TOPICAL NALTREXONE ACCELERATES FULL THICKNESS WOUND HEALING IN NORMAL AND TYPE 1 DIABETIC RATS

A Thesis in
Anatomy
by
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Abstract

Diabetes Mellitus affects almost 20 million people in the United States and 6% of the total world’s population. It results in a reduced life expectancy and an overall poor quality of life, requiring patients to be dependent on regular medication, supplements and altered diets.

Full thickness wound healing presents a number of complications to diabetic patients. Often, due to neuropathy, a wound can go unnoticed increasing its severity and risk of infection. Moreover, even when wounds are found and treated, a slow rate of wound closure is observed further increasing the risk of higher severity and infection level. Treatments are typical for epithelial defects such as bandaging and antibiotic ointments but they leave out a key element to the wounding process in diabetes, increased levels of plasma [Met$^5$]-enkephalin, or OGF.

OGF interacts specifically with its receptor OGFr, which is a nuclear receptor, and together they act directly on DNA to tonically inhibit DNA synthesis and thus cell proliferation. To counteract this effect, naltrexone (NTX) serves as a long acting opioid antagonist and through high affinity binding with the OGFr it blocks the effect of OGF, increasing DNA synthesis and cell proliferation. NTX has been shown to be an effective treatment in re-epithelialization of the rat and rabbit cornea in vivo and human cornea in tissue culture. In the current study 10$^{-5}$ M NTX is shown to increase both DNA synthesis in the epithelium of normal and diabetic rats, and to increase wound closure rates in normal and diabetic rats.
Initially a topical dose and a proper vehicle were selected. In order to test the doses and vehicles, Sprague-Dawley rats were rendered diabetic by Streptozotocin injections and maintained hyperglycemic for 5 weeks. Topical applications of $10^{-4}$M, $10^{-5}$M, and $10^{-6}$M NTX in Sorenson’s phosphate buffer, DMSO, Neutrogena moisturizing cream, and KY jelly were administered to unwounded skin of both normal and diabetic (glucose > 350mg/dL) rats. Animals received BrdU injections and DNA synthesis was calculated in the basal layers of epithelium. In comparison to DNA labeling indexes in untreated rats, as well as though receiving only vehicles, dosages and vehicles were selected that significantly increased labeling indexes. Although all of the vehicles significantly increased cell proliferation at the basal layer of epithelium with at least one of the doses mentioned, moisturizing cream and KY jelly vehicles were shown to be the most effective at a concentration of $10^{-5}$M NTX in both normal and diabetic rats.

In the second study, 6 mm full thickness wounds were surgically created on the dorsum of normal and diabetic rats. The wound were treated 3 times daily with topical applications of moisturizing cream, moisturizing cream + $10^{-5}$M NTX, KY jelly or KY jelly + $10^{-5}$M NTX beginning immediately after surgery and lasting until complete wound closure (no more than 13 days in any treatment group). Wounds were photographed and areal analyses conducted. Data revealed that in comparison to animals receiving only vehicle, wound closure of NTX treated animals was increased on day 3 in normal rats and significantly increased on days 3, 5, 7 and 9 in diabetic rats. On day 7 in diabetic rats wound closure was accelerated by more than 2 fold in topical moisturizing cream + $10^{-5}$M NTX when
compared to topical moisturizing cream alone. By day 9, wound closure in diabetic rats was accelerated by more than 2.8 fold in topical KY jelly + $10^{-5}\text{M}$ NTX when compared to topical KY jelly control.

Additional studies demonstrated that the type of moisturizing cream had no significant effect on wound closure rate. No differences in wound closure rates were noted between Oil of Olay moisturizing cream and Neutrogena moisturizing cream on days 3, 5, 7 and 9 in normal animals and days 5, 7 and 9 in diabetic animals.

The number of applications of moisturizing cream plus NTX was studied. There were no significant differences in rates of wound closure between diabetic animals receiving 1x daily or 2x daily application and 3x daily application at day 3, 5, 7 or 9. In normal animals there were no significant differences in rates of wound closure between diabetic animals receiving 1, 2, or 3 applications daily of NTX + moisturizing cream at days 3, 5 or 9.

Along with being an effective treatment for increasing wound closure rates, NTX was also shown to be non-toxic. Gross observation of unwounded skin showed no redness or irritation. Animals display unusual behavior after receiving topical treatment. Topical applications of cream and NTX did not result in qualitative increases in redness, infection, discharge, or swelling of the wounded or surrounding areas. Histopathological studies supported that the processes of wound healing were similar between untreated rats and those receiving topical applications. Furthermore, apoptosis was not observed in the epidermis or dermis of skin sections from any treatment group.
In conclusion, these studies demonstrate a new and novel treatment for wound healing that involves the disruption of biological processes invoked by the OGF-OGFr pathway.
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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>β</td>
<td>beta</td>
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<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
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<tr>
<td>°C</td>
<td>degrees centigrade</td>
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<td>cm</td>
<td>centimeter</td>
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<td>cm²</td>
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<td>DAB</td>
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<td>et al.</td>
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<td>FDA</td>
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<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<td>heat shock protein</td>
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<td>OGF</td>
<td>opioid growth factor</td>
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<td>OGFr</td>
<td>opioid growth factor receptor</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>POD</td>
<td>peroxidase</td>
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<td>S.E.M.</td>
<td>standard error of the mean</td>
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<td>STZ</td>
<td>streptozotocin</td>
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<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase mediated dUTP nick end labeling</td>
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Chapter 1

Introduction

1.1. Diabetes mellitus

Diabetes mellitus is one of the most common endocrine diseases, affecting almost 6% of the world’s population (Adeghate et al., 2006). Diabetes is associated with reduced life expectancy and increased morbidity and mortality as well as a lower quality of life (Cohen et al., 2007). Often diabetic patients have to regularly take medication, insulin supplementation and/or control their diet. There are two different types of diabetes, type 1 (juvenile) and type 2. Clinically type 1 is least common and usually presents during early childhood. Some common symptoms of diabetes type 1 are extreme hunger and thirst, fatigue, irritability, and impaired vision. Other symptoms in type 1 include frequent infections, abrasions that heal very slowly, numbness of the hands or feet, tingling of the hands or feet and recurrent bladder infections according to studies done by the FDA.

1.2. Type 1 Diabetes

Type 1 diabetes is an immune mediated destruction of islet β cells of the pancreas (Cohen et al., 2007). This leads to an inability to produce endogenous insulin. Insulin is a hormone responsible for signaling liver and muscle cells to take up glucose from the blood and store it in the form of glycogen (Adeghate et al., 2006). This causes the patient to experience abnormally high levels of blood glucose, a condition defined as hyperglycemia. Type 1 diabetes typically has
several problematic conditions associated with it including polyuria, polydypsia, and ketoacidosis (Reaven et al., 1964). Polyuria is an increase in urine volume or an increase in the frequency of urination. Polydypsia is a dramatic weight loss. Ketoacidosis is a type of metabolic acidosis in which there are elevated levels of ketone bodies. There is no known way to prevent this chronic disorder, treatment requires regular insulin supplementation for survival. It has commonly been called childhood or juvenile diabetes but this terminology is giving way to type 1 or insulin-dependent diabetes mellitus (Cohen et al., 2007). Though later in life it is still an autoimmune disorder, it can be brought on because of a similarity in a foreign antigen to the antigens expressed on the surface of the pancreatic beta cell. Type 1 diabetes is not a genetically inherited disease but risk factors can be inherited giving an individual a higher predisposition for the disorder (Cohen et al., 2007). Diabetes mellitus (DM) has a long list of complications associated with the disorder outside of those previously mentioned. Some of these complications include microvascular disease due to sustained periods of high glucose levels, kidney disorders such as glomerulosclerosis, which is a hardening of the glomeruli thus interfering with proper filtration. Others include neural pathologies such as an inability to detect injury especially in the distal lower extremity, and ocular disorders such as retinopathy to name a few (Reaven et al., 1964). Of particular interest to this study is the prolonged healing period in a wide range of different types of skin wounds that are observed in the diabetic patient. Having an appropriate treatment for these types of wounds is essential to prevent infection of injuries or
to enhance proper wound closure following surgery. Also, more effective wound healing for the diabetic patient could prevent symptoms such as reduced vascularity, poor blood flow and insufficient circulation and could even lead to the prevention amputations in more severe cases.

1.3. Wound Models

There are two main types of wound models called (1) incisional wounds and (2) excisional wounds. Each of these two contain several subcategories such as tape stripping, blisters and split thickness wounds, and full thickness wounds (Davidson, 1998). Incisional wounds are described as use of a blade to cut the skin resulting in a disruption in the organization of tissue such that there is minimal collateral damage (Davidson, 1998). A scalpel is used to longitudinally slice the skin with the intention of disrupting epidermal, dermal and subcutaneous layers in an isolated area. This technique can successfully initiate the inflammatory response and subsequent reepithelialization and vasculogenesis of the epidermis and dermis in the periphery of the wound. Incisional wounds cause a rapid aggregation of erythrocytes and plasma cells to the site of injury, which form a fibrin blood clot with the intention of sealing the wound margin (Davidson, 1998). However, incisional wound models do not imitate the most disruptive and problematic types of wounds because they do not require 360° wound contraction nor do they require replacement of large sections of tissue.

An excisional wound is one described as a removal of a large section of tissue from the animal (Davidson, 1998). Punches or scalpels can be used to
section a set area of skin, resulting in the important addition of wound contracture as another element of the wound healing process that is unique to an excisional wound. Excisional wounds that require full scale replacement of the dermis and epidermis are called full-thickness wounds. Full-thickness wounds are representative of the most severe disruptions in the skin and incorporate the most diverse set of tissues for repair. In full-thickness wounds much more is demanded from the cells making up the tissue in the wound periphery and in the newly forming scar tissue that is soon to restore the lesioned area. Fibroblasts need to contract the wound on a broader scale, leukocytes must respond to a larger area of potential infection, neovascularization must occur as well as proliferation of epithelial cells. Sections of tissue can then be removed and analyzed in a variety of ways to determine cell content, RNA, proteins, and cytokines among many other things that are not as readily harvested in other wounding techniques (Witte et al., 1997). Other excisional wound techniques are more specialized to specific types of wounds. Tape stripping is one type of excisional wound in which the epidermis is partially removed using an adhesive material. This, in turn, reveals the basal layer of epidermis and is significant enough to induce the activation of epidermal repair processes while implementing only a mild stress on the animal (Davidson, 1998). There are many benefits to using the tape stripping method in the appropriate experimental situation. The most prominent benefit is it is a relatively mild injury causing no blood loss and leaving the basement membrane intact, its only compromise to the animal is a possible disruption of the water barrier (Davidson, 1998). Also,
the procedure is simple and can be done without anesthetizing the animal, and the same animal can experience several tape stripping experiments. In tape stripping procedures all of the hair and 75-80% of the cornified layer of epidermis is removed while leaving intact the underlying epidermal layers (Wilson et al., 1998).

Yet another type of excisional wound is blistering which is the separation of the epidermis from the dermis. It has been determined, in the case of skin blistering, that the epidermis must be five to six cell layers thick to avoid necrosis (Mertz, 2001). The significance of the thickness of the epidermal layer has to do with adequate transport of vital cellular metabolic elements. A split thickness or partial thickness wound is a lesion in which the skin is shaved away removing the epidermis and parts of the dermal layer (Davidson, 1998). It is possible by this method to leave behind such structures as sebaceous glands and hair follicles, and it is a good experimental representation of the common scraped knee or elbow. This wide range of possible skin wounding techniques represents many if not all clinical scenarios involving damaged skin with the exception of burns.

1.4. Wound Healing

The skin is the body's outermost barrier against the environment and plays a pivotal role in homeostasis from water retention, to thermoregulation. The skin contains nerve endings that allow for sensation and control of secretory glands and hair follicles among others. A deficient wound healing process leads to many medical and surgical complications such as those in diabetes, cancer, and the immunocompromised patient (Garcia-Esteo et al., 2007). Wound healing of
the skin is commonly described as having 3 stages. These three stages are (1) inflammation, (2) tissue formation, also known as proliferation (Witte et al., 1997), and (3) tissue remodeling or maturation (Davidson, 1998; Witte et al., 1997). These three stages overlap in time and involve soluble mediators, blood cells, extracellular matrix and parenchymal cells (Singer et al., 1999).

1.4.1. Inflammation

The cascade of events that initiates proper wound closure begins with inflammation accompanied by an increase in permeability of vascular endothelium, chemotaxis of cells from the circulation, and importantly, release of cytokines such as platelet derived growth factor that attract and activate macrophages and fibroblasts (Singer et al., 1999). The release of cytokines and growth factors also contributes to the formation of the matrix-like fibrin clot that gives cells such as neutrophils, fibroblasts, and monocytes a route to the wound (Witte et al., 1997). It is important to note that injuries may occur without hemorrhage in which case platelets are not mandatory for the wound healing process because activated complement pathways and parenchymal cells are able to assume the responsibility of recruiting leukocytes to the site of the lesion (Singer et al., 1999). After the release of cytokines, neutrophils are the first migrating cells to arrive at the wound and are responsible for destroying foreign particles such as bacteria later to be phagocytosed by macrophages (Singer et al., 1999). Macrophages arise from the infiltration of monocytes into the tissue from the blood stream. It is this distinction that defines the state of a monocyte or macrophage. Macrophages are essential not only for the phagocytosis of foreign
material but also for their release of monocyte and macrophage derived growth factor that stimulates the transition from inflammation to repair (Leibovich et al., 1975). Among the necessary and helpful duties of cytokines there can also be adverse effects of these signaling molecules. Cytokines can cause cell and tissue damage on a larger scale and in some instances such as the NFκB nuclear receptor pathway need to have a mechanism for feedback inhibition.

1.4.2. Tissue Formation

In the tissue formation stage fibroblasts and endothelial cells are the primary cell types that proliferate (Witte et al., 1997). However, they come to occupy the wound via different pathways. Fibroblasts need to be activated initially to a cellular state that accommodates replication, this is achieved by recognition cytokines or other small molecule signals (Witte et al., 1997). Differentiation takes the normally quiescent fibroblast and causes it to change to an actively replicating myofibroblast. Myofibroblasts are responsible for depositing much of the collagen necessary for proper scar formation as well as providing some of the contractile properties necessary for excisional wound healing. Contraction of the wound occurs along an axis of contraction which may not be symmetrical but rather in alignment with previously existing and newly formed collagen fibers. Myofibroblasts are normally removed via the apoptotic mechanism after the wound healing process is complete. However, if myofibroblasts do persist after wound closure and scar formation is complete, complications such as fibrosis and over-contraction of the epithelium may occur. Endothelial cells proliferate from intact venules and enter the site of the wound to
form new capillaries in the process of angiogenesis (Witte et al., 1997). The process by which epidermal cells become activated has not yet been fully described but it has been suggested that the lack of neighboring cells to those adjacent to the wound, in itself, causes cellular changes and proper division (Singer et al., 1999).

1.4.3. Remodeling

The maturation and remodeling phase is marked mainly by the deposition of collagen in the wound, this is very important clinically because the matrix deposition is responsible for the rate, quality, and strength of the scar. Fibroblasts are the epidermal cells responsible for the production of collagen and thus play a pivotal role in not only the subsequent tensile strength of the wound but the rate at which the wound closes (Witte et al., 1997).

1.5. Wound Healing and Diabetes Mellitus

The most common types of lesions affected by loss of sensation in the diabetic patient are those in the distal lower extremity. Poor glucose control leads to a decrease in peripheral circulation attributed to arteriosclerosis, neuropathic changes leading to a loss in sensation and intrinsic defects in the wound healing process resulting in poor wound closure (Davidson, 1998). The normal healing process in healthy human patients has been observed to take place at a much higher rate and of higher quality than that of diabetic patients (Darby et al., 1997), in which case diabetic patients present with a wound healing process that may occur at an impaired rate or may even be completely disrupted (Qui et al., 2007). Though the exact wound healing processes that diabetes
mellitus hinders are not clearly understood, it is believed that all stages of the complex wound healing process are affected in some way (Qui et al., 2007). Due to slow and insufficient wound closure infection often occurs as yet another complication. It has been suggested that wounds will fail to heal if contamination is greater than $10^5$ organisms per gram of tissue involved (Robson, 1988). Diabetic wounds are often described as having poor tensile strength in comparison to normal skin (Darby et al., 1997). In animal models an increase in apoptosis is observed which was determined to be a result of disruption of controlled cell survival late in the healing process specifically during the period of scar formation (Darby et al., 1997). In humans additional complications including loss of sensory function in distal extremities and decreases in peripheral blood perfusion are observed (LoGerfo et al., 1984). It is suggested that additional complications initially caused by diabetes are aberrant growth factor expression and cross-linking of matrix proteins (Vlassara et al., 1994). In a more recent review article it is mentioned that direct effects on the wound healing process include a reduced abundance and response of cellular growth factors (Davidson, 1998). In other animal models specifically, genetically induced diabetic rats, added complications are due to not only the biochemical factors but physical inability of wound contraction to take place because of excess fat usually associated with these animals (Davidson, 1998). In these cases it is imperative that the wound heal by way of granulation tissue formation.
1.6. Opioids, Opioid Antagonists and the OGF-OGFr Axis

Opioid growth factor (OGF) and other endogenous opioid peptides were discovered in 1975. Since this time advancements in the study of opioid peptides have shown that there are three identified opioid peptide gene families including proopiомelanocortine (POMC), proenkephalin A, proenkephalin B synonymous with prodynorphin (Akil et al., 1984). It is also important to note that opioid peptides have a morphine-like action in the body and are primarily found in nervous tissue and the gastrointestinal tract. There are seven peptides associated with proenkephalin having the [Met]- or [Leu]-enkephalin active core. Focusing in on one in particular, the opioid growth factor, [Met\(^5\)]-enkephalin, is identified as a negative growth factor in both neural and non-neural tissues (Zagon et al., 1991). It has been identified as the most potent opioid relating to growth (Zagon et al., 1989). Also, it is important to note that there are several characteristics of the opioid growth factor receptor that differentiate it from previously understood opioid receptors (Zagon et al., 1991).

There were several important findings regarding opioids and their function during the early 1980s (Zagon et al., 1989). These include alterations in cell growth, the importance of duration of receptor blockage in comparison to dosage, increases in cell growth when the interaction of opioid and receptor is interrupted, increases in opioid and receptor leading to an increase in sensitivity and negative growth regulation, identification of opioid peptides in early stage cells of both neural and non-neural tissues, increased levels of [Met\(^5\)]enkephalin during development of the cerebellum (Zagon et al., 1989). OGF functions in
development, cellular renewal, cancer, wound healing and angiogenesis (Zagon et al., 2002). It was discovered that a blockade of the interaction between opioid and receptor leads to an acceleration in tumor growth (Zagon et al., 1983). This blockade is accomplished by NTX or NAL.

NTX and NAL are opioid antagonists that block the interaction of OGF and its receptor causing increases in cell proliferation (Zagon et al., 1983). NTX blocks the interaction by associating with the OGF receptor in turn activating the cyclin-dependent inhibitory kinases and nucleocytoplasmic transport (Klocek et al., 2007). NTX has been shown to have a high affinity for the OGF receptor resulting in disruption of the relationship between OGF and OGFr with the consequence of accelerating cell proliferation and growth (Zagon et al., 1984). It is also important to note that NTX is a long acting opioid antagonist, which differs from NAL, a short acting opioid antagonist. NTX has the ability to block OGF-OGFr interaction for 24 hours, NAL is only capable of a fraction of that time. NTX and NAL have been administered via IP injection resulting in increased cell proliferation on a large scale (Zagon et al., 1990; Zagon et al., 1998). Topical applications of NTX and NAL have been shown to increase cell proliferation in repair of corneal wounds in both diabetic and normal animals (Zagon et al., 2006).

1.7. OGF-OGFr Axis and Wound Healing: Corneal Wounds in Normal Subjects

It has been shown that reepithelialization of the human cornea is improved with the addition of NTX. Human corneas, in organ culture, showed complete healing 48 hours sooner than controls in one experiment (Zagon et al., 2000).
Tissue culture experiments have shown that damaged rabbit corneas healed significantly faster over a seven day period when compared with controls as well as showing that the addition of [Met\textsuperscript{5}]enkephalin (OGF) slowed the healing process over a seven day period (Zagon et al., 1995). Results from this tissue culture experiment indicated that OGF and OGFr are present in the epithelium of mammalian corneas functioning as modulators of cell proliferation and possibly organization and migration (Zagon et al., 1995). In experiments involving reepithelialization of the rat cornea, it was observed that systemic application of NTX via IP injection significantly increased the rate of healing in normal rats (Zagon et al., 1998). These rats experienced 4.7-fold increases in rate of healing when compared with controls and rats receiving topical treatments of NTX experienced 2.8-fold increases in the rate of healing versus controls (Zagon et al., 1998). These experiments proved that the opioid growth factor and its receptor serve an inhibitory function in the reepithelialization of the rat cornea and that blockade of this pathway allows the subject to heal at a much higher rate.

1.8. OGF-OGFr Axis and Wound Healing: Corneal Wounds in Type 1 Diabetes

The OGF-OGFr axis has been shown to be an important factor in the healing process of the cornea (Klocek et al., 2007; Zagon et al., 1998). In diabetic subjects the cornea experiences a slow rate of wound closure as well as a lack of tensile strength and poor overall scar quality. Several experiments had shown increases in the rate, strength, and quality of wound closure in normal individuals, but it has also been shown that type 1 diabetic rats experienced a
more rapid healing process with the addition of NTX, in fact there were 25%-83% smaller defects, faster closure time by 33% and 20%-42% higher healing rates (Klocek et al., 2007). It has been shown that NTX can be safely added to the rat cornea in concentrations of $10^{-4}$M or $10^{-5}$M without increases in pain sensitivity or any other deleterious effects on the subject (Zagon et al., 2006). Through this study NTX was proven to be a practical and safe method for treating corneal wounds across normal rats, induced diabetic rats and induced diabetic rats receiving insulin treatments (Zagon et al., 2006). These experiments have shown that NTX has the ability to increase cell proliferation in the healing of the rat cornea, a vital step in the healing process especially in the diabetic rat which faces a greater challenge throughout the wound repair process. With this knowledge, and the knowledge of the efficacy of systemic injections of NTX on corneal wound repair, there is reason to believe that NTX may indeed have positive effects on the wound healing process in the skin of both diabetic and non-diabetic subjects (Zagon et al., 2007). NTX applied topically to skin wounds is the next logical step in determining the extent to which NTX can be used in a localized manner.

1.9. OGF-OGFr Axis and Wound Healing: Cornea and Epidermis

Systemic insulin has long been a treatment for type 1 diabetes. Exogenous insulin is needed because of the autoimmune targeting and destruction of the beta cells of the pancreas. These beta cells located in the Islets of Langerhan’s are responsible for endogenous insulin production. The addition of insulin to an open wound significantly increases the rate at which the
wound heals. Epidermal closure occurs sooner than controls and restructuring of the underlying vasculature in the dermis occurs at a faster rate (Greenway et al., 1999). A method of applying topical insulin to wound includes the mixture of zinc which is used to crystallize insulin (Greenway et al., 1999). It has been suggested that zinc also has some wound healing properties independent of insulin but insulin increases wound healing quality in both the diabetic and non-diabetic patient (Greenway et al., 1999). It has been shown that topical administration of insulin normalizes corneal reepithelialization in diabetic rats.

Rabbit models have shown that NTX aids in the reepithelialization process of the cornea during healing (Zagon et al., 1998) as well as the fact that OGF has been shown to return the damaged surface epithelium of the rat cornea to homeostasis (Zagon et al., 1997). These results in corneal wounding should be applied to epithelial wound healing with similar results because increased DNA synthesis at the site of an epithelial wound will aid in both the quality and time of closure in normal and markedly, diabetic patients.

NTX provided to animals systemically via intraperitoneal injection has been shown to improve complete re-epithelialization of the rat cornea (Zagon et al., 1997). It was shown that the OGF and OGFr axis acts tonically to inhibit DNA synthesis in the epidermis (Zagon et al., 1996). With the fact that the OGF and OGFr axis is responsible for negative control of DNA synthesis, it can be hypothesized that treatment of skin wounds with NTX can increase DNA synthesis necessary during the healing process just as it has been extensively shown in the cornea.
Opioid growth factor when in contact with its receptor acts to inhibit cell proliferation (Zagon et al., 2000). In the wound healing process it is necessary for cells to replicate at a rate higher than that of normal, undamaged skin in order to restore the body's natural barrier. Cell proliferation is markedly most important during the second phase of the wound healing process - the proliferative phase also known as the tissue formation phase (Witte et al., 1997). The specific cells called upon to replicate quickly are fibroblasts and endothelial cells. Fibroblasts that differentiate into myofibroblasts are the most notable cells responsible for proper wound closure, it is their collagen production that determines the rate, quality and strength of the forming scar (Witte et al., 1997). It has been shown that NTX has the ability to increase cell division in the cornea (Zagon et al., 2007), developing nervous system (Zagon et al., 1991), and even neuroblastoma and fibrosarcoma culture cells (Zagon et al., 1990). Therefore, application of NTX whether it be systemically or topically to an isolated wound site should increase cell proliferation. Once these fibroblasts enhance their rate of replication theoretically the total amount of fibroblasts would increase causing an increase in collagen production and ultimately a faster healing wound. An experiment aimed at epithelial wound healing would serve to answer one of the next questions regarding cell proliferation and NTX - does it have the ability to increase cell proliferation in the skin, specifically at the site of injury. It becomes apparent that NTX, given its effects on cell division through blockade of the OGF-OGFr pathway, has the ability to perhaps speed cell proliferation of the collagen-
depositing fibroblasts, which in turn may increase the rate of closure, tensile strength, and overall quality of the scar.

1.10. **Topical Applications of Naltrexone**

NTX can be administered several different ways; systemically, topically in liquid form, topically in dimethyl sulfoxide (DMSO), or topically in KY jelly. Topical application of NTX is advantageous because it targets one specific area for cell proliferation such as a surgical incision, corneal wounds (Zagon et al., 2006), or in traumatic injuries. The disadvantage of applying NTX systemically to patients would be that the entire body is subject to the effects of NTX (i.e. cell proliferation). In the case of a patient with a known or unknown tumor or autoimmune disorder, cell proliferation on a large scale would not be ideal treatment because of the adverse effects it would have on these other pathologies. One can see how important it will be to find a proper vehicle of delivery for NTX if it is to be effective to all patients.

The first route of delivery to be explored is through dimethyl sulfoxide (DMSO), which is derived from wood pulp and is often harvested during paper processing. DMSO has the ability to penetrate the skin barrier due to its small molecular structure and ability to dissolve organic and inorganic molecules. It also has the ability to carry substances along with it.
These characteristics make it possible to add NTX to make it applicable in a topical form. The same concept applies to KY jelly, which shares some of the same properties of DMSO. KY jelly is a water soluble lubricant that shares some of the same advantageous properties that DMSO does in that it can penetrate the water barrier of the skin and ideally carry NTX with it. Other topical administration techniques include solutions with varying concentrations of NTX in saline solution, Sorenson's phosphate buffer and moisturizing cream. Sorenson's phosphate buffer is a common buffer that mimics certain components of the extracellular matrix. It can be problematic because it makes a suitable environment for microorganisms, which could be problematic in a wounding study. The moisturizing cream chosen for this study was Neutrogena Oil free sensitive skin moisturizing cream. The ingredients are water, glycerin, ethylhexyl palmitate, dimethicone, petrolatum, cyclomethicone, soy sterol, isopropyl isostearate, cetyl alcohol, PEG 10 soy sterol, glyceryl stearate, PEG 100 stearate, C12 15 alkyl benzoate, carbomer, tetrasodium EDTA, sodium hydroxide, diazolidinyl urea, ethylparaben, methylparaben, propylparaben. In a preliminary experiment the best topical administration was determined using BrdU staining for cell proliferation.

1.11. Skin

Skin is composed of two main layers, the epidermis and dermis. Subcutaneous tissue is also sometimes considered part of the skin in the scalp. In regard to the facial hair of males, hair follicles have roots extending into the subcutaneous tissue. The epidermis is composed of four layers including the
**stratum corneum**, the **stratum granulosum**, the **stratum spinosum**, and the **stratum basale** or basal layer. The **stratum corneum** is made up of anuclear cells that become flattened and dried out. These are the most differentiated cells of the epidermis and no longer contain organelles, they are composed almost entirely of keratin fibers. The deeper keratinized cells have very thick plasma membranes that form the water barrier of the skin. This cell layer can vary in thickness depending on where it is located on the body. For instance, the **stratum corneum** is thicker in the palm of the hand than it is on the ventral side of the forearm. The **stratum granulosum** is the second most superficial layer of the epidermis and is identified by the presence of keratohyaline granules. These granules contain cysteine-rich and histidine-rich proteins that ultimately give rise to filaggrin proteins that help to aggregate the keratin cells of the stratum corneum. It is important to note that this skin layer is less prominent in rats than humans. In rats it often seems that the basal layer gives way to only a few migrating cells (2-3 layers) then the keratinized layer becomes quite pronounced. The **stratum spinosum** is the third most superficial layer of the epidermis and is identified by the presence of large cells that contain very pronounced cytoplasmic constituents. The name spinosum comes from presence of all of the cytoplasmic processes or "spines." The final, deepest layer of the epidermis is the stratum basale or basal layer. This layer of cells is only represented by a single cell layer that rests on the surface of the basal lamina. These cells of the **stratum basale** are stem cells that retain the ability to differentiate further into the more superficial cell layers of the epidermis. For this same reason it is common that
the *stratum basale* to also be referred to as the *stratum germinativum*. Several distinguishing factors of this cell layer are that the nuclei of the basal layer are packed more tightly, there are very tight intercellular junctions preserving the integrity of the one layer boundary, and the cells themselves are columnar to cuboidal in shape as appose to more differentiated keratinocytes that have a flattened appearance histologically (Ross et al., 2006).

The dermis is the next layer of skin deep to the epidermis. It remains in contact with the dermis by the presence of dermal papillae that interact with corresponding projections and recessions in the epidermis called *rete ridges*. Together these two structures help to keep the tight union between the epidermis and dermis and serve to prevent sliding of the epidermis with respect to the dermis. The dermis contains two subdividing layers, the *papillary dermis* and the reticular dermis. The papillary dermis is composed mostly of type 1 and type 3 collagen whereas the *reticular dermis* contains only type 1 collagen. These are of interest because type 3 collagen is present in granulation tissue and is present preceding the tougher, more abundant type 1 collagen. Type 1 collagen is present in scars, muscle, tendon, artery walls and other areas requiring a high resistance to physical stresses. The papillary layer of dermis houses blood vessels that are responsible for providing oxygenated blood to the epidermis. It is important to note that these vessels do not cross the basal lamina to the epidermis, the epidermis receives its nutrients and oxygen via diffusion through the extracellular matrix. The papillary dermis also contains nerve endings that sometimes penetrate further but are not required to. The reticular layer of dermis
has fewer cellular components when compared to the papillary layer, instead this layer has a high density of regularly oriented collagen and elastic fibers. Important to note in the study of skin wounding is the fact that wounds made parallel to these fibers heal with less scarring (Ross et al., 2006).

1.12. Conclusions

OGF has been shown to interact with OGFr and modulate DNA synthesis. NTX is an opioid antagonist and has been shown to effectively block the OGF-OGFr interaction and increase cell proliferation by accelerating DNA synthesis. Addition of NTX to corneal wounds has been shown to significantly increase the rate of wound closure in both normal and diabetic rats. Thus it is proposed that NTX appropriately applied in a topical agent to full-thickness wound should increase cell replication and proliferation by increasing DNA synthesis.
Chapter 2

Objectives

Blockade of the OGF-OGFr axis using NTX results in an increase in cell proliferation. This paradigm of enhanced cell proliferation has been demonstrated in corneal keratopathy in normal and diabetic rats and rabbits where NTX was administered systemically (normal rat eyes) or topically (diabetic rats and rabbits). Treatment of abrasions with NTX has also revealed enhanced cell proliferation following systemic administration of NTX. Little or no information is available on the blockade of the OGF-OGFr axis with NTX in a full-thickness wound model. In a logical series of experiments, the optimal route of administration of NTX, as well as an optimal dosage of NTX were determined and then investigated using full thickness circular wounds on the dorsum of normal type 1 diabetic rats. The hypothesis of this research is that blockade of the OGF-OGFr axis enhances wound healing by upregulation of DNA synthesis. This hypothesis will be tested by completion of the following specific aims:

Specific Aim 1: To establish the optimal dose and route of administration of NTX in promoting cell proliferation in normal and diabetic rats.

Specific Aim 2: To evaluate wound closure in normal and diabetic rats receiving topical applications of the optimal NTX dosage and vehicle. Wound closure will be monitored by evaluation of vascularization (α-smooth muscle actin),
inflammation (Hematoxylin and eosin, Masson's Trichrome), collagen regeneration (Sirius red), and epithelialization. In addition, the mechanisms of action will be studied by examination of DNA synthesis (BrdU) and apoptosis (TUNEL).
Chapter 3
Methodology

3.1. Animals

Male Sprague-Dawley rats (Charles River Laboratories) were used for all experiments in this study. After clearance from quarantine the rats were randomly assigned to a diabetic or normal group. They were housed in an atmosphere of 50 +/- 10% relative humidity, a complete exchange of air 12-15 times per hour, and a 12-hour light-dark cycle with no twilight. Water and Purina 5010 Rodent Chow were fed ad-libitum. All studies were conducted under the approved IACUC protocol 2004-044 and all experiments were according to guidelines set forth by the National Institute of Health and the Pennsylvania State University College of Medicine.

3.2. Diabetes

Six week old Sprague-Dawley rats were rendered diabetic using STZ (minimum 98% with HPLC) by injecting 40mg/kg STZ. The STZ was made fresh no more than thirty minutes prior to injection of the rats. The STZ was dissolved in sodium citrate buffer (ph=4.5) at room temperature. The animals received IP injections on two consecutive days. Five days after injection weekly blood glucose readings were taken and additional STZ injections were given as needed. Any animals failing to reach blood glucose levels of 250 mg/dL were not used for this experiment.
3.3. **Blood Glucose Measurements**

Rats had blood glucose levels analyzed using a True Track Smart System glucometer© (Home Diagnostics, Inc.; Ft. Lauderdale FL) on a weekly basis to ensure maintenance of the diabetic state. Blood drawn from the animals was the minimum amount needed for the blood glucose measurement device. The tail prick technique entails a quick puncture of the tail vein using a small gauge needle drawing a single drop of blood. Blood glucose measurements over 250mg/dL were considered diabetic, animals failing to meet this blood glucose level after two injections received an additional injection of 40mg/kg STZ. If after this third injection animals did not reach the 250mg/dL blood glucose level they were discarded from the experiment. Blood glucose measurements were taken weekly for 4 weeks. At that time, a time known to result in impaired corneal wound healing, full thickness wounds were made.

3.4. **Body weight**

Body weights were recorded on a weekly basis using a Mettler Toledo PB 3001-S scale made in Switzerland.

3.5. **Experiment I: Determination of Dose and Vehicle**

Rats received topical NTX in 1 of 4 different topical vehicles. The topical vehicles were SPB, DMSO, moisturizing cream (Neutrogena), and KY jelly. Each rat received topical treatment at 3 different concentrations of NTX in the topical agent, those concentrations were $10^{-4}$M, $10^{-5}$M, and $10^{-6}$M. The topical applications were given on the animal's back 5cm, 7cm and 9cm caudal to the occipital notch and 1cm off the midline to the left. The region 5cm from the
occipital notch and 1 cm to the left of the vertebral column was a $10^{-4}$M concentration of NTX in the given topical vehicle. The region 7 cm from the occipital notch and 1 cm off the vertebral column was a $10^{-5}$M concentration of NTX in the given topical vehicle. The region 9 cm from the occipital notch and 1 cm to the left of the vertebral column was a $10^{-6}$M concentration of NTX in the given vehicle. Each of these applications was controlled for on the opposite side of the vertebral column at the same distances from the occipital notch. These control areas received only vehicle and no NTX. The animals received NTX at 0800 hours, 1200 hours and 1600 hours. All animals were terminated between 1700 and 1800 hours. Animals were terminated using 0.5 mL Euthasol (Virbac, Fort Worth, TX), the active ingredients include 195mg sodium pentobarbital and 25 mg sodium phenytoin for this dose. All animals received IP injection of BrdU (100mg/kg) at 6 and 3 hours prior to euthanasia. After the animals were terminated a 2cm$^2$ section of skin was removed from the site of topical applications and tissue was then placed in a cassette (Sakura Uni-Cassette, Sakura Finetek USA Inc., Torrance CA) and in 10% Neutral Buffered Formalin (Richard Allan Scientific, Kalamazoo MI) for 24 hours. Tissue was then placed in 70% ethanol for 48 hours. Tissue was then manually processed using alcohols, Clear-rite 3 (Richard Allan Scientific, Kalamazoo MI), and paraffin wax (Fisherbrand Paraplast X-TRA Tissue embedding medium, Fisher HealthCare, Houston TX). The alcohol regimen was 1 hour in each of the following alcohols; 50%, 50%, 70%, 70%, 95, 95%, 100%, 100% followed by two hour long immersions in clear-rite 3 and two hour long immersions in 60° paraffin wax.
Tissue was then embedded using a Shandon Embedding Center S-EC (Thermo Fisher Scientific Inc., Waltham MA) for later analysis.

3.6. **Experiment II: Full-Thickness Wounds**

64 Sprague-Dawley rats, 16 weeks of age, were anesthetized by IP injection of ketamine (60 mg/kg), xylazine (10mg/kg), and acepromazine, (1 mg/kg). A full-thickness excisional skin wound made to the level of panniculus muscle was made with a 6mm Acupunch biopsy punch (Acuderm Inc., Fort Lauderdale, FL). Prior to surgery and after administration of anesthetic, animals were shaved using a Remington Precision Model HC-817 60Hz hair clipper (Remington Precision, Bridgeport CT), this ensured that animals were in the same hair cycle prior to surgery. Animals were marked using a washable marker prior to surgery in order to consistently position the wounds on the animal's dorsum. The sites of the wounding were over each scapula and cranial to the iliac wings bilaterally, 3 wounds per rat were created. The first wound was made 5 cm posterior to the occipital notch at the posterior of the animal's cranium and 1 cm off the midline. The second wound was made 7 cm posterior to the occipital notch at the posterior of the animal's cranium and 1 cm off the midline on the opposite side from the first wound. The third wound was made 9 cm posterior to the occipital notch at the base of the animal's cranium and 1 cm off the midline on the same side as the first wound. Excisional wounds minimize repopulation of the epidermis from hair follicles or adnexal structures. Circular wounds are preferred over square or rectangular wounds for these investigations because the degree of wound contraction is less and re-epithelialization is greater with
circular wounds. To minimize the effects of diurnal rhythm surgery was performed between 0800-1000 hours. Wounds were left uncovered because with the removal of a dressing followed by application of NTX and recovering may cause stimulation of the healing processes of the skin. Administration of NTX applied topically occurred 3 times daily at 0800, 1200, and 1600, control rats received vehicle at the same time points, unless otherwise stated. Studies were done to compare efficacy of topical agents in Oil of Olay moisturizing cream and compare it to Neutrogena. These studies were done in rats n = 4 per group. OGF was combined with Neutrogena in a $10^{-5}$M concentration and added to wounds the same as before mentioned topical treatments. In another experiment to test the potency of $10^{-5}$M NTX in moisturizing cream, topical applications were made 1x/day, 2x/day or 3x/day. The topical applications were given at the early timepoints such that animals receiving topical moisturizing cream + $10^{-5}$M NTX only once received it at 0800 hr.

3.6.1. Wound Analysis

Photography was done using a Kodak EasyShare Z712IS Zoom Digital Camera (Eastman Kodak Company, Rochester NY.) On this camera there is an adjustment wheel that allows for different settings, in all photographs taken for these experiments the "P" setting was used. The close up setting was used, which is indicated by a flower icon on the top of the camera. The camera was mounted on a Bencher Copymate II 900-20/21 (Bencher Inc, Antioch IL). The camera was positioned 20 cm from the surface of the Bencher II stand. During photography a 15 cm ruler was held at the height of the wound for later
calibration during computer analysis. No flash was used on the camera due to a glare produced by the calibration ruler. During photography of the wounds a small IKEA 77 BJ-80,659 lamp was used to provide proper lighting. Animals were secured during photography by placement of the left hand on the animal's dorsum, pictures could then be taken without the use of anesthesia. If hair regrowth occurred to an extent which impeded the ability to determine wound boundaries then a Remington Precision Model HC-817 60Hz hair clipper (Remington Precision, Bridgeport CT) was used over the wounded areas. It is important that a razor is not used because of microabrasions caused by its interaction with the skin and the possibility that this could initiate a wound healing response and alter results.

Wound size was monitored at seven time points. The first photograph was taken just after surgery and prior to the first treatment of topical vehicle or topical vehicle with NTX. The second photograph was taken 72 hours following surgery. Subsequent photographs were taken every 48 hours up until complete wound closure occurred and there was no adherent scab.

Photographs were analyzed using ImageProPlus 6.2. Open wound areas were calculated to the cm$^2$. These values were then divided by the original wound size and a percentage was generated for comparison. The optimal original wound size was 28.26cm$^2$. Any wounds that varied from this value by more than 2.00cm$^2$ were not used for the experiment. Photographs were taken until there was no adherent scab present on the wound site and no redness indicating future scab formation was present.
3.6.2. **Termination and Tissue Harvesting**

Rats were terminated using 0.5 mL Euthasol (Virbac, Fort Worth, TX), the active ingredients include 195mg sodium pentobarbital and 25 mg sodium phenytoin for this dose. This was administered via IP injection and occurred between the hours of 1600-1800.

3.6.3. **Tissue Fixation, Processing and Sectioning**

After euthanasia, for non-wounded studies a cm$^2$ of tissue was removed over the treated site, for wounding studies a square piece of tissue was removed leaving 0.5 cm between the edge of the wound and the edge of the tissue. Tissue was then placed in a cassette (Sakura Uni-Cassette, Sakura Finetek USA Inc., Torrance CA) and in 10% Neutral Buffered Formalin (Richard Allan Scientific, Kalamazoo MI) for 24 hours. Tissue was then placed in 70% ethanol for 48 hours. Tissue was then processed in a Shandon Citadel 1000 Tissue processor (Thermo Fisher Scientific Inc., Waltham MA). It was programmed for the following regimen (1 hour in each solution) 50% ethanol, 50% ethanol, 70% ethanol, 80% ethanol, 95% ethanol, 95% ethanol, 100% ethanol, 100% ethanol, Clear-rite 3 (Richard Allan Scientific, Kalamazoo MI), Clear-rite 3, paraffin wax (Fisherbrand Paraplast X-TRA Tissue embedding medium, Fisher HealthCare, Houston TX), paraffin wax. Tissue was then embedded using a Shandon Embedding Center S-EC (Thermo Fisher Scientific Inc., Waltham MA).

Tissue sections were made using a microtome (Spencer Lens Co., Buffalo NY) the sections were cut to 5μm. Sections were made to include the very center of the wound or visible scar tissue, at its greatest diameter. Tissue was
placed in a warm water bath at 38°C then spread onto a subbed microscope slide (VWR International, West Chester PA). Tissue was then allowed to dry for 72 hours and then flash heated on a hot plate to ensure adhesion to the slide. From this point slides were stained using techniques to analyze cell proliferation, toxicity, and collagen formation.

3.7. **Cell Proliferation**

To determine cell proliferation a technique used to identify DNA synthesis was used. Animals were injected with BrdU (100 mg/kg) at a time point 6 hours prior to euthanasia and again 3 hours prior to euthanasia. Tissue sections were then deparaffinized and stained with anti-BrdU-POD, reacted with DAB, and counterstained with hematoxylin. The numbers of labeled and unlabeled cells in the basal layer of the epithelium were counted from 2 grids in unwounded skin and 4 grids from wounded skin. Grids were standardized by use of the same grid eyepiece for all counts. The grids in wounded skin were divided into two categories. The first grid was directly adjacent to the wound and n=2 because both sides of the wound were used. The second grid was at the outer extent of the section at least five grid lengths away from the first one and no more than seven grid lengths away. Unwounded skin two adjacent grids were counted in the center of the removed skin section.

3.8. **Histopathology**

3.8.1. **Hematoxylin and Eosin**

Skin sections from both wounded and unwounded skin were deparaffinized and stained with hematoxylin and eosin to identify inflammatory
infiltrates and cells undergoing necrosis. Basophilic nuclei are stained blue and eosin stained cytoplasm are stained pink or red. This stain allows for detection of inflammation or to determine the integrity of a tissue.

3.8.2. **Sirius Red**

As discussed earlier collagen formation plays a pivotal role in proper scar formation. Collagen was analyzed using Sirius Red stain. 0.1% Sirius Red in Saturated Picric Acid (Electron Microscopy Sciences, Hatfield PA) was used to identify both the presence and fiber direction of type 1 collagen. Sirius Red stains type 1 collagen fibers red, orange or green. Longer collagen fibers stain red, this is indicative of older more mature collagen. Intermediate length collagen fibers stain yellow to orange and are indicative of developing collagen fibrils that are not yet fully elongated but are longer than new fibers. Short collagen fibers stain green and this is indicative of new collagen growth.

3.8.3. **Masson's Trichrome**

Masson's trichrome stain distinguishes cells and extracellular proteins from each other. Muscle fibers and keratin stain red, collagen and bone stain blue or green, cytoplasm stains light red or pink and nuclei stain dark brown to black.

3.8.4. **Alpha Smooth Muscle Actin**

Alpha smooth muscle actin is present in the cytoplasm so the cell membrane must be dissolved prior to staining. In this case HSP-linked secondary antibody was used to indicate the presence of alpha smooth muscle actin, DAB was used as an indicator.
3.9. **Statistical Analysis**

Body weights, blood glucose, wound size and cell proliferation were analyzed by t-tests using GraphPad Prizm 5. In dose and vehicle selection experiments the values used were the percentages of labeled cells out of the total amount. In wounding experiments the values used for t-tests were percentages of residual wound area. In unwounded experiments an ANOVA was used to compare efficacy of both topical vehicles and identify the most effective dose.
Chapter 4

Results

4.1. Gross Observations

No animals died unexpectedly prior to surgery or throughout baseline blood glucose and body weight measurements. In the unwounded study, all normal and diabetic animals survived through the extent of the experiment and showed no signs of infection or irritation at the treated sites or elsewhere. In the wounding studies one animal died post-surgery from an adverse reaction to anesthesia. All other animals survived until termination. Also, in the wounding studies, no animals indicated infection of the wounded area or excessive irritation, there was no evidence of animals tampering with a wound on another animal or itself. Also, there was no witnessed removal of topical agents by the animals. However, if topical KY jelly was rubbed off on the wall of the housing units it was observed that animals did ingest it, this was not observed for moisturizing cream based topical treatments.
4.2. **Experiment 1: Determination of Dose and Vehicle**

4.2.1. **Body Weight**

Baseline body weights for all rats were approximately 250 g at the time of STZ injection (FIG. 4.3). Within one week, STZ injected rats weighed significantly less than sterile saline injection control animals (266 ± 4 g versus 292 ± 4 g; p<0.01). Throughout the duration of the experiment, diabetic rats weighed significantly less than controls. At week 8, Normal animals weighed 462 ± 12 g in comparison to DB rats that weighed 323 ± 10 g; p<0.0001.
Figure 4.1. Represents body weight of rats used in dose determination and vehicle selection experiments. Body weights normal and diabetic (DB) rats were measured every 7 days starting just prior of the first STZ injections (time = 0). Each timepoint represents mean ± S.E.M. Week 1 indicates one week after induction of diabetes. Statistically different from normal rats at ***p<0.001.
4.2.2. Hyperglycemic Induction

Animals became diabetic within one week of STZ injection. Seven days after injection of STZ rats had significantly higher (p<0.0001) glucose levels of 568.6 ± 9.3 mg/dL or relative to those of control animals (119.7 ± 4.2 mg/dL). The blood glucose levels of diabetic animals significantly differed from normal animals throughout the 8-week period (p<0.001). At week 8, normal animals had a glucose reading of 114.4 ± 6.0 mg/dL while diabetic rats had blood glucose levels of 547.7 ± 21.5 mg/dL (FIG. 4.1 and 4.2)
Figure 4.2. Blood glucose levels of Normal and diabetic (DB) rats used in Experiment I to determine optimal dose and vehicle. STZ injections were given on day one of week 0. Values represent means ± S.E.M. for 16 animals/group. Significantly different from Normal rats at p<0.001 (***).
4.2.3. Rates of DNA Synthesis in Untreated Normal and Diabetic Rats

In untreated animals it was shown that diabetic animals 8.0 ± 0.004% experienced a significantly lower level of basal layer cell proliferation than normal animals 9.8 ± 0.1% (p<0.05).
Labeling Indexes of Basal Layer Cell Proliferation in Untreated Normal and Diabetic Rats

Figure 4.3. Labeling indexes represent those of normal and diabetic animals receiving proper clearance and cleaning of dorsum. Data represent means ± S.E.M. Significantly different from their normals at p<0.05.
4.2.4. Selection of Vehicle and Dose by DNA Synthesis

Four different compounds were tested for their efficacy as topical applications for NTX – DMSO, Sorenson’s phosphate buffer (SPB), commercially available moisturizing cream, and KY jelly. Each compound had different textures and absorbancy rates; NTX was readily dissolved in all compounds. Different concentrations of NTX were dissolved in each vehicle and topically applied to skin. The rate of DNA synthesis was used as a marker for determining the optimal vehicle and dosage.
4.2.4.1. Selection of Dose and Vehicle by DNA Synthesis in Normal Rats

Each of the vehicles was analyzed separately to identify an ideal NTX dose appropriate for enhanced cell proliferation. No differences in DNA synthesis were noted between any of the vehicles, and no differences were noted between untreated skin of normal rats (8.1 ± 0.8%) and any vehicle (Fig. 4.5).

Sorenson’s phosphate buffer: Basal cell proliferation in skin treated with SPB alone was 9.1 ± 0.7%. DNA synthesis in skin treated with 10^{-4} M, 10^{-5}M, and 10^{-6} M NTX dissolved in SPB was elevated approximately 5% (FIG. 4.5).

DMSO: Basal cell proliferation in skin treated with DMSO alone was 8.7 ± 0.5%. DNA synthesis in skin treated with 10^{-4} M, 10^{-5}M, and 10^{-6} M NTX dissolved in DMSO was elevated to 15 – 20 % (FIG. 4.5).

KY Jelly: In comparison to a basal level in DNA synthesis of 8.6 ± 0.7% in skin treated with KY jelly alone, the DNA synthesis rates in skin treated with topical applications of 10^{-4} M, 10^{-5}M, and 10^{-6} M NTX dissolved in KY jelly were 16 -19% (p0.001).

Moisturizing cream: DNA synthesis in the basal layer of skin treated with moisturizing cream was 8.2 ± 0.8%. Relative to these levels, DNA synthesis rates were 14.1 ± 0.9% in skin receiving 10^{-4} M NTX and 19.0 ± 0.9% in skin treated with 10^{-5} M or 10^{-6} M NTX.
Labeling Indexes in Selection of Dose and Vehicle Experiment in Normal Rats

Figure 4.4. The percent DNA labeling indexes in skin from Normal rats treated topically with $10^{-4}$ M, $10^{-5}$ M, and $10^{-6}$ M NTX dissolved in Sorenson's phosphate buffer (SPB), DMSO, KY Jelly, or Moisturizing Cream. Data represent means ± S.E.M. Significantly different from their respective vehicle only group at $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***)
4.2.4.2. Selection of Vehicle and Dose by DNA Synthesis in Diabetic Rats

The same 4 compounds (i.e., DMSO, Sorenson’s phosphate buffer (SPB), commercially available moisturizing cream, and KY jelly) were tested for their efficacy as vehicles for topical application of NTX in diabetic rats (FIG. 4.6). Different concentrations of NTX were dissolved in each vehicle and topically applied to DB skin. The rate of DNA synthesis was used as a marker for determining the optimal vehicle and dosage. Each of the vehicles was analyzed separately to identify an ideal NTX dose appropriate for enhanced cell proliferation. DNA synthesis rates were comparable among all of the vehicles alone, and did not differ from the labeling index in untreated DB skin (7.0 ± 0.8%). However, the rate of DNA synthesis in untreated DB skin was significantly (p<0.05) lower than that of Normal skin.

Sorenson’s phosphate buffer: Basal cell proliferation in skin treated with SPB alone was 8.0 ± 0.8%. DNA synthesis rates in skin treated with $10^{-4}$ M and $10^{-5}$M NTX were 9 and 11.1% and did not differ from the vehicle alone. However, skin treated with $10^{-6}$ M NTX dissolved in SPB displayed an elevated labeling index of 12.8 ± 0.8%; this rate was significantly higher than vehicle alone (p<0.01). (FIG. 4.6).

DMSO: Basal cell proliferation in skin treated with DMSO alone was 8.6 ± 0.9%. Labeling indexes in DB skin treated with $10^{-4}$ M, $10^{-5}$M, and $10^{-6}$ M NTX dissolved in DMSO was elevated to 11 to 18%, a 28% to 2-fold increase over baseline levels (FIG. 4.5).
KY Jelly: In comparison to a basal level in DNA synthesis of 9.5 ± 0.1.1% in DB skin treated with KY jelly alone, the labeling indexes in skin treated with topical applications of $10^{-4}$ M, $10^{-5}$M, and $10^{-6}$ M NTX dissolved in KY jelly were 19-23.5% which represented 100-247% increases over basal levels of DNA synthesis ($p<0.001$).

Moisturizing cream: DNA synthesis in the basal layer of DB skin treated with moisturizing cream was 8.4 ± 1.1%. Relative to these levels, DNA synthesis rates were 15.4 ± 1.0% in skin receiving $10^{-4}$ M NTX and 15.5 ± 1.2% in skin treated with $10^{-5}$ M NTX and 15.8 ± 1.0% in skin treated with $10^{-6}$ M NTX. These DNA synthesis rates represented increases of at least 85% ($p<0.001$).

Based on the data gathered from DNA synthesis studies in both Normal and diabetic rats in Experiment I, two optimal vehicles were chosen - moisturizing cream and KY jelly, and an optimal dosage of $10^{-5}$ M NTX was selected for the wound healing studies described in Experiment II.
Labeling Indexes in Selection of Dose and Vehicle Experiment in Diabetic Rats

Figure 4.5. The percent DNA labeling indexes in skin from Diabetic rats treated topically with $10^{-4}$ M, $10^{-5}$M, and $10^{-6}$ M NTX dissolved in Sorenson’s phosphate buffer (SPB), DMSO, KY Jelly, or Moisturizing Cream. Data represent means ± S.E.M. Significantly different from their respective vehicle only group at $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***).
4.3. **Experiment II: Efficacy of Topical Naltrexone to Enhance Full-thickness Cutaneous Wound Healing**

In wounding experiments there were no pathologies such as excessive redness or infection. There was no swelling of the wounded areas and bleeding did not occur after scar formation occurred which happened before day 3 photographs were taken. There was no abnormal healing, wounds did not bunch up in a way that made elevated scar tissue and they all healed in a circular fashion. There were no aberrations such as increases in wound size or infectious discharge. There also did not appear to be any side effects to the topical treatment vehicles or NTX.
Figure 4.6. Digital photographs of normal control, normal treated ($10^{-5}$M NTX), diabetic control and diabetic treated ($10^{-5}$M NTX) animals. Photographs were taken at timepoints 0 (directly after surgery) 3, 5, 7, 9, and 13 days. Diabetic animals were diabetic for 5 weeks prior to surgery. The rats received topical NTX in moisturizing cream 3 times daily until day 13. Control wounds received only moisturizing cream.
4.3.1. Body Weight in Efficacy of Topical Naltrexone to Enhance Full-Thickness Cutaneous Wound Healing

Baseline body weights for all rats were approximately 250 g at the time of STZ injection (FIG. 4.3). Within one week, STZ injected rats weighed significantly less than sterile saline injection control animals (206 ± 4 g versus 224 ± 4 g; p<0.01). Throughout the duration of the experiment, diabetic rats weighed significantly less than controls. At week 8, Normal animals weighed 477 ± 8 g in comparison to DB rats that weighed 339 ± 8 g; p<0.0001.
Figure 4.7. Represents body weight of rats used in wounding experiments. Body weights normal and diabetic (DB) rats were measured every 7 days starting just prior to the first STZ injections (time = 0). Each timepoint represents mean ± S.E.M. Week 1 indicates one week after induction of diabetes. Significantly different from normal rats at **p<0.01 and ***p<0.001.
4.3.2. **Hyperglycemic Induction in Efficacy of Topical Naltrexone to Enhance Full-Thickness Cutaneous Wound Healing**

Animals became diabetic within one week of STZ injection. Seven days after injection of STZ rats had significantly higher (p<0.0001) glucose levels of 477.8 ± 25.1 mg/dL or relative to those of control animals (121.9 ± 2.5 mg/dL). The blood glucose levels of diabetic animals significantly different from normal animals throughout the 8-week period (p<0.001). At week 8, normal animals had a glucose reading of 122.2 ± 2.1 mg/dL while diabetic rats had blood glucose levels of 589.4 ± 5.4 mg/dL (FIG. 4.1 and 4.2)
Hyperglycemic Induction in Full-Thickness Wounding Experiment

Figure 4.8. Graph represents mean ± S.E.M. of blood glucose levels at different times before or after STZ injections for rats used in wounding experiments. STZ injections were given on day one at week 0. The values indicate means ± S.E.M. for 48 animals/group for both diabetic (DB) and normal rats. Statistically different from normal rats at ***p<0.001.
4.3.3. **Full-Thickness Wound Healing Rates in Normal Rats**

On day 3 and of wounding studies normal animals showed a significant difference between moisturizing cream treated wounds which had a mean % residual defect of $75.9 \pm 2.7 \text{mm}^2$ when compared to moisturizing cream + $10^{-5}\text{M}$ NTX treated wounds which had a mean % residual defect of $68.5 \pm 2.1 \text{mm}^2$ ($p < 0.05$). On day 5 and of wounding studies normal animals showed a significant difference between moisturizing cream treated wounds which had a mean % residual defect of $55.3 \pm 3.5 \text{mm}^2$ when compared to moisturizing cream + $10^{-5}\text{M}$ NTX treated wounds which had a mean % residual defect of $46.3 \pm 2.4 \text{mm}^2$ ($p < 0.05$). On day 7 and of wounding studies normal animals showed a significant difference between moisturizing cream treated wounds which had a mean % residual defect of $38.4 \pm 3.4 \text{mm}^2$ when compared to moisturizing cream + $10^{-5}\text{M}$ NTX treated wounds which had a mean % residual defect of $29.7 \pm 1.8 \text{mm}^2$ ($p < 0.05$). On day 9 and of wounding studies normal animals showed a significant difference between moisturizing cream treated wounds which had a mean % residual defect of $9.9 \pm 1.5 \text{mm}^2$ when compared to moisturizing cream + $10^{-5}\text{M}$ NTX treated wounds which had a mean % residual defect of $6.4 \pm 1.0 \text{mm}^2$ ($p < 0.05$).

On day 3 and of wounding studies normal animals showed a significant difference between KY jelly treated wounds which had a mean % residual defect of $75.9 \pm 2.7 \text{mm}^2$ when compared to KY jelly + $10^{-5}\text{M}$ NTX treated wounds which had a mean % residual defect of $68.5 \pm 2.1 \text{mm}^2$ ($p < 0.05$). On day 5 of wounding studies normal animals showed a significant difference between KY
jelly treated wounds which had a mean % residual defect of 50.9 ± 3.7mm² when compared to KY jelly + 10^{-5}M NTX treated wounds which had a mean % residual defect of 42.1 ± 1.8mm² (p < 0.05).
Figure 4.9. Percentage of residual defect in full thickness wounds in Normal rat skin after formation of a 6 mm wound. Rats were topically treated with $10^{-5}$ M NTX in either moisturizing cream (MC) or KY jelly (KY) three times daily over the 9 day period. Wounds were photographed with a Kodak EasyShare &Z712IS Zoom digital camera, and areas analyzed using ImageProPlus 6.2 Data are expressed as means ± S.E.M. Significantly different from respective vehicle controls at p<0.05 (*).
4.3.4. **Full-Thickness Wound Healing Rates in Diabetic Rats**

On day 3 of wounding studies diabetic animals showed a significant difference between KY jelly treated wounds which had a mean % residual defect of $81.5 \pm 2.7$ mm$^2$ when compared to KY jelly + $10^{-5}$M NTX treated wounds which had a mean % residual defect of $68.9 \pm 2.1$ mm$^2$ ($p < 0.001$). There was also a difference between moisturizing cream treated wounds which had a mean % residual defect of $88.6 \pm 1.4$ mm$^2$ when compared to moisturizing cream + $10^{-5}$M NTX which had a mean % residual defect of $77 \pm 1.4$ mm$^2$ ($p < 0.001$).

On day 5 of wounding studies diabetic animals showed a significant difference between KY jelly treated wounds which had a mean % residual defect of $71.5 \pm 3.6$ mm$^2$ and KY jelly + $10^{-5}$M NTX treated wounds which had a mean % residual defect of $58.2 \pm 2.8$ mm$^2$ ($p < 0.05$). There was also a difference between moisturizing cream treated wounds which had a mean % residual defect of $68.2 \pm 4.5$ mm$^2$ and moisturizing cream + $10^{-5}$M NTX treated wounds which had a mean % residual defect of $51.5 \pm 3.1$ mm$^2$ ($p < 0.01$).

On day 7 of wounding studies diabetic animals showed a significant difference between KY jelly treated wounds which had a mean % residual defect of $44.2 \pm 5.0$ mm$^2$ and KY jelly + $10^{-5}$M NTX treated wounds which had a mean % residual defect of $28.7 \pm 2.3$ mm$^2$ ($p < 0.01$). There was also a difference between moisturizing cream treated wounds which had a mean % residual defect of $48.1 \pm 5.6$ mm$^2$ and moisturizing cream + NTX treated wounds which had a mean % residual defect of $23.6 \pm 2.2$ mm$^2$ ($p < 0.001$).
On day 9 of wounding studies diabetic animals showed a significant difference between KY jelly treated wounds which had a mean % residual defect of 19.8 ± 3.2mm² and KY jelly + 10^-5M NTX treated wounds which had a mean % residual defect of 7.0 ± 1.6mm² (p < 0.001). There was also a difference between moisturizing cream treated wounds which had a mean % residual defect of 17.3 ± 2.8mm² and moisturizing cream + NTX treated wounds which had a mean % residual defect of 7.6 ± 1.6mm² (p < 0.01).
Figure 4.10. Histograms of residual defect (%) in full thickness wounds in diabetic rat skin after formation of a 6mm wound tracked for 9 days. Four groups of rats were included: diabetic moisturizing cream treated (Normal MC), diabetic moisturizing cream + 10^-5 M NTX treated (Normal MC + NTX), diabetic KY jelly treated (Normal KY) and diabetic KY jelly + 10^-5 M NTX treated (Normal KY + NTX). Photographs were captured with a Kodak EasyShare Z712IS Zoom Digital Camera, and areas analyzed using ImageProPlus 6.2. Residual defects are presented as a percentage of the original wound. Data are expressed as means ± S.E.M. Significantly different from control vehicle at *p<0.05, **p<0.01, ***p<0.001.
4.3.5. **Number of Daily Applications of Naltrexone in Moisturizing Cream in Normal and Diabetic Rats**

To determine whether 3 topical applications of naltrexone in moisturizing cream were necessary for the accelerated wound healing rates documented above, 6 mm wounds in both Normal and DB rats were treated once (0800) or twice (0800 and 1200) daily for 9 days. Photographs of wounds were analyzed with ImageProPlus 6.2 and percentage of residual defect graphed in Figures 4.9 and 4.10.
Figure 4.11. Histograms of residual defect (%) in full thickness wounds in normal rat skin after formation of a 6mm wound tracked for 9 days. Three groups of rats were included: normal moisturizing cream + 10⁻⁵M NTX treated 1x/day (1x), normal moisturizing cream + 10⁻⁵M NTX treated 2x/day (2x), normal moisturizing cream + 10⁻⁵M NTX treated 3x/day (3x). Photographs were captured with a Kodak EasyShare Z712IS Zoom Digital Camera, and areas analyzed using ImageProPlus 6.2. Residual defects are presented as a percentage of the original wound. Data are expressed as means ± S.E.M. Significantly different from 1x/day treatment at *p<0.05, **p<0.01 significantly different from 2x/day treatment at *p<0.05. Significant differences are according to respective controls.
Figure 4.12. Histograms of residual defect (%) in full thickness wounds in diabetic rat skin after formation of a 6mm wound tracked for 9 days. Three groups of rats were included: diabetic moisturizing cream + $10^{-5}$M NTX treated 1x/day (1x), diabetic moisturizing cream + $10^{-5}$M NTX treated 2x/day (2x), diabetic moisturizing cream + $10^{-5}$M NTX treated 3x/day (3x). Photographs were captured with a Kodak EasyShare Z712IS Zoom Digital Camera, and areas analyzed using ImageProPlus 6.2. Residual defects are presented as a percentage of the original wound. Data are expressed as means ± S.E.M. Significantly different from 1x/day treatment at *p<0.05, significantly different from 2x/day treatment at **p<0.01, significantly different from 3x/day treatment at ***p<0.001. Significant differences are according to respective controls.
In normal rats 1x daily treatment with moisturizing cream + 10^{-5}M NTX differed significantly from 1x daily moisturizing cream treatments at days 3, 7, and 9. In normal rats 2x daily treatment with moisturizing cream + 10^{-5}M NTX did not differ significantly from 2x daily moisturizing cream treatments. In normal rats 3x daily treatment with moisturizing cream + 10^{-5}M NTX differed significantly from 3x daily moisturizing cream treatments at day 3, 5, 7 and 9.

In diabetic rats 1x daily treatment with moisturizing cream + 10^{-5}M NTX differed significantly from 1x daily moisturizing cream treatments at days 5 and 7. In diabetic rats 2x daily treatment with moisturizing cream + 10^{-5}M NTX differed significantly from 2x daily moisturizing cream treatments at days 5, 7 and 9. In diabetic rats 3x daily treatment with moisturizing cream + 10^{-5}M NTX differed significantly from 3x daily moisturizing cream treatments at days 3, 5, 7 and 9.
4.3.6. **Topical Application of Opioid Growth Factor (OGF) and Wound Healing in Normal and Diabetic Rats**

OGF is an autocrine produced inhibitory growth factor that has been widely documented to inhibit DNA synthesis in epithelial tissues (Wilson et al., 1998)(Zagon et al., 1996). To determine whether the effects of NTX may be working through the OGF-OGFr axis and thus blocking the interactions of the inhibitory peptide-receptor regulatory pathway, exogenous OGF was added in moisturizing cream and applied 3 times daily to 6 mm wounds in Normal (FIGURE 4.11) and diabetic (FIGURE 4.12) rats. There was no statistical difference in rate of wound closure between moisturizing cream + $10^{-5}$M OGF and moisturizing cream control.
% Residual Defect of Full-Thickness Wounds in Normal Rats with Neutrogena + OGF 3x Daily

Figure 4.13. Histograms of residual defect (%) in full thickness wounds in normal rat skin after formation of a 6mm wound tracked for 9 days. Two groups of rats were included: normal moisturizing cream control (NMC) normal moisturizing cream + 10^{-5}M OGF treated (NMC + OGF. Photographs were captured with a Kodak EasyShare Z712IS Zoom Digital Camera, and areas analyzed using ImageProPlus 6.2. Residual defects are presented as a percentage of the original wound. Data are expressed as means ± S.E.M. There are no significant differences.
Figure 4.14. Histograms of residual defect (%) in full thickness wounds in diabetic rat skin after formation of a 6mm wound tracked for 9 days. Two groups of rats were included: diabetic moisturizing cream control (NMC) diabetic moisturizing cream + 10^{-5}M OGF treated (NMC + OGF). Photographs were captured with a Kodak EasyShare Z712IS Zoom Digital Camera, and areas analyzed using ImageProPlus 6.2. Residual defects are presented as a percentage of the original wound. Data are expressed as means ± S.E.M. There are no significant differences.
4.3.7. Analysis of Different Moisturizing Creams as Optimal Vehicle

To assess whether Neutrogena moisturizing cream was the only moisturizing cream that was useful, Oil of Olay was tested as a vehicle. Concentrations of $10^{-5}$ M NTX were added to Oil of Olay or Neutrogena creams and topically applied 3 times daily for 9 days to 6 mm wounds in Normal (FIGURE 4.13) and Diabetic (FIGURE 4.14) rats. Comparisons of Oil of Olay and Neutrogena in Normal (FIGURE 4.15) and Diabetic (FIGURE 4.16) revealed that both creams were satisfactory and optimal vehicles for delivery of NTX to full thickness cutaneous wounds.

Oil of Olay moisturizing cream was substituted for Neutrogena in this study. Residual wound size of wounds treated with Oil of Olay moisturizing cream + $10^{-5}$M NTX significantly differed from wounds treated with Oil of Olay moisturizing cream only on days 5 and 9 in normal animals. On day 5 wounds treated with Oil of Olay moisturizing cream + $10^{-5}$M NTX had a mean size of 47.7 ± 1.0% which was statistically different from wounds treated with Oil of Olay moisturizing cream only which had a mean size of 62.8 ± 2.0% (p<0.001). On day 9 wounds treated with Oil of Olay moisturizing cream + $10^{-5}$M NTX had a mean size of 7.2 ± 1.3% which was statistically different from wounds treated with Oil of Olay moisturizing cream only which had a mean size of 20.0 ± 3.5% (p<0.05).

Residual wound size of wounds treated with Oil of Olay moisturizing cream + $10^{-5}$M NTX significantly differed from wounds treated with Oil of Olay moisturizing cream only on days 5, 7 and 9 in diabetic animals. On day 5
wounds treated with Oil of Olay moisturizing cream + $10^{-5}$M NTX had a mean size of 50.4 ± 2.5% which was statistically different from wounds treated with Oil of Olay moisturizing cream only which had a mean size of 70.6 ± 7.0% ($p<0.05$). On day 7 wounds treated with Oil of Olay moisturizing cream + $10^{-5}$M NTX had a mean size of 19.9 ± 2.3% which was statistically different from wounds treated with Oil of Olay moisturizing cream only which had a mean size of 51.2 ± 10.8% ($p<0.05$). On day 9 wounds treated with Oil of Olay moisturizing cream + $10^{-5}$M NTX had a mean size of 4.1 ± 1.8% which was statistically different from wounds treated with Oil of Olay moisturizing cream only which had a mean size of 20.5 ± 5.2% ($p<0.05$).
Figure 4.15. Histograms of residual defect (%) in full thickness wounds in normal rat skin after formation of a 6mm wound tracked for 9 days. Two groups of rats were included: normal Oil of Olay moisturizing cream control (Oil of Olay) normal Oil of Olay moisturizing cream + \(10^{-5}\)M NTX treated (Oil of Olay + NTX). Photographs were captured with a Kodak EasyShare Z712IS Zoom Digital Camera, and areas analyzed using ImageProPlus 6.2. Residual defects are presented as a percentage of the original wound. Data are expressed as means ± S.E.M. Significantly different from Oil of Olay control treatment at *p<0.05, ***p<0.001.
Figure 4.16. Histograms of residual defect (%) in full thickness wounds in diabetic rat skin after formation of a 6mm wound tracked for 9 days. Two groups of rats were included: diabetic Oil of Olay moisturizing cream control (Oil of Olay) diabetic Oil of Olay moisturizing cream + 10^{-5}M NTX treated (Oil of Olay + NTX). Photographs were captured with a Kodak EasyShare Z712IS Zoom Digital Camera, and areas analyzed using ImageProPlus 6.2. Residual defects are presented as a percentage of the original wound. Data are expressed as means ± S.E.M. Significantly different from Oil of Olay control treatment at *p<0.05.
% Residual Defect of Full-Thickness Wounds in Normal Rats With Neutrogena + NTX 3x Daily vs. Oil of Olay + NTX 3x Daily

Table showing the residual defect (%) in full thickness wounds in normal rat skin after formation of a 6mm wound tracked for 9 days. Two groups of rats were included: normal Neutrogena moisturizing cream + 10^{-5}M NTX (Neutrogena + NTX) normal Oil of Olay moisturizing cream + 10^{-5}M NTX treated (Oil of Olay + NTX). Photographs were captured with a Kodak EasyShare Z712IS Zoom Digital Camera, and areas analyzed using ImageProPlus 6.2. Residual defects are presented as a percentage of the original wound. Data are expressed as means ± S.E.M. There are no significant differences in this graph.
% Residual Defect of Full-Thickness Wounds in Diabetic Rats
With Neutrogena + NTX 3x Daily vs. Oil of Olay + NTX 3x Daily

Figure 4.18. Histograms of residual defect (%) in full thickness wounds in diabetic rat skin after formation of a 6mm wound tracked for 9 days. Two groups of rats were included: diabetic Neutrogena moisturizing cream + 10^{-5}M NTX (Neutrogena + NTX) diabetic Oil of Olay moisturizing cream + 10^{-5}M NTX treated (Oil of Olay + NTX). Photographs were captured with a Kodak EasyShare Z712IS Zoom Digital Camera, and areas analyzed using ImageProPlus 6.2. Residual defects are presented as a percentage of the original wound. Data are expressed as means ± S.E.M. Significantly different from Neutrogena moisturizing cream + 10^{-5}M NTX treatment at *p<0.05.
4.3.8. **Mechanisms of Action for NTX Acceleration of Full-Thickness Wound Healing**

To begin to assess mechanisms underlying the accelerated wound closure observed in both Normal and DB rats that had wounds treated with NTX, DNA synthesis rates were examined by measurement of BrdU incorporation. Wounds and surrounding tissue were collected 5 and 10 following initiation of treatment. These time points were selected because it was hypothesized that cell proliferation should be one of the first responses that could be measured. BrdU labeling was counted in areas immediately adjacent to the wound (Region 1) and also in areas distal to the wound (Region 2).

On day 5 BrdU labeling in normal animals from region 1 in moisturizing cream control sections differed significantly from moisturizing cream + $10^{-5}$M NTX sections. Moisturizing cream controls had a mean of 23.0 ± 1.8% labeling, moisturizing cream + $10^{-5}$M NTX sections had a mean of 30.3 ± 2.3% labeling ($p < 0.05$). On day 5 region 2 showed no significance between moisturizing cream controls and moisturizing cream + $10^{-5}$NTX. The mean for moisturizing cream controls was 18.8 ± 0.1% which did not differ from moisturizing cream + $10^{-5}$M NTX which had a mean of 20.6 ± 0.3%.

On day 5 BrdU labeling in diabetic animals from region 1 in moisturizing cream control sections differed significantly from moisturizing cream + $10^{-5}$M NTX sections. Moisturizing cream controls had a mean of 18.2 ± 1.4% labeling, moisturizing cream + $10^{-5}$M NTX sections had a mean of 30.6 ± 1.9% labeling ($p < 0.01$). On day 5 region 2 showed no significance between moisturizing cream
controls and moisturizing cream + $10^{-5}$NTX. The mean for moisturizing cream controls was $15.0 \pm 1.8\%$ which did not differ from moisturizing cream + $10^{-5}$M NTX which had a mean of $18.5 \pm 1.4\%$.

On day 10 BrdU labeling in normal animals from region 1 in moisturizing cream control sections differed significantly from moisturizing cream + $10^{-5}$M NTX sections. Moisturizing cream controls had a mean of $13.5 \pm 1.2\%$ labeling, moisturizing cream + $10^{-5}$M NTX sections had a mean of $22.6 \pm 2.4\%$ labeling ($p < 0.05$). On day 10 region 2 showed no significance between moisturizing cream controls and moisturizing cream + $10^{-5}$NTX. The mean for moisturizing cream controls was $11.9 \pm 0.7\%$ which did not differ from moisturizing cream + $10^{-5}$M NTX which had a mean of $13.8 \pm 1.3\%$.

On day 10 BrdU labeling in diabetic animals from region 1 in moisturizing cream control sections differed significantly from moisturizing cream + $10^{-5}$M NTX sections. Moisturizing cream controls had a mean of $19.4 \pm 2.0\%$ labeling, moisturizing cream + $10^{-5}$M NTX sections had a mean of $25.7 \pm 0.1\%$ labeling ($p < 0.05$). On day 10 region 2 showed no significance between moisturizing cream controls and moisturizing cream + $10^{-5}$NTX. The mean for moisturizing cream controls was $17.0 \pm 3.0\%$ which did not differ from moisturizing cream + $10^{-5}$M NTX which had a mean of $20.3 \pm 2.5\%$. 
Labeling Indexes in Full-Thickness Wound Experiment

Figure 4.19. Labeling indexes in cells of the basal layer of dorsal epithelium from Normal and DB rats treated topically with 10^{-5} M NTX in moisturizing cream (MC) or vehicle alone (MC). Tissues were collected 5 and 10 days following the creation of a 6 mm wound. Region 1 was an area directly on the edge of the wound; region 2 was 5 grid lengths from the edge of the wound. Values represent means ± S.E.M. Significantly different from vehicle alone at p<0.05 (*) and p<0.01 (**).
4.4. **Histopathology**

To ascertain whether topical application of NTX directly to full-thickness cutaneous wounds resulted in any deleterious side effects or caused any delays in wound healing such as inflammation or infection, epithelial tissues were processed for histopathology. Tissues were collected 5, 10, and 20 days after wounding, and sections were stained with hematoxylin and eosin, Sirius Red, and Masson’s Trichrome. Some sections were also immunohistochemically evaluated with α-smooth muscle actin.

4.4.1. **Hematoxylin and Eosin**

Hematoxylin and eosin was used to assess the structural integrity of the epithelium surrounding the wound at 5, 10 and 20 days. The epithelium of all the experimental groups is stratified squamous epithelium which is expected of this type of tissue. The outermost layer of cells are keratinized, thus anucleate and flattened. These cells provide a boundary for water, pathogens and other materials, they also provide some level of protection from abrasions. The epithelium is stained purple by hematoxylin and the connective tissue is stained pink with eosin. The boundary between the epithelium and connective tissue is the basal lamina and appeared intact for all experimental groups. Other histological features observed included glandular tissue as well as blood vessels and capillaries and hair follicles. These occurred at a consistent level throughout the experimental groups. Also of note is the lack of cells undergoing necrosis, this is indicative of the non-toxic properties of the topical agents both NTX and moisturizing cream. Also there was no observed lymphocytes at this point (day
20) meaning that there was no prolonged incidence of infection of the wounds across experimental groups.

It is also important to note that the skin sections display the typical characteristics of rat epithelium. There is a defined keratinized layer, a lack of prominent stratum lucidum which could also be due to the location of the wound. For instance, the epithelium on the back of the animal does not require the thickness of the pad of the animal’s foot. The basal layer is very definitive and characteristic of epithelium.
Figure 4.20. Hematoxylin and eosin pictures adjacent to the wounded region of moisturizing cream + 10^-5 M NTX wounds, control wounds in normal animals and moisturizing cream + 10^-5 M NTX wounds, control wounds in diabetic animals at days 5, 10, and 20. Qualitative evaluation indicates no difference in presence of lymphocytes or necrosis between any NTX treatment group or condition. Photographs were taken at 20x.
4.4.2. **Alpha Smooth Muscle Actin**

To evaluate the relative amounts of myofibroblasts in and around the site of the wound. Though no real differences across treatment groups can be determined, the presence of myofibroblasts was observed in each of the treatment groups.

4.4.3. **Masson's Trichrome**

To evaluate the different elements of extracellular and cellular components that make up of epithelium across treatment groups. Muscle fibers appear red as well as keratin in all experimental treatment groups. The density of collagen in the dermis is evident by the deep blue stain of the connective tissue layers deep to the epithelium. The collagen density does not appear to vary across treatment group. Nuclei are stained with Weigert's iron hematoxylin and appear as expected in epithelial cells; however it is difficult to locate fibroblast nuclei and differentiate it in the deep blue stain of the dermal collagen.

4.4.4. **Sirius Red**

To evaluate collagen type, size and age approximations across treatment groups. Sections stained showed colors ranging from red, to orange/yellow, to green. Red stained fibers indicate those fibers that are larger and more regularly arranged. Orange/yellow stained fibers indicate those fibers that are of an intermediate size and are not fully organized. Green fibers are smaller collagen fiber fragments that tend to be irregular. Collagen fiber size is often indicative of the age of collagen; therefore red fibers can, but are not definitely older fibers. Day 20 normal animals show more red fibers and orange/yellow fibers. Day 20
diabetic animals show a more significant amount of green fibers. Since the wound was made 20 days prior to the tissue fixation green fibers in the diabetic animal pictures are most likely new fibers. The normal animals do not display a large amount of green fibers indicating that the healing process may progress at a more rapid pace in the normal animals.

4.4.5. **Apoptosis**

TUNEL staining was used to measure apoptosis in all treatment groups at day 20. In day 20 animals there was no sign of apoptosis of cells of the epidermis or dermis across treatment group. The positive control makes disruptions in the DNA of the cell resulting in the blue/green stain observed in the figures. The experimental groups show day 20 sections from normal animals with moisturizing cream only, moisturizing cream + $10^{-5}\text{M}$ NTX and diabetic animals with moisturizing cream only and moisturizing cream + $10^{-5}\text{M}$ NTX. None of these groups indicate apoptosis of the cells in the epidermis or dermis of the experimental groups as indicated by visual comparison with the positive control.
Histopathology: Alpha Smooth Muscle Actin, Masson’s Trichrome, Sirius Red and Apoptosis (TUNEL)

Figure 4.21. Alpha smooth muscle actin, Masson’s trichrome, Sirius Red, and Apoptosis (TUNEL) pictures taken adjacent to the wound. Photographs were of moisturizing cream + 10^{-5}M NTX wounds, control wounds in normal animals and moisturizing cream + 10^{-5}M NTX wounds, control wounds in diabetic animals at day 20. Qualitative evaluation indicates no difference in presence of myofibroblasts in alpha smooth muscle actin sections between any NTX treatment group or condition (photographs taken at 20x). Qualitative analysis of Masson’s trichrome shows no difference in collagen density in the dermis or in staining of nuclei in the epithelium between any NTX treatment group or condition (photographs taken at 10x). Qualitative analysis of Sirius red indicates that normal animals show more red fibers and orange/yellow fibers. Diabetic animals show a more significant amount of green fibers, normal animals do not display a large amount of green fibers (photographs taken at 10x). Qualitative analysis of apoptosis via TUNEL stain shows no difference in presence of cells undergoing apoptosis between any NTX treatment group or condition (photographs taken at 20x).
Chapter 5

Discussion

Through this work an optimal dose and route of administration of NTX was determined in the form of a $10^{-5}$M NTX concentration in Neutrogena© moisturizing cream or in KY jelly. This dose in moisturizing cream vehicle increased cell proliferation in the basal layer of skin epithelium by 2.3 times in normal rats and it increased cell proliferation in the basal layer of skin epithelium by 1.9 times in diabetic animals. This dose in KY jelly vehicle increased cell proliferation in the basal layer of skin epithelium by 1.9 times in normal rats and it increased cell proliferation in the basal layer of skin epithelium by 2.5 times in diabetic rats. All topical vehicles + $10^{-5}$M NTX showed a significant increase in basal layer cell proliferation with the exception of Sorenson’s Phosphate buffer + $10^{-5}$M NTX. With moisturizing cream and KY jelly vehicles + $10^{-4}$M, $10^{-5}$M, and $10^{-6}$M NTX concentrations were very statistically different from controls ($p<0.01$).

Previous studies involving tape stripping of mouse tails showed that normal homeostatic basal layer cell proliferation occurred at a rate 7.6% in controls (Wilson et al., 1998). Control labeling indexes of basal layer cell proliferation in this study ranged from 8.0 – 9.5%. This difference can be attributed to the location of skin sections taken, as it has been noted that the timing of peak DNA synthesis and mitotic activity varies according to body location (Potten, 1971). Also the procedures taken to clear hair, and clean the
area of topical administration could have caused irritation or stimulation of increased cell proliferation. Hair clipping may have played a role in stimulating cell proliferation in the basal layer of animals in dose and vehicle determination experiments. The data from dose and vehicle selection experiments show some interesting results because in most cases $10^{-4}$M NTX, $10^{-5}$M NTX and $10^{-6}$M NTX did not statistically differ, in some cases the highest concentration used ($10^{-4}$M NTX) was the least effective at increasing cell proliferation. This may be due to the consistent location of topical treatments on the rats, meaning that perhaps cell proliferation naturally occurs at a lower rate the more cranial the observed section is taken from as suggested in previous studies (Potten, 1972).

$10^{-5}$M NTX and moisturizing cream vehicle applications to full thickness wounds were also shown to increase cell proliferation in the wound site. These increases in normal animals reached over 130% by the fifth day after wound induction. In diabetic rats, $10^{-5}$M NTX and moisturizing cream vehicle increased cell proliferation in the wound site by more than 165% by the fifth day after wound induction. NTX treatments only increased cell proliferation on the edge of the wound site.

Several interesting control experiments included controlling for the effect of Neutrogena moisturizing cream may have had on the rate of wound closure. This was controlled for by using a different brand Oil of Olay moisturizing cream for topical vehicle and it was found that in 7 out of 8 experiments across both normal and diabetic animals there was no significant difference in wound closure rate. Experiments involving Oil of Olay moisturizing cream + $10^{-5}$M NTX only had
a possible n of 4 meaning that in the experiment that showed a difference between moisturizing cream vehicle a single rat could have had any number of uncharacteristic healing processes. Separate from comparative studies between the moisturizing cream vehicles, Oil of Olay + $10^{-5}$M NTX was shown to increase wound closure rate significantly over controls at days 5 and 9 in normal rats and days 5, 7 and 9 in diabetic rats.

Furthermore, experiments showed that $10^{-5}$M NTX in Neutrogena moisturizing cream may be effective at only 1 application per day. Normal animals showed no significant difference in wound closure rate with 1 daily application, 2 daily applications, or 3 daily applications. Diabetic rats also showed that 1 daily application of moisturizing cream + $10^{-5}$M NTX was just as effective if not more effective than 2 or 3 daily applications. This also brings about the possibility of incorporation of the animal’s diurnal rhythm because every animal in this study received topical application at the 0800 hr suggesting that maybe this is the point at which NTX blockade is the most necessary.

In OGF studies there was no significant difference in wound closure rate between moisturizing cream + $10^{-5}$M OGF treated wounds and moisturizing cream control treated wounds. This was an unexpected result because additional OGF peptide should add to endogenous levels of OGF in the skin and prevent wound closure as shown in diabetes. There are several possible reasons for this finding. One, the peptide may be degraded or altered in the moisturizing cream in such a way that prevents it from binding to OGFr or prevents nuclear localization after binding. Another reason may be that the
peptide is removed somewhere in the absorption process from topical vehicle to cell to cytoplasm.

Cell proliferation several grid lengths away from the wound showed no significant increase at day 5 or day 10 in both normal and diabetic animals. This finding is important because it is a testament to the site-specific activity of topical NTX. It has been observed that systemic NTX increases cell proliferation throughout the body due to the fact that OGF and OGFr are present in all tissues of the body. Systemic NTX treatment of full thickness wounds and other site-specific epithelial defects is not an ideal treatment because of the risk of raising cell proliferation levels in tissue to which it may be harmful such as in unknown tumors. Evidence from previous studies has shown that NTX can stimulate cell proliferation in human and animal neuroblastoma and human fibrosarcoma cells in culture (Zagon et al., 1990). Therefore the fact that topical $10^{-5}$M NTX in moisturizing cream and topical $10^{-5}$M NTX in KY jelly have been shown to have a localized activity is truly an important finding.

Wound closure also occurred at an accelerated rate when $10^{-5}$M NTX and either moisturizing cream vehicle or KY jelly vehicle were applied directly to the wound site. In diabetic rats wound closure occurred up to 283% faster as on day 9 and no less than 115% faster on day 3. Though only day 3 wound analysis showed significant improvements in wound closure time in normal animals, this may be due to the overall faster wound closure time that is associated with normal rats in comparison to diabetics. This finding may support the proposed mechanism by which wound healing is disturbed in diabetes, that increased
plasma levels of OGF in diabetic rats can cause disruptions in one or several of the necessary processes in wound closure.

Diabetes leads to poor glucose control and elevated levels of blood glucose. This poor glucose control can lead to a decrease in peripheral circulation attributed to arteriosclerosis, and neuropathic changes causing loss of sensation. This loss of sensation leads to a lack of awareness and proper care of wounds leading further to infection and other disruptions of the normal healing process. These disruptions can lead to poor scar formation and decreased tensile strength (Ehrlich, 2004). As suggested earlier, another problem could be that plasma levels of Met-enkephalin are elevated in human diabetic patients (Negri et al., 1992). Increased levels of OGF in the human cornea have shown that re-epithelialization occurs at a slower rate but that re-epithelialization can be accelerated by blockade of the opioid receptors by NTX (Zagon et al., 1998). Also it was discovered that OGF [Met⁵]-Enkephalin and the zeta opioid receptor are present in both human and mouse skin and act as inhibitors of cell proliferation via decreased DNA synthesis in the epidermis (Zagon et al., 1996). The mechanism for increased [Met⁵]-enkephalin levels as a cause for increases in wound closure time is supported by findings in the previous study because the addition of NTX has increased not only DNA synthesis in the skin epithelium but has also wound closure time. Furthermore, the greatest increases in rate of wound closure were found in diabetic rats, and as evidenced before diabetic animals display an elevated level of plasma [Met⁵]-enkephalin. This means that the disruption of the OGF-OGFr axis can be used to increase wound closure rate
and therefore provide an effective treatment for full-thickness and other types of skin wounds in the diabetic patient without disrupting the replication rates of the cells elsewhere in the body. This means that the same mechanism for accelerated wound repair in the human tissue culture samples (Zagon et al., 1995), rabbit (Zagon et al., 1998) and rat cornea (Zagon et al., 1998) could possibly be applied to full thickness skin wounds. By blocking OGF-OGFr and removing it as a trans-acting factor cell proliferation can occur at a greater rate by decreases in the duration of DNA synthesis and mitotic phases of the cell cycle, which can promote wound repair (Zagon et al., 1998).

Collagen deposition is an important factor in wound healing as well as fibroblast differentiation and presence of myofibroblasts. In order to assess the relative levels of collagen across treatments Masson’s trichrome and Sirius Red staining procedures were used. The results from Masson’s trichrome suggest that collagen deposition was relatively constant across treatment groups in both diabetic and normal animals in this study. However, Sirius Red staining showed an increased number of green fibers indicating that smaller less organized collagen fibers were present in the dermis of the diabetic rats. Since wounding had occurred 20 days prior to tissue harvesting for these sections it is fair to say that this was indicative of newer collagen fibers. In 20 day normal treated and control sections fibers were orange/yellow indicating that these were older fibers probably because of the increased rates that normal skin undergoes during the healing process. Again, normal animals may show increased wound closure rates because of decreased plasma OGF relative to diabetics. Alpha smooth
muscle actin stain was used to show presence of myofibroblasts which contain high levels of alpha smooth muscle actin and are responsible in large part for the contractile activity of the skin upon wound healing. The results of this qualitative study showed fairly consistent presence of myofibroblasts across treatment group in both normal and diabetic rats.

Aside from increasing basal layer cell proliferation and increasing wound closure rates NTX showed no adverse effects throughout any of these studies. Throughout dose and vehicle selection studies there were no observations of redness or irritation of the areas to which topical vehicle and NTX were applied. Animals were not observed to react to topical applications by pawing at them or attempting to remove the topical agents. In wounding studies there was no observed infection of any wounds, this includes no abnormal redness or swelling, no abnormal discharge or signs of increased irritation. There was no observed tampering of the wounds by individual rats or cage mates throughout the wounding studies. There were no qualitative increases in apoptosis, necrosis, lymphocyte infiltration or scar tissue in NTX treated wounds versus control wounds. These observations are consistent with studies done in the eye showing that there are no increases in intraocular pressures, corneal thickness, endothelial cell number or epithelial apoptosis, necrosis or organization reiterating the non-toxic effects of NTX (Zagon et al., 2006).
REFERENCES


