J. (2012). The role of estrogen deficiency in skin ageing and wound healing. *Biogerontology*, 3-20.

Endrich, B., & Menger, M. D. (2000). Regeneration of the microcirculation during wound healing. *Unfallchirurg*, 1006-1008.

Enoch, S., & Leaper, J. D. (2005). Basic science of wound healing. *Surgery (Oxford)*, 37-42.

Enoch, S., & Price, P. (2004). *Cellular Molecular and biochemical differences in the pathophysiology of healing between acute wounds chronic wounds and wounds*

in the aged. Retrieved from World Wide Wounds: http://www.worldwidewounds.com/2004/August/Enoch/Pathophysiology-Of-Healing.html

Erbguth, F. J. (2004). Historical notes on botulism, Clostridium botulinum, botulinum toxin, and the idea of the therapeutic use of the toxin. *Movement Disorderss*, S2-S6.

Esteves Junior, I., Tacani, P. M., Liggieri, V. C., Ruggi, B. G., Ferreira, L. M., & Liebano, R. E. (2009). Histamine iontophoresis on the viability of random skin flap in rats. *Acata Cirurgica Brasileira*, 48-51.

Fernandes, E. S., Fernandes, M. A., & Keeble, J. E. (2012). The functions of TRPA1 and TRPV1: moving away from sensory nerves. *British Journal of Pharmaocology*, 510-521.

Ferreira, L. M., Gragnani, A., Furtado, F., & Hochman, B. (2009). Control of the skin scarring response. *Anais De Academia de Ciencias*, 623-629.

Field, F. K., & Kerstein, M. D. (1994). Overview of wound healing in a moist environment. *American Journal of Surgery*, 2S-6S.

Finnerty, C. C., Herndon, D. N., Przkora, R., Pereira, C. T., Oliveria, H. M., Queiroz, D. M., . . . Jeschke, M. G. (2006). Cytokine expression profile over time in severely burned pediatric patients. *Shock*, 13-19.

Flynn, T. C. (2009). Use of Intraoperative Botulinum Toxin in Facial Reconstruction . *Dermatologic Surgery*, 182-188.

Fore-Pfliger, J. (2004). The Epidermal Skin Barrier: Implications for the Wound Care Practitioner, Part I Adv Skin Wound Care. *Advances in Skin and Wound Care*, 417-425.

Freedberg, I. M., Tomic-Canic, M., Komine, M., & Blumenberg, M. (2001). Keratins and the keratinocyte activation cycle. *The Journal of Investigative Dermatology*, 633-640.

Fukai, T., Takeda, A., & Uchinuma, E. (2005). Wound healing in denervated rat skin. *Wound Repair and Regeneration*, 175-180.

Galiano, R. D., Tepper, O. M., Pelo, C. R., Bhatt, K. A., Callaghan, M., Bastidas, N., . . . Gurtner, G. C. (2004). Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by mobilizing and recruiting bone marrow derived cells. *The American Journal of Pathology*, 1935-1947.

Ganz, T. (2002). Epithelia: not just physical barriers. *Proceedings of the National Academy of Sciences of the United States of America*, 3357-3358.

Gassner, H. G., Sherris, D. A., & Otley, C. C. (2000). Treatment of facial wounds with botulinum toxin A improves cosmetic outcome in primates. . *Plastic and Reconstructive Surgery*, 1948-1955.

Genirini, S., Tuveri, M. A., Matucci Cerinic, M., Mastinu, F., Manni, L., & Aloe, L. (2004). Topical application of nerve growth factor in human diabetic foot ulcers. *Experimental and Clinical Endocrinology and Diabetes*, 542-544.

Gil, D. W., Wang, J., Gu, C., Donello, J. E., Cabrera, S., & Al-Chaer, E. D. (2016). Role of sympathetic nervous system in rat model of chronic visceral pain. *Neurogastroenetrology and Motility*, 423-431.

Glotzbach, J. P., Levi, B., Wong, V. W., Longaker, M. T., & Gurtner, G. C. (2010). The basic science of vascular biology: implications for the practicing surgeon. *Plastic and Reconstructive Surgery*, 1528-1538.

Gomez, R., & Canico, L. C. (2007). Management of burn wounds in the emergency department. *Emergency Medicine Clinics of North America*, 135-146.

Goretsky, M. J., Harriger, M. D., Supp, A. P., Greenhalgh, D. G., & Boyce, S. T. (1996). Expression of interleukin-1alpha, interleukin-6, and basic fibroblast growth factor by cultured skin substitutes before and after grafting to full-thickness wounds in athymic mice. *The Journal of Trauma*, 894-899.

Gozsy, B., & Kato, L. (1966). Role of norepinephrine and 5-hydroxytryptamine in the delayed phase ofthe inflammatoryreaction in rats. *International Archives of Allergy and Applied Immunology*, 553-560.

Graiani, G., Emanueli, C., Desortes, E., Van Linthout, S., Pinna, A., Fiqueroa, C. D., . . . Madeddu, P. (2004). Nerve growth factor promotes reparative angiogenesis and inhibits endothelial apoptosis in cutaneous wounds of Type 1 diabetic mice. *Diabetologia*, 1047-1054.

Green, K. L. (1974). Role of endogenous catecholamines in the anti-inflammatory activity ofalpha-adrenoceptor blocking agents. *British Journal of Pharmacology*, 45-53.

Greenhalgh, D. G. (1998). The role of apoptosis in wound healing. *International Journal of Biochemistry and Cell Biology*, 1019-1030.

Gregory, M. S., Duffner, L. A., Faunce, D. E., & Kovacs, E. J. (2000). Estrogen mediates the sex difference in post-burn immunosuppression. *The Journal of Endocinology*, 129-138.

Guadry, M., Bregerie, O., Andrieu, V., El Banna, J., Pocidalo, M. A., & Hakim, J. (1997). Intracellular pool of vascular endothelial growth factor in human neutrophils. *Blood*, 4153-4163.

Günter, C., & Machens , H. (2012). New Strategies in Clinical Care of Skin Wound Healing. *European Surgical Research*, 16-23.

Gurtner, G. C., & Evand, G. D. (2000). Advances in head and neck reconstruction. *Plastic and Reconstructive Surgery*, 672-682.

Gurtner, G. C., Werner, S., Barrandon, Y., & Longaker, M. T. (2008). Wound repeair and regeneration. *Nature*, 314-321.

Gyulai, R., Hunyadi, J., Kenderessy-Szabo, A., Kemeny, L., & Dobozy, A. (1994). Chemotaxis of freshly separated and cultured human keratinocytes. *Clinical and Experimental Dermatology*, 309-311.

Hammond, D. L., & Ruda, M. A. (1991). Developmental alterations in nociceptive threshold, immunoreactive calcitonin gene-related peptide and substance P, and fluoride-resistant acid phosphatase in neonatally capsaicin-treated rats. *The Journal of Comparative Neurology*, 436-450.

Harsum, S., Clarke, J. D., & Martin, P. (2001). A reciprocal relationship between cutaneous nerves and repairing skin wounds in the developing chick embryo. *Developmental Biology*, 27–39.

Hasan, W., Zhang, R., Liu, M., Warn, J. D., & Smith, P. G. (2000). Coordinate expression of NGF and α-smooth muscle actin mRNA and protein in cutaneous wound tissue of developing and adult rats. *Cell and Tissue Research*, 97-109.

Haubner, F., Ohmann, E., Muller-Vogt, U., Kummer, P., Strutz, J., & Gassner, H. G. (2012). Effects of Botulinum Toxin A on Cytokine Synthesis in a Cell Culture Model of Cutaneous Scarring. *Archives of Facial Plastic Surgery*, 122-126.

Haulten, L. (1994). Dressings for surgical wounds. *American Journal of Surgery*, 42S-45S.

Hebda, P. A., Klingbeil, C. K., Abraham, J. A., & Fiddes, J. C. (1990). Basic fibroblast growth factor stimulation of epidermal wound healing in pigs. *The Journal of Investigative Dermatology*, 626-631.

Helme, R. D., & Andrews, P. V. (1985). The effect of nerve lesions on the inflammatory response to injury. *Journal of Neuroscience Research*, 453-459.

Hermans, M. H. (2005). A general overview of burn care. *International Wound Journal*, 206-220.

Hinman, C. D., & Maibach, H. (1963). Effect of air exposure and occlusion on experimental human skin wounds. *Nature*, 377-378.

Hinz, B. (2007). Formation and function of the myofibroblast during tissue repair. *The Journal of Investigative Dermatology*, 526-537.

Holman, D. M., & Kalaaji, A. N. (2006). Cytokines in dermatology. *Journal of Drugs in Dermatology*, 520-524.

Hu, X., Sun, H., Han, C., Wang, X., & Yu, W. (2011). Topically applied rhGM-C SF for the wound healing: a systematic review. *Burns*, 729-741.

Huang, J. S., Wang, Y. H., Ling, T. Y., Chuang, S. S., Johnson, F. E., & Huang, S. S. (2002). Synthetic TGFbeta antagonist accelerates wound healing and reduces scarring. *FASEB Journal*, 1269-1270.

Igrashi, M., Finch, P. W., & Aaronson, S. A. (1998). Characterization of recombinant human fibroblast growth factor (FGF)-10 reveals functional similarities with keratinocyte growth factor (FGF-7). *Journal of Biological Chemistry*, 13230-13235.

Jacinto, A., Martinez-Arias, A., & Martin, P. (2001). Mechanisms of epithelial fusion and repair. *Nature Cell Biology*, 117-123.

Jameson, J., Ugarte, K., Chen, N., Yachi, P., Fuchs, E., Boismenu, R., & Havran, W. L. (2002). A role for skin gammadelta T cells in wound repair. *Science*, 747-749.

Jancso, N. (1960). Role of the nerve terminals in the michanism of inflammatory reactions. *Bull Millard Fillmore Hospital*, 53-77.

Jankovic, J. (2004). Botulinum toxin in clinical practice. *Journal of Neurology, Neurosurgery and Psychiatry*, 951-957.

Jaschke, E., Zabernigg, A., & Gattringer, C. (1999). Recombinant human granulocyte-macrophage colony-stimulating factor applied locally in low doses enhances healing and prevents recurrence of chronic venous ulcers. *International Journal of Dermatology*, 380-386.

Johnson, E. A. (1999). Clostridial toxins as therapeutic agents: benefits of nature's most toxic proteins. *Annual Reviews of Microbiology*, 551-575.

Jonkman, M. F., Hoeksma, E. A., & Nieuwenhuis, P. (1990). Accelerated epithelization under a highly vapor-permeable wound dressing is associated with increased precipitation of fibrin(ogen) and fibronectin. *The Journal of Investigative Dermatology*, 477-484.

Jurjus, A., Atiyeh, B. S., Abdallah, I. M., JUrjus, I. A., Hayek, S. N., Jaoude, M. A., . . . Tohme, R. A. (2007). Pharmacological modulation of wound healing in experimental burns. *Burns*, 892-907.

Kawamoto, K., & Matsuda, H. (2004). Nerve growth factor and wound healing. *Progress in Brain Research*, 369-384.

Kerstein, M. D. (1995). Moist wound healing: the clinical perspective. *Ostomy/Wound Management*, 37S-44S.

Kiernan, J. A. (1974). Action of adenosine triphosphate on Mast Cells in Normal and Denervated skin. *Archiv fur Dermatologische Forschung*, 83-86.

Kiernan, J. A. (1975). A pharmacological and histological investigation of the involvement of mast cells in cutaneous axon reflex vasodilatation. *Quarterly Journal of Experimental Physiology & Cognate Medical Sciences*, 123-130.

Kitzis, V., Engrav, L. H., & Quinn, L. S. (1999). Transient exposure to tumor necrosis factor-alpha inhibits collagen accumulation by cultured hypertrophic scar fibroblasts. *The Journal of Surgical Research*, 134-141.

Kiwanuka, E., Junker, J., & Eriksson, E. (2012). Harnessing growth factors to influence wound healing. *Clinics in Plastic Surgery*, 239-248.

Knabl, J. S., Bayer, G. S., Bauer, W. A., Schwendenwein, I., Dado, P. F., Kucher, C., . . . Meissl, G. (1999). Controlled partial skin thickness burns: an animal model for studies of burnwound progression. *Burns*, 229-235.

Koh, T. J., & DiPietro, L. A. (2011). Inflammation and wound healing: the role of the macrophage. *Expert Reviews in Molecular Medicine*, 13e23.

Koh, T. W., & Bellen, H. J. (2003). Synaptotagmin I, a Ca2+ sensor for neurotransmitter release. *Trends in Neurosciences*, 413-422.

Kopin, I. J., & Gordon, E. K. (1963). Metabolism of administered and drug-released norephrine-7-H3 in rat. *Journal of Pharmacology and Experimental Therapeutics*, 207.

Korting, H. C., Schollmann, C., & White, R. J. (2011). Management of minor acute cutaneous wounds: importance of wound healing in a moist environment. *Journal of the European Academy of Dermatology & Venereology*, 130-137.

Krafts, K. P. (2010). Tissue repair: the hidden drama. *Organogenesis*, 225-233.

Lambiase, A., Manni, L., Rama, P., & Bonini, S. (2003). Clinical application of nerve growth factor on human corneal ulcer. *Archives Italiennes de Biologie*, 141-148.

Landi, F., Aloe, L., Russo, A., Cesari, M., Onder, G., Bonini, S., . . . Bernabei, R. (2003). Topical treatment of pressure ulcers with nerve growth factor: a randomized clinical trial. *Annals of Internet Medicine*, 635-641.

Lapin, G. A., Hochman, B., Nishioka, M. A., Maximino, J., Chadi, G., & Ferreira, L. M. (2015). A Research Design for the Quantification of the Neuropeptides Substance P and Calcitonin Gene-Related Peptide in Rat Skin Using Western Blot Analysis. *Advances in Skin and Wound Care*, 259-265.

Lau, k., Paus, R., Tiede, S., Day, P., & Bayat, A. (2009). Exploring the role of stem cells in cutaneous wound healing. *Experimental Dermatology*, 921-933.

Lazarus, G. S., Cooper, D. M., Knighton, D. R., Margolis, D. J., Percoraro, R. E., Rodeheaver, G., & Robson, M. C. (1994). Definitions and guidelines for assessment of wounds and evaluation of healing. *Wound Repair and Regeneration*, 165-170.

Lee, A., Coderre, T. J., Basbaum, A. I., & Levine, J. D. (1991). Sympathetic neuron factors involved in bradykinin-induced plasma extravasation in the rat. *Brain Research*, 146-148.

Leibovich, S. J., & Ross, R. (1975). The role of macrophage in wound repair. A study with hydrocortison and anitmacrophase serum. *American Journal of Pathology*, 71-100.

Levine, J. D., Dardick, S. J., Roizen, M. F., Helms, C., & Basbaum, A. I. (1986). The contribution of neurogenic inflammationin experimental arthritis. *The Journal of Neuroscience*, 3423-3429.

Lewin, G. R., & Mendell, L. M. (1993). Nerve growth factor and nociception. *Trends in Neurosciences*, 353-359.

Li, A. G., Wang, D., Feng, X. H., & Wang, X. J. (2004). Latent TGFbeta1 overexpression in keratinocytes results in a severe psoriasis-like skin disorder. *The EMBO Journal*, 1770-1781.

Li, A. K., Koroly, M. J., Schattenkerk, M. E., Malt, R. A., & Young, M. (1980). Nerve growth factor: acceleration of the rate of wound healing in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 4379-43781.

Liang, Z., Engrav, L. H., Muangman, P., Muffley, L. A., Zhu, K. Q., Carrougher, G. J., . . . Gibran, N. S. (2004). Nerve quantification in female red Duroc pig (FRDP) scar compared to human hypertrophic scar. *Burns*, 57-64.

Liechty, K. W., Crombleholme, T. M., Cass, D. L., Martin, B., & Adzick, N. S. (1998). Diminished interleukin-8 (IL-8) production in the fetal wound healing response. *The Journal of Surgical Research*, 80-84.

Linge, C., Richardson, J., Vigor, C., Clayton, C., Hardas, B., & Rolfe, K. (2005). Hypertrophic scar cells fail to undergo a form of apoptosis specific to contractile collagen-the role of tissue transglutaminase. *Journal of Investigative Dermatoloy*, 72-82.

Loh, L., & Nathan, P. W. (1978). Painful peripheral states and sympathetic blocks. *Journal of Neurology Neurosurgery and Psychiatry*, 664-671.

Loots, M. A., Lamme, E. N., Zeegelaar, J., Mekkes, J. R., Bos, J. D., & Middelkoop, E. (1998). Differences in cellular infiltrate and extracellular matrix of chronic diabetic and venous ulcers versus acute wounds. *The Journal of Investigative Dermatology*, 850-857.

Maden, J. W., & Peacock, E. E. (1971). Study on the biology of collagen during wound healing. 3. Dynamic metabolism of scar collagen and remodeling of dermal wounds. *Annals of Surger*, 511-520.

Maggi, C. A. (1991). Thepharmacology ofthe efferent function of sensory nerves. *Journal of Autonomic Pharmacology*, 173-208.

Malik, I. A., Zahid, M., Haq, S., Syed, S., Moid, I., & Waheed, I. (1998). Effect of subcutaneous injection of granulocyte-macrophage colony stimulating factor (GM-CSF) on healing of chronic refractory wounds. *European Journal of Surgery*, 737-744.

Martin, P. (1997). Wound healing - aiming for perfect skin regeneration. *Science*, 75-81.

Martinez-Martinez, E., Galvan-Hernandez, C. I., Toscano-Marquez, B., & Gutierrez-Ospina, G. (2012). Modulatory Role of Sensory Innervation on Hair Follicle Stem Cell Progeny during Wound Healing of the Rat Skin. *PLoS One*, e36421.

Matsuda, H., Koyama, H., Sato, H., Sawada, J., Itakura, A., Tanaka, A., . . . Matsuda, K. (1998). Role of nerve growth factor in cutaneous wound healing: accelerating effects in normal and healing-impaired diabetic mice. *The Journal of Experimental Medicine*, 297-306.

Maurer, M., Theoharides, T., Granstein, R. D., Bischoff, S. C., Bienenstock, J., Henz, B., . . . Galli, S. (2003). What is the physiological function of mast cells? . *Experimental Dermatology*, 886–910.

McCawley, L. J., O'Brien, P., & Hudson, L. G. (1998). Epidermal growth factor (EGF)-and scatter factor/hepatocyte growth factor (SF/HGF)-mediated keratinocyte

migration is coincident with induction of matrix metalloproteinase (MMP)-9. *Journal of Cellular Physiology*, 255-265.

Micera, A., Puxeddu, I., Aloe, L., & Levi-Schaffer, F. (2003). New insightson the involvement of nerve growth factor in allergic inflammation and fibrosis. *Cytokine and Growth Factor Reviews*, 369-374.

Miller, S. j., Bruke, E. M., Rader, M. D., Coulombe, P. A., & Lavker, R. M. (1998). Re-epithelialization of porcine skin by sweat apparatus. *The Journal of Investigative Dermatology*, 13-19.

Misery, L. (2011). Atopic dermatitis and the nervous system. *Clinical Reviews in Allergy and Immunology*, 259-266.

Moore, E. L., & Salvatore, C. A. (2012). Targeting a family B GPCR/RAMP receptor complex: CGRP receptor antagonists and migraine. *British Journal of Pharmacology*, 66-78.

Moran, M. M., McAlexander, M. A., Bíró, T., & Szallasi, A. (2011). Transient Receptor potantial channels as therapeutic targets. *Nature Reviews Drug Discovery*, 601-620.

Moss, L. S. (2010). Treatment of the burn patient in primary care. *Advances in Skin & Wound Care*, 517-24.

Muangman, P., Muffley, L. A., Anthony, J. P., Spenny, M. L., Underwood, R. A., Olerud, J. E., & Gibran, N. S. (2004). Nerve growth factor accelerates wound healing in diabetic mice. *Wound Repair and Regeneration* , 44-52.

Muller, S. A. (1971). Hair Neogenesis . *Journal of Investigative Dermatology*, 1-9.

Mustoe, T. (2004). Understanding chronic wounds: a unifying hypothesis on their pathogenesis and implications for therapm. *American Journal of Surgery*, 65S-70S.

Nauta, A., Gurtner, G., & Longaker, M. (2011). Wound healing and regenerative strategies. *Oral Diseases*, 541-549.

Nickoloff, B. J., & Naidu, Y. (1994). Perturbation of epidermal barrier function correlates with initiation of cytokine cascade in human skin. *Journal of the American Academy of Dermatology*, 535-546.

Niemann, H., Blasi, J., & Jahn, R. (1994). Clostridial neurotoxins: new tools for dissecting exocytosis. *Trends in Cell Biology*, 179-185.

Niessen, F. B., Andriessen, M. P., Schalkwijk, J., Visser, L., & Timens, W. (2001). Keratinocyte-derived growth factors play a role in the formation of hypertrophic scars. *the Journal of Pathology*, 207-216.

Nilius, B., & Owslanik, G. (2010). Transient receptor potential channelopathies. *Pflugers Archiv - European Journal of Physiology*, 437-450.

Nissen, N. N., Polverini, P. J., Koch, A. E., Volin, M. V., Gamelli, R. L., & DiPietro, L. A. (1998). Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. *The American Journal of Pathology*, 1445-1452.

Ossipov, M. H., Bian, D., Malan Jr, T. P., Lai, J., & Porreca, F. (1999). Lack of involvement of capsaicin-sensitive primary afferents in nerve-ligation injury induced tactile allodynia in rats. *Pain*, 127-133.

Oyler, G. A., Higgins, G. A., Hart, R. A., Battenberg, E., Billingsley, M., Bloom, F. E., & Wilson, M. C. (1989). The identification of a novel synaptosomalassociated protein, SNAP-25, differentially expressed by neuronal subpopulations. *The Journal of Cell Biology*, 3039-3052.

Paus, R., Theoharides, C., & Arck, P. (2006). Neuroimmunoendocrine circuitry of the'brain-skin connection'. *Trends in immunology*, 32-39.

Pavlovic, S., Liezmann, C., Blois, S. M., Joachim, R., Kruse, J., Romani, N., . . . Peters, E. M. (2011). Substance P is a key mediator of stress-induced protection from allergic sensitization via modified antigen presentation. *Journal of Immunology*, 848-855.

Peeper, M. S. (1992). Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. *bicohme*, 24-39.

Pepper, M. S., Ferrara, N., Orchi, L., & Montesano, R. (1992). Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. *Biochemical and Biophysical Research Communications*, 824-831.

Peters, E. M., Ericson, M. E., Hosoi, J., Seiffert, K., Hordinsky, M. K., Ansel, J. C., . . . Scholzen, T. E. (2006). Neuropeptide control mechanisms in cutaneous biology: physiological and clinical significance. *The Journal of Investigative Dermatology*, 1937-1947.

Peters, E. M., Kuhlmei, A., Tobin, D. J., Muller-Rover, S., Klapp, B. F., & Arck, P. C. (2005). Stress exposure modulates peptidergic innervation and degranulates mast cells in murine skin. *Brain, Behavior, & Immunity*, 252-262.

Pincelli, C. (2000). Nerve growth factor and keratinocytes: a role in psoriasis. *European Journal of Dermatology*, 85–90.

Pincelli, C., & Marconi, A. (2000). Autocrine nerve growth factor in human keratinocytes. *Journal of Dermatological Science*, 71-79.

Profyris, C., Tziotzios, C., & Do Vale, I. (2012). Cutaneous scarring: Pathophysiology, molecular mechanisms, and scar reduction therapeutics Part I. The molecular basis of scar formation. *Journal of the American Academy of Dermatology.*, 1-10.

Radtke, C., Rennekampff, H. O., Reimers, K., Vogt, P. M., & Kocsis, J. D. (2013). Paracrine loop of keratinocyte proliferation and directed neuritic outgrowth in a neuroepithelial coculture. *Annals of Plastic Surgery*, 162-167.

Raychaudhuri, S. K., Raychaudhuri, S. P., Weltman, H., & Farber, E. M. (2001). Effect of nerve growth factor on endothelial cell biology: proliferation and adherence molecule expression on human dermal microvascular endothelial cells. *Archives of Dermatological Research*, 291-295.

Redd, M. J., Cooper, L., Wood, W., Stramer, B., & Martin, P. (2004). Wound healing and inflammation: embryos reveal the way to perfect repair. *Philosophical Transactions of the Royal Society of London*, 777-784.

Reinke, J. M., & Sorg, H. (2012). Wound Repair and Regeneration. *European Surgical Research*, 35-43.

Rheinwald, J. G., & Green, H. (1977). Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature*, 421-424.

Richards, A. M., Floyd, D. C., Terenghi, G., & McGrouther, D. A. (1999). Cellular changes in denervated tissue during wound healing in a rat model . *The British Journal of Dermatology*, 1093-1099.

Robson, M. C. (1991). Robson MC. Growth factors as wound healing agents. *Current Opinion in Biotechnology*, 863-867.

Robson, M. C., Steed, D. L., & Franz, M. G. (2001). Wound healing: biological features and approaches to maximize healing trajectories. *Current Problems in Surgery*, 72-140.

Roh, C., & lyle, S. (2006). Cutaneous stem cells and wound healing. *Pediatric Research*, 100R-103R.

Roosterman, D., Goerge, T., Schneider, S. W., Bunnett, N. W., & Steinhoff, M. (2006). Neuronal control of skin function: the skin as a neuroimmunoendocrine organ. *Physiological Reviews*, 1309-1379.

Rumalla, V. K., & Borah , G. L. (2001). Cytokines, growth factors and plastic surgery. *Plastic and Reconstructive Surgery*, 719-733.

Saab, C. Y., Shamaa, F., El Sabban, M. E., Safie-Garabedian, B., Jabbur S, J., & Saade, N. E. (2009). Transient increase in cytokines and nerve growth factor in the rat dorsal root ganglia after nerve lesion and peripheral inflammation. *Journal of Neuroimmunology*, 94-103.

Saade, N. E., Massaad, C. A., Ochoa-Chaar, C. I., Jabbur, S. J., Safieh-Garabedian, B., & Atweh, S. F. (2002). Upregulation of proinflammatory cytokines and nerve growth factor by intraplantar injection of capsaicin in rats. *The Journal of Physiology*, 241-253.

Safieh-Garabedian, B., Poole, S., Allchorne , A., Winter, J., & Woolf, C. J. (1995). Contribution of interleukin-1β to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia. *British Journal of Pharmacology*, 1265-1275.

Sanz Gracia, S., Santos Hereder, X., Izquierdo Hernandez, A., Pascual Pena, E., Bilbao de Aledo, G., & Hamann, C. (2000). Experimental model for local application of growth factors in skin re-epithelialisation. *Scandinavian Journal of Plastic and Reconstructive Surgery and Hand Surgery*, 199-206.

Saraceno, R., Kleyn, C. E., Terenghi, G., & Griffiths, C. E. (2006). The role of neuropeptides in psoriasis. *The British Journal of Dermatology*, 876-882.

Sarrazy, V., Billet, F., Micallef, L., Coulomb, B., & Desmoulière, A. (2011). Mechanisms of pathological scarring: role of myofibroblasts and current developments. *Wound Repair and Regeneration*, s10-s15.

Sauder, D. N., Kilian, P. L., McLande, J. A., Quick, T. W., Jakubovic, H., Davis, S. C., . . . Mertz, P. M. (1990). Interleukin-1 enhances epidermal wound healing. *Lymphokine Research*, 465-473.

Schiavo, G., Matteoli, M., & Montecucco, C. (2000). Neurotoxins affecting neuroexocytosis. *Physiological Reviews*, 717-766.

Schiavo, G., Santucci, A., Dasgupta, B. R., Mehta, P. P., Jontess, J., Benfenati, F., . . . Montecucco, C. (1993). Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds. *FEBS Letters*, 99-103.

Schmelz, M., & Petersen, L. J. (2001). Neurogenic inflammation in human and rodent skin. *News in Physiological Sciences*, 33-37.

Scott, A. B. (2004). Development of botulinum toxin therapy. *Dermatologic Clinics*, 131-133.

Shi, C. M., Qu, J. F., & Cheng, T. M. (2003). Effects of the nerve growth factor on the survival and wound healing in mice with combined radiation and wound injury. *Journal of Radiation Research*, 223-228.

Shi, T., & Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell*, 685-700.

Siebenhaar, F., Magerl, M., Peters, E. M., Hendrix, S., Metz, M., & Maurer, M. (2008). Mast cell-driven skin inflammation is impaired in the absence of sensory nerves. *Journal of Allergy & Clinical Immunology*, 955-961.

Singer, A. J., & Clarl, R. A. (1999). Cutaneous wound healing. *The New England Journal of Medicine*, 738-746.

Singer, A. J., & Dagum, A. B. (2008). Current management of acute cutaneous wounds. *The New England Journal of Medicine*, 1037-1046.

Singer, A. J., McClain, S. A., Hacht, G., Batchkina, G., & Simon, M. (2006). Semapimod reduces the depth of injury resulting in enhanced reepithelialization of partial-thickness burns in swine. *Journal of Burn Care and Research*, 40-49.

Singh, L. K., Pang, X., Alexacos, N., Letourneau, R., & Theoharides, T. C. (1999). Acute immobilization stress triggers skin mast cell degranulation via corticotropin releasing hormone, neurotensin and substance P: a link to neurogenic skin disorders. *Brain Behav. Immun*, 225–239.

Smith, P. G., & Liu, M. (2002). Impaired cutaneous wound healing after sensory denervation in developing rats: effects on cell proliferation and apoptosis. *Cell and Tissue Research*, 281-291.

Sofroniew, M. V., Howe, C. L., & Mobley, W. C. (2001). Nerve growth factor signaling, neuroprotection, and neural repair. *Annual Reviews of Neuroscience*, 1217-1281.

Souza, B. R., Cardoso, J. F., Amadeu, T. P., Desmouliere, A., & Costa, A. M. (2005). Costa Sympathetic denervation accelerates wound contraction but delays reepithelialization in rats . *Wound Repair and Regeneration*, 498-505.

Steiling, H., & Werner, S. (2003). Fibroblast growth factors: key players in epithelial morphogenesis, repair and cytoprotection. *Current Opinion in Biotechnology*, 533-537.

Steinhoff, M., Stander, S., Seeliger, S., Ansel, J. C., Schmelz, M., & Luger, T. (2003). Modern aspects of cutaneous neurogenic inflammation. *Archives of Dermatology*, 1479-1488.

Steinle, J. J., Pierce, J. D., Clancy, R. L., & Smith, G. P. (2002). Increased Ocular Blood Vessel Numbers and Sizes Following Chronic Sympathectomy in Rat. *Experimental Eye Research*, 761-768.

Sudhof, T. C. (2004). The synaptic vesicle cycle. *Annual Reviews in Neuroscience*, 509-547.

Sutton, R. B., Fasshauer, D., Jahn, R., & Brunger, A. T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature*, 347-353.

Svensjo, T., Pomahac, B., Yao, F., Slama, J., & Eriksson, E. (2000). Accelerated healing of full-thickness skin wounds in a wet environment. *Plastic and Revonstructive Surgery*, 602-612.

Taylor, G. I., & Palmer, J. H. (1987). The vascular territories (angiosomes) of the body: Experimental study and clinical applications. *British Journal of Plastic Surgery*, 113-141.

Tender, G. C., Li, Y., & Cui, J. (2013). The Role of Nerve Growth Factor in Neuropathic Pain Inhibition Produced by Resiniferatoxin Treatment in the Dorsal Root Ganglia. *Neurosurgery*, 158-166.

Theoharides, T. C., Singh, T. C., Boucher, W., Pang, X., Letourneau, R., Webster, E., & Chorousos, G. (1998). Corticotropin-releasing hormone induces skin mast cell degranulation and increased vascular permeability, a possible explanation for its proinflammatory effects. *Endocrinology*, 403-413.

Tian, M., Qing, C., Niu, Y., Dong, J., Cao, X., Song, F., . . . Lu, S. (2016). The Relationship Between Inflammation and Impaired Wound Healing in a Diabetic Rat Burn Model. *Journal of Burn Care and Research*, e115-124.

Tomlinson, D. R., Mayor, D., Mitchel, M., & Banks, P. (1971). Ultrastructural changes in constricted sympathetic nerves maintained in vitro. *Journal of Anatomy*, 612.

Trengove, N. J., Bieliefedt-Ohmann, H., & Stacey, M. C. (2000). Mitogenic activity and cytokine levels in nonhealing and healing chronic leg ulcers. *Wound Repair and Regeneration*, 13-25.

Tziotzios, C., Profyris, C., & Sterling, J. (2012). Cutaneous scarring: Pathophysiology, molecular mechanisms, and scar reduction therapeutics Part II. Strategies to reduce scar formation after dermatologic procedures. *Journal of the American Academy of Dermatology.*, 13-24.

Uutela, M., Wirzenius, M., Paavonen, K., Rajantie, I., He, Y., Karpanen, T., . . . Alitalo, K. (2004). PDGF-D induces macrophage recruitment, increased interstitial pressure, and blood vessel maturation during angiogenesis. *Blood*, 3198-3204.

Van De Water, L., Varney, S., & Tomasek, J. J. (2013). Mechanoregulation of the Myofibroblast in Wound Contraction, Scarring, and Fibrosis: Opportunities for New Therapeutic Intervention. *Advances in Wound Care*, 122-141.

Verhaegen, P. D., van Zuijlen, P. P., Pennings, N. M., van Marle, J., Niessen, F. B., vad der Horst, C. M., & Middelkoop, E. (2009). Differences in collagen architecture between keloid, hypertrophic scar, normotrophic scar, and normal skin: an objective histopathological analysis. *Wound Regpair and Regeneration*, 649-656.

Vogt, P. M., Lehnhardt, M., Wagner, D., Jansen, V., Krieg, M., & Steinau, H. (1998). Determination of endogenous growth factors in human wound fluid: temporal presence and profiles of secretion. *Plastic and Reconstructive Surgery*, 117-123.

Wang, X. P., Schunck, M., Kallen, K. J., Neumann, C., Trautwein, C., Rose-John, S., & Proksch, E. (2004). The interleukin-6 cytokine system regulates epidermal permeability barrier homeostasis. *The Journal of Investigative Dermatology*, 124-131.

Wehrens, K. E., Arnoldussen, C. W., Booi, D. I., & van der Hulst, R. R. (2016). Clinical evaluation of wound healing in split-skin Graft donor sites using microscopic quantificatio of reepethelialization. *Advances in Skin and Wound Care*, 254-260.

Werner, S., & Grose, R. (2003). Regulation of wound healing by growth factors and cytokines. *Physiological Reviews*, 835-870.

Whitby, D. J., & Ferguson, M. W. (1991). Immunohistochemical localization of growth factors in fetal wound healing. *Developmental Biology*, 207-215.

Wiechula, R. (2003). The use of moist wound-healing dressings in the management of split-thickness skin graft donor sites: a systematic review. *International Journal of Nursing Practice*, S9-S17.

Winter, G. D. (1962). Formation of the scab and the rate of epithelization of superficial wounds in the skin of the young domestic pig. *Nature*, 293-294.

Woo, Y. C., Park, S. S., Subeita, A. R., & Brennan, T. J. (2004). Changes in tissue pH and temperature after incision indicate acidosis may contribute to postoperative pain. *Anesthesiology*, 468-475.

Wood, L. C., Elias, P. M., Calhoun, C., Tsai, J. C., Grunfeld, C., & Feingold, K. R. (1996). Barrier disruption stimulates interleukin-1 alpha expression and release from a pre-formed pool in murine epidermis. *The Journal of Investigative Dermatology*, 397-403.

Wood, L. C., Jackson, S. M., Elias, P. M., Grunfeld, C., & Feingold, K. R. (1992). Cutaneous barrier perturbation stimulates cytokine production in the epidermis of mice. *The Journal of Clinical Investigation*, 482-487.

Xi-Qiao, W., Ying-Kai, L., Chun, Q., & Shu-Liang, L. (2009). Hyperactivity of fibroblasts and functional regression of endothelial cells contribute to microvessel occlusion in hypertrophic scarring. *Microvascular Research*, 204-211.

Yagmur, C., Akaishi, S., Oqawa, R., & Guneren, E. (2010). Mechanicalreceptor-related mechanisms in scar management: a review and hypothesis. *Plastic and Reconstructive Surgery*, 426–434.

Yarden, Y. (2001). The EGFR family and its ligands in human cancer. Signalling mechanisms and therapeutic opportunities. *European Journal of Cancer*, S3-S8.

Younan, G., Ogawa, R., Ramirez, M., Helm, D., Dastouri, P., & Orgill, D. (2010). Analysis of nerve and neuropeptide patterns in vacuum-assisted closure-treated diabetic murine wounds. *Plastic and Reconstructive Surgery*, 87-96.

Zhu, K. Q., Engrav, L. H., Gibran, N. S., Cole, J. K., Matsumura, H., Piepkorn, M., . . . Yang, T. M. (2003). The female, red Duroc pig as an animal model of hypertrophic scarring and the potential role of the cones of skin. *Burns*, 649–664.

Ziche, M., Morbidelli, L., Masini, E., Amerini, S., Granger, H. J., Maggi, C. A., . . . Ledda, F. (1994). . Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. *The Journal of Clinical Investigation*, 2036-2044.