Metabolic Therapy for Age-Dependent Impaired Wound Healing

Shannon Lynn Kesl

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Metabolic Therapy for Age-Dependent Impaired Wound Healing

by

Shannon Lynn Kesl

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Date of Approval:
December 09, 2015

Keywords: Aging, Chronic Wounds, Ketosis, Exogenous Ketone Supplementation

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ACKNOWLEDGEMENTS

The research included in this dissertation was made possible by support from our funding sources, including the James A. Haley Veterans Hospital’s Merit Review, the Department of Molecular Pharmacology and Physiology, Scivation Inc., and the Office of Naval Research (ONR).

I would like to express my heartfelt gratitude to everyone who has supported me in my pursuit of my doctoral degree. Firstly, I want to acknowledge the contribution from all of the ratticans that have sacrificed their lives to further scientific research.

To my mentor, Dr. Dominic D’Agostino thank you for your relentless encouragement, guidance, and always going above and beyond for me no matter what was going on around you. You are one of the hardest working people I know. Thank you for always being just an email or phone call away, and always working diligently through the night for the betterment of our lab, my career, and my life. Thank you for instilling in me your passion and drive for helping others through our research and for helping me to realize why our job as scientists is so important. To my Co-mentor, Dr. Mack Wu, thank you for investing in me when I needed someone and for your continuous direction and support. To Dr. Lisa Gould, thank you for believing in me as your first Ph.D. student, for always being there even when we are far apart, for giving me an opportunity to see the clinical aspect of our research, and especially for challenging me and making me a better scientist.
To the members of my dissertation committee, Dr. Thomas Taylor-Clark, Dr. Paula Bickford, Dr. Kenneth Ugen, Dr. Patrick Bradshaw for their scientific guidance and support over the past five plus years. To my outside chair, Dr. Stanley Stevens Jr., thank you for stepping in last minute and handling everything efficiently. I wish I had gotten to know you earlier in my career, but am hopeful for a continued relationship in the future.

To my lab family, Carol Landon, Dr. Jay Dean, Geoffrey Ciarlone, Jacob Sherwood, Dr. Christopher Rogers, Dr. Csilla Ari, Andrew Koutnik, and Nathan Ward, thank you for making work such an entertaining but reassuring environment even when I needed to order something last minute, we have had a midnight time point, or when I have forced you to listen to musicals. I will be forever grateful for the time we have shared. To Dr. Andrea Trujillo for your continued guidance and support, thank you for teaching me so much. I want to give a special recognition and thank you to my wonderful friend and colleague, Dr. Angela Poff, from my whole heart I wish to thank you for your unwavering love, support, and encouragement, none of this would have been possible without you.

To my school family, Dr. Franklin Poff, Randi McCallian, Dr. Chase Lambert, and Dr. Jillian Whelan, who have always been there with wine, excellent dinners, wine, relaxing company, wine, ridiculous conversation, and wine to ease the stress of this journey. To Shelby Evans and Marilyn Rodriguez, who always listened when I gushed about science even when they didn’t understand and thought it was boring, thank you for being there whether near or far. To Myshell (Michelle) Jung, thank you for being a source of constant inspiration and love even when we didn’t get to see each other every day or get to braid hair during incubation times. To my friends and family who have always loved and encouraged me throughout my life, Beth Webster, Joshua Smith, Katie Rader, Chad Rader, Harper Rader, June Guy, Merle Guy, John
Fry, Michelle Adkins, Kyle Adkins, Travis Adkins, Amanda Fox, James Fox, Keith Waldron, Jeanette Kesl, and Robert Kesl. To my Presley Rader for always being enthusiastic to learn about science, watching your face light up when you looked into a microscope for the first time restored my wonderment for science. To my hippo and chicken, thank you for your unconditional love and for always being there to melt my stress away with snuggles on the couch.

To my best friend, my heart, my husband, Jason, I know it has been hard being apart for the majority of my dissertation work and prep, but know that I felt your unconditional love and support even from Germany. Thank you for the countless times of reassurance, encouragement, laughter, and inspiration. I look forward to many more years of our life together. Last but not least to my mother, Jennifer Fry, thank you for always believing in me and pushing me to achieve my dreams. None of this would have been possible without your continual words of encouragement, your unconditional love, and your unwavering support no matter where life has taken me or how long it has taken me to get there. I am forever grateful.
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### LIST OF ABBREVIATIONS

- \( \alpha \)-SMA: **Alpha-smooth muscle actin**
- \( \beta \)HB: **Beta-hydroxybutyrate**
- AcAc: **Acetoacetate**
- AD: **Alzheimer’s disease**
- ADH: **Aldehyde dehydrogenase**
- ADP: **Adenosine diphosphate**
- ALS: **Amyotrophic lateral sclerosis**
- AMP: **Adenosine monophosphate**
- ARE: **Antioxidant or electrophilic response element**
- ATP: **Adenosine triphosphate**
- BD: **R, S-1, 3-Butanediol**
- BMI: **Body Mass Index**
- BMS: **\( Na^+ / K^+ \) \( \beta \)HB Salt**
- BMS+MCT: **\( Na^+ / K^+ \) \( \beta \)HB Salt: Medium Chain Triglyceride Oil**
- CAT: **Catalase**
- CD31: **Cluster of differentiation 31**
- CD68: **Cluster of differentiation 68**
- CD206: **Cluster of differentiation 206**
CMS ................................................................. Centers for Medicare & Medicaid Services
CNS .................................................................. Central Nervous System
CRP .................................................................. Cysteine-rich protein
CVD .................................................................. Cardiovascular disease
DHE .................................................................. Dihydroethidium
DMEM ........................................................... Dulbecco’s Modified Eagle Medium
ECM .................................................................. Extracellular matrix
EGF .................................................................. Endothelial growth factor
EMT .................................................................. Electromagnetic therapy
eNOS ................................................................. Endothelial nitric oxide synthase
ET-1 .................................................................. Endothelin-1
ETC .................................................................. Electron transport chain
FADH₂ .............................................................. Flavin adenine dinucleotide
FDA ................................................................. US Food and Drug Administration
FGF .................................................................. Fibroblast growth factor
FOXO3A ............................................................ Forkhead box O3a
G6P .................................................................. Glucose-6-phosphate
G6PDH ................................................................ Glucose-6-phosphate dehydrogenase
GC/MS ............................................................. Gas chromatography - mass spectrometry
GI ...................................................................... Gastrointestinal
GLUT .................................................................. Glucose transporter
GNG .................................................................. Gluconeogenesis
GSH .................................................................. Reduced glutathione
GSSG ................................................................. Oxidized glutathione
H$_2$O$_2$ ............................................................... Hydrogen peroxide
HBOT ............................................................... Hyperbaric oxygen therapy
HDAC .............................................................. Histone deacetylase
HDACI ............................................................. Histone deacetylase inhibitor
HDF ................................................................. Primary human dermal fibroblast
HDL ................................................................. High density lipoprotein
HKE ................................................................. High Dose Ketone Ester
I-CAM ............................................................. Intercellular adhesion molecule
IFN-$\gamma$ .......................................................... Interferon gamma
IGF-1 ............................................................... Insulin like growth factor-1
IL-1 ................................................................. Interleukin-1
IL-1$\beta$ ............................................................ Interleukin-1 beta
IL-4 ................................................................. Interleukin-4
IL-6 ................................................................. Interleukin-6
IL-8 ................................................................. Interleukin-8
IL-10 ............................................................... Interleukin-10
IL-13 ............................................................... Interleukin-13
IL-18 ............................................................... Interleukin-18
IRF-5 .............................................................. Interferon regulatory factor-5
KD ................................................................. Ketogenic diet
KE ................................................................. R, S-1, 3-butanediol diacetoacetate ketone ester
KEAP-1 .......................................................... Kelch-like ECH-associated protein -1
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBM</td>
<td>Lean body mass</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography with tandem mass spectrometry</td>
</tr>
<tr>
<td>LCFA</td>
<td>Long chain fatty acid</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LKE</td>
<td>Low dose ketone ester</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M1</td>
<td>Pro-inflammatory macrophage</td>
</tr>
<tr>
<td>M2</td>
<td>Anti-inflammatory macrophage</td>
</tr>
<tr>
<td>MCFA</td>
<td>Medium chain fatty acid</td>
</tr>
<tr>
<td>MCT</td>
<td>Medium chain triglyceride</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>Mg-ATP</td>
<td>Magnesium adenosine triphosphate</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metallopeptidase</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>MRC</td>
<td>Mitochondrial respiratory chain</td>
</tr>
<tr>
<td>MT2</td>
<td>Metallothionein-2</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>NAD+</td>
<td>Oxidized nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NLR family, pyrin domain containing 3</td>
</tr>
</tbody>
</table>
NO ........................................................................................................ Nitric oxide
NOS ........................................................................................................ Nitric oxide synthase
NPWT ........................................................................................................ Negative Pressure Wound Therapy
NQO1 ........................................................................................................ NAD(P)H dehydrogenase [Quinone]-1
Nrf2 ........................................................................................................ Nuclear factor (erythroid-derived 2)-like 2
O$_2^-$ ........................................................................................................ Superoxide anion
PAI-1 ......................................................................................................... Plasminogen activator inhibitor-1
PCOS ........................................................................................................... Poly cystic ovary syndrome
PDGF ......................................................................................................... Platelet-derived growth factor
PDH ............................................................................................................ Pyruvate dehydrogenase
PEM ............................................................................................................ Protein energy malnutrition
PHMB ......................................................................................................... Polyhexamethylene Biguanide
PO2 ............................................................................................................. Partial pressure of oxygen
PMN ............................................................................................................ Polymorphonuclear leukocytes
PPP ............................................................................................................. Pentose phosphate pathway
QUICKI ..................................................................................................... Quantitative insulin-sensitivity check index
RANTES ................................................................................................. Regulated on activation, normal T cell expressed and secreted
ROS ............................................................................................................ Reactive oxygen species
S-BHB ......................................................................................................... Na$^+$/Ca$^{+2}$-β-hydroxybutyrate mineral salt
S/MCT ......................................................................................................... Na$^+$/Ca$^{+2}$-β-hydroxybutyrate mineral salt: Medium Chain Triglyceride Oil
SAD ............................................................................................................. Standard American diet
SD ................................................................................................................ Standard diet
SOD ............................................................................................................. Superoxide dismutase
Skin substitutes
Type 2 diabetes mellitus
Tert-butyl hydrogen peroxide
Traumatic brain injury
Transforming growth factor-beta
Tissue, infection, moisture, and wound edge
Tumor necrosis factor-alpha
Virgin coconut oil
Vascular endothelial growth factor
ABSTRACT

Chronic wounds represent an under-acknowledged socioeconomic epidemic, affecting 1.8 million new patients per year and costing the US health care system upwards of $25 billion annually. This substantial cost is rapidly growing due to a disproportionate occurrence in the ever-aging population. Key features associated with age-related impairment of wound healing include limited energy and nutrient exchange, unremitting inflammations, increased reactive oxygen species (ROS), and diminished blood flow. Most chronic wound therapies target specific molecular mechanisms; however, there are often multiple mitigating factors that prevent normal wound closure. This is likely one reason most wound therapies are minimally effective. In the standard American diet, carbohydrates are broken down for fuel (glucose). While fasting, starvation, and calorie or carbohydrate restriction, beta-oxidation of stored fats in the liver produces ketone bodies (primarily acetoacetate (AcAc) and β-hydroxybutyrate (βHB) to serve as energy metabolites for extra-hepatic tissues. In addition to enhancing metabolic physiology, ketone bodies have recently been discovered to have signaling properties that are independent of their function as energy metabolites. Here we present the evidence for a novel method of inducing therapeutic ketosis via exogenous ketone supplementation to promote enhanced ischemic wound healing in young and aged Fischer 344 rats. Preliminary mechanistic studies demonstrated that exogenous ketone supplementation enhanced wound healing via increasing proliferation and migration, decreasing lactate production, and decreasing ROS production as
well as affecting inflammatory cytokines and growth factors. We conclude that exogenous ketone supplementation will be an effective, cost efficient, low toxicity therapy to promote enhancement of wound healing in an aged population.
CHAPTER 1: WOUND HEALING PHYSIOLOGY

1.1. Chapter Synopsis

The following is a brief review of skin morphology, wound classification and types of wound healing, and the classic wound healing cascade. It is important to understand the complexity of the orchestration in conjunction with the magnitude of participants and the vast number of interactions it takes to heal a wound effectively. Even though most of the time wound healing proceeds to completion without complication, the majority of this chapter addresses what happens when things do not follow the standard progression. The current topical approach to wound healing treatments has failed not only in reversing chronic wound stagnancies but has cost the US alone billions of dollars in health care costs. There is a dire need to discover novel and effective treatments for chronic wounds. The aged population has a high prevalence of chronic wounds due to comorbidities in combination with physiological changes of aging. Increasing evidence shows that unresolved inflammation, increased reactive oxygen species (ROS), diminished blood flow, and decreased metabolism and ATP production are key physiological features associated with age-related impairment of chronic wound healing. In this chapter, we concentrate on the importance of these factors as well as how age affects them in a wound-healing environment. Additionally, these are the four physiological features that we will seek to target in our proposed treatment, which will be discussed in later chapters. A good understanding of the information presented in this chapter will be critical to interpreting the
rationale behind our proposed therapies and the data represented in the subsequent chapters of this dissertation.

1.2. Skin Morphology

The skin is the largest organ in the human body, comprising up to 12-15% of total body weight and covering 1.5-2m² of surface area [1]. There are three distinct layers of the skin: the epidermis, the dermis, and the hypodermis. The epidermis is the avascular outermost layer, primarily populated by keratinocytes, which regulates body temperature, absorbs nutrients, and defends against pathogens. The dermis, the thicker layer deep to the epidermis, contains an extracellular matrix (ECM) that provides tensile strength and flexibility. It also includes fibroblasts, nerve endings, hair follicles, sweat glands, sebaceous glands, apocrine glands, lymphatic vessels, and blood vessels. Though they are only present in the dermis, the blood vessels supply nutrients to both the dermis and the epidermis [2-4]. The deepest layer, the hypodermis, is mainly made up of adipose tissue and serves as an anchor, permitting most areas of the skin to move freely over the deeper tissues [1]. These distinct layers each play a unique role in wound healing and add to the heterogeneity of wound classification.

1.3. Wound Classification and Closure Types

Wound classification takes into account the nature and the severity of the wound to determine an appropriate treatment plan. First, the wound is classified as acute or chronic. Acute wounds achieve sustained restoration of structure and function in an orderly and timely
reparative manner. In contrast, a chronic wound does not proceed through the healing process in a timely fashion and does not advance to closure [5]. Discussion of the classification of chronic wounds and treatment protocols will occur later in this chapter. The rest of this section will focus mostly on acute wound closure.

Initially, acute wounds are classified as being open or closed. Open or penetrating wounds are those with exposed underlying tissue and/or organs. These include stab, gunshot, and surgical wounds. Closed or non-penetrating wounds do not break through the skin. These include abrasions, lacerations, contusions, and concussions. There are also some wounds that do not fit into either of these categories; these include thermal (burns or frostbite) and chemical wounds [6]. Each type of wound has an additional classification for severity. For example, open surgical wounds are further classified into four different classes based on surgical location and level of contamination [6].

Wound healing, or cicatrization, is the process by which tissues restore anatomical structure and function after an injury [7, 8]. Factors that affect the wound closure process include the type of wound, size, depth, location, the age of wound, presence of infection, condition of the patient, and urgency of closure [9]. Wound depth is classified as partial thickness or full thickness. Partial thickness wounds are shallow and involve epidermal loss and partial loss of the dermal layer. Full thickness wounds involve a total loss of the epidermal and dermal layers and extend to at least the subcutaneous tissue layer and possibly as deep as the fascia, muscle layer, and the bone. Partial thickness wounds heal primarily by re-epithelialization from the remaining dermis with minimal scar formation. There are three methods of open wound closure: primary, secondary, and tertiary repair [5, 7, 9]. In primary closure or first intention, the wounds are sealed immediately with simple suturing, skin graft placement, or flap closure. Wound edges
are brought close together, and the wound heals spontaneously with scar formation oriented along the Langer’s lines (collagen fiber alignment within the dermis) [5]. Closure by secondary or spontaneous intention involves no active intent to seal the wound. This type of healing is associated with large, highly contaminated wounds, coupled with extensive tissue loss. They close by the natural wound-healing cascade, which results in contraction and increased scar formation. Failure to heal within a month can lead to a chronic state [5]. Wound closure by tertiary intention or delayed primary closure occurs when wounds are infected or contain foreign debris and cannot be closed until the complications are resolved. A contaminated wound is initially treated by various methods for several days to control infection. Once the wound is ready for closure, surgical intervention allows the wound to heal by first intention. Again, failure to heal within a month can lead to a chronic state. Primary or tertiary wound healing is preferred to secondary since secondary involves a more severe wound contraction and scar formation.

1.4. Acute Wound Healing Cascade

All wounds undergo the same basic steps of repair regardless of tissue type or nature of the injury. Full thickness wounds damage many structures within in the skin. These include epidermal keratinocytes and appendages (sweat glands, sebaceous glands, and hair follicles), the basement membrane, and dermal fibroblasts and appendages (ECM, nerves, and blood vessels) [10]. To heal the gamut of damaged structures, the classic wound-healing model consists of four distinct but overlapping phases: hemostasis, inflammation, proliferation, and maturation [11-14].
1.4.1. Hemostasis

Hemostasis or the coagulation phase is the initiator of healing and occurs immediately following an injury [15, 16]. Ruptured cells and damaged blood vessels activate the blood-clotting cascade, which promotes platelet activation, adhesion, and aggregation at the site of injury to form a fibrin clot. This fibrin clot prevents exsanguination, creates a protective layer to minimize infection and further injury, and provides a provisional matrix of support as the wound heals [3, 7, 16-18]. Activated platelets are a major source of growth factors that play a significant role in the subsequent phases of wound healing [10].

1.4.2. Inflammation

Inflammation, the next phase of wound healing, begins within the first 24 hours of injury and can last up to two weeks in the normal wound healing cascade [16]. Physically, inflammation is associated with signs of redness, swelling, heat, and pain caused by the release of histamine and other active amines by mast cells [16]. The priority of the inflammatory phase is to neutralize infection and prepare the wound bed for repair; therefore, it is not the active stage of wound closure. Nevertheless, it is the most critical phase of the wound-healing cascade. Successful wound repair requires effective and timely resolution of inflammatory responses to activate subsequent steps of the wound healing process. Incomplete resolution of the inflammatory phase leads to a chronic state [8, 19].

The initial vasoconstriction needed to prevent excess blood loss is closely followed by vasodilation and increased capillary permeability. The increased capillary permeability and
chemotactic factors facilitate diapedesis of neutrophils into the wound bed. Likewise, there is an influx of pro-inflammatory cytokines (IL-1, IL-6, IL-8, and TNF-α) and growth factors (PDGF, TGF-β, IGF-1, and FGF) into the wound bed [16]. Polymorphonuclear neutrophils (PMNs) are the most abundant cells in the wound site during the first two days post initial wounding [8]. They generate a respiratory burst to destroy phagocytized bacteria by the release of reactive oxygen species (ROS) [2, 10, 20, 21]. Electrons donated by the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) are transported across the membrane into lysosomes, and superoxide anion (O2⁻) is produced [22]. O2⁻ is bactericidal but is also toxic to neutrophils and surrounding viable tissue if not stringently regulated. In healthy young cells, downstream antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase mitigate ROS production by catalyzing the formation of H₂O₂ [23-25]. Neutrophils utilize myeloperoxidase to combine H₂O₂ with Cl⁻ to form hypochlorite, which plays a supplementary role in eliminating bacteria [3]. Additionally, neutrophils release high levels of proteases (elastase, neutrophil collagenase, and neutrophil collagenase MMP-8) and remove necrotic debris and foreign material [16]. Once the neutrophils complete their function, usually after 2-3 days, they are extruded in the eschar (the crust containing dead cells and degraded products of the wound) or phagocytized by macrophages.

Monocytes from the bloodstream infiltrate into avascular hypoxic areas of the wound site and mature into macrophages by stimulation from pro-inflammatory cytokines produced by the neutrophils [3, 26]. Macrophages in the wound bed can exhibit two distinct functional phenotypes: M1 (classically activated, pro-inflammatory) and M2 (alternatively activated, anti-inflammatory). Early during the inflammation phase, macrophages that are activated by lipopolysaccharide (LPS) or inflammatory cytokines like interferon gamma (IFN-γ) continue the
job of neutrophils by phagocytizing bacteria and damaged tissue. Additionally, the M1 macrophages release pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) [3]. Later during inflammation, initiated by the phagocytosis of apoptotic cells, M1 macrophages phenotypically convert to M2 macrophages [27]. M2 macrophages, activated by interleukin-4 (IL-4) and interleukin-13 (IL-13), play a critical role in the resolution of inflammation. Additionally, they are a predominant component of the transition to the proliferative phase by promoting angiogenesis, tissue remodeling, and repair [3, 28-35]. Lucas et al. demonstrated that a depletion of macrophages during the inflammatory phase significantly delayed wound repair in a mouse model; thus, they demonstrated the critical need for macrophages for wound healing completion [36].

1.4.3. Proliferation

Until now, the wound bed has been prepping for the active repair phase by preventing exsanguination, accomplishing wound debridement, and controlling the bacterial load. Proliferation or the reparative phase is subdivided into different stages including granulation tissue formation, collagen deposition, re-epithelialization, and angiogenesis [7, 15, 16]. The initiation of the proliferative phase is evident by the infiltration of dermal fibroblasts into the wound bed. Fibroblasts in the non-wounded dermis are typically dormant and sparsely populated; therefore, fibroblasts are recruited from neighboring uninjured cutaneous tissues and migrate to the wound bed [16]. A concentration gradient provided by chemotactic growth factors, cytokines, and chemokines in conjunction with the alignment of collagen fibrils in the provisional ECM govern the direction of fibroblast migration [16, 37]. Once the fibroblasts have
reached the wound bed, they anchor in the scaffold of the interim ECM and begin to proliferate and synthesize granulation tissue components such as collagen, elastin, and proteoglycans. The creation of granulation tissue, named for its irregular grainy appearance, slowly replaces the temporary fibrin clot. Initially collagen, predominantly Type III collagen, is laid down in irregular bundles providing moderate stability and tensile strength. In the later part of proliferation, fibroblasts assume the myofibroblast phenotype, expressing α-smooth muscle actin (α-SMA) [2, 38]. The myofibroblast phenotype enables the wound to contract but is dependent upon growth factors PDGF and TGF-β [39].

Simultaneously, the epidermis must replace the initial wound seal, the fibrin clot, with a new epithelial barrier to protect the newly forming ECM. Growth factors released during the healing process stimulate the migration and proliferation of keratinocytes. Actively migrating cells are incapable of proliferating; therefore, the neo-epithelium is created using new cells from the proliferating basal layer of the epithelium near the wound margin. The normal cuboidal basal epithelial cells flatten in shape and begin to migrate as a monolayer over the newly deposited granulation tissue. The epithelial cells move in a tumbling fashion (also known as the epithelial tongue) until the edges establish contact and form a confluent sheet. Once the newly formed monolayer of keratinocytes covers the wound’s surface, migration stops, and the sheet enters a proliferative state to re-establish the stratified layers of the epidermis and restore barrier function. This process has to happen succinctly with ECM foundation or else the wound can easily reopen.

As dermal and epidermal cells migrate and proliferate within the wound bed, there is a substantial need for adequate blood supply for nutrient delivery and gas and metabolite exchange. Due to vascular disruption, ischemia, and high oxygen consumption by metabolically
active cells, the microenvironment of the early wound is hypoxic, oxygen depleted. Compared to normal tissue PO$_2$ levels of 45–50 mm Hg, wound oxygen tension is 6 –7 mm Hg after five days [40]. An inadequately replenished oxygen supply suppresses wound-healing processes; as an example, fibroblasts can survive in a low-oxygen state but cannot replicate or synthesize [16, 41]. Angiogenesis restores tissue nutrient exchange, reestablishes microcirculation, and increases oxygen tension to 30 – 40 mm Hg. Ischemia and hypoxia are critical inducers of angiogenic factors to the wound site [40]. Binding of angiogenic factors such as VEGF, FGF, and TGF-β cause microvascular endothelial cells of the blood vessels next to the wound site to migrate in the same fashion as the fibroblasts. Once in the wound bed, the microvascular endothelial cells proliferate to form buds or sprouts. As sprouts grow and encounter other sprouts, they develop a cleft that subsequently becomes the lumen of the new vessel and complete a new vascular loop. This process continues until the capillary system is sufficiently repaired for nutrient delivery and oxygenation [16]. VEGF is noted to be the most potent angiogenic factor for its effects on multiple components of the angiogenesis cascade: VEGF is an endothelial cell mitogen, chemotactic agent, and inducer of vascular permeability [3, 42]. However, the angiogenic effect of VEGF appears to be dependent on nitric oxide (NO). It has been shown that VEGF increases NO production by up-regulating endothelial NOS (eNOS). Conversely, it has been shown that when blocking eNOS, VEGF angiogenic properties such as endothelial migration and mitogen activities are prevented [43, 44].
1.4.4. Maturation

The maturation or remodeling phase is different from the previous phases in that it does not have a time sensitive pressure and can take from months to years to complete. During this phase cell proliferation slows, protein synthesis decreases, and most endothelial cells, macrophages, and fibroblasts undergo apoptosis or exit the wound. The goal of the remodeling phase is to convert the new ECM, with a tensile strength of only 25-30% compared to normal skin, to the a final scar with maximum tensile strength. Though tensile strength increases, the maximum a wound can achieve is 80% of unwounded skin’s tensile strength. Collagen is remodeled, and Type III collagen that is predominant in the proliferation stage is replaced by stronger Type I collagen. The collagen is further strengthened by cross-links between collagen fibrils. The final result is a scar that is more brittle and less elastic than normal skin. It should be noted that scar tissue lacks the appendages (sweat glands) seen in the undamaged skin. Thus, scar represents wound repair rather than regeneration.

1.5. Cellular Energy Metabolism

Cellular energy metabolism extracts a utilizable form of energy, adenosine triphosphate (ATP) from the nutrition we eat via an elaborate set of biochemical reactions. There are two categories of metabolic pathways: catabolism and anabolism. Catabolism includes the digestion of ingested macromolecules (carbohydrates and fats) into smaller components (sugars and fatty acids), which are either fully metabolized for energy or stored for later use. As a result of these pathways, the energy released (7.3 kCal/mole) from the hydrolysis of the tertiary (ATP to ADP) and secondary (ADP to AMP) phosphate bonds is used to execute all biologically necessary
reactions in the body. In contrast, anabolism uses the newly created ATP to synthesize essential biomolecules such as proteins, lipids, and nucleic acids (DNA and RNA). The ratio between anabolism and catabolism is an important factor in determining wound-healing progression, which will be discussed later in this section.

1.5.1. Catabolism

In a standard American diet (SAD), ingested carbohydrates are metabolized into monosaccharides, predominantly glucose, as the main fuel source for ATP. Glucose is transferred into the cellular cytosol by low affinity, high capacity membrane transporters (GLUTs). While in the cytosol, the process of glycolysis, ten enzymatic steps involving a number of intermediates and specific enzymes, converts the 6-carbon glucose into two molecules of 3-carbon pyruvate. Glycolysis results in the net gain of 2 ATP (2 ATP are invested in reactions 1-5 and 4 ATP are produced in reactions 6-10) and the formation of 2 molecules of NADH, an electron carrier that will eventually play an important role in the production of additional ATP. Glycolysis is the only pathway to produce ATP without the presence of oxygen. The flux through glycolysis is enough to sustain both ATP generation and fueling of the biosynthetic pathways. Additionally, glycolytic intermediates can funnel into multiple biosynthetic pathways. As an example glucose-6-phosphate (G6P) can enter the pentose phosphate pathway, which generates NADPH to maintain antioxidant capacity and nucleic acid synthesis.

The fate of pyruvate is dependent on the cellular oxygen concentration. In the absence of oxygen, lactate dehydrogenase (LDH) converts pyruvate to lactate in a process known as
anaerobic respiration or fermentation. Coupled to this reaction are the formation of another ATP and the oxidation of NADH to NAD\(^+\), which is cycled back to glycolysis. In the presence of oxygen, the cells will use aerobic respiration. The two molecules of pyruvate are shuttled into the mitochondrial matrix where they are oxidized into acetyl-CoA by the pyruvate dehydrogenase complex (PDH). Two additional NADH molecules are created during this step. Acetyl-CoA is joined to oxaloacetate, beginning the Krebs cycle. The Krebs cycle or citric acid cycle completely oxidizes the remaining carbons from glucose, generates additional electron carriers (6 NADH and 2 FADH\(_2\)) to feed the electron transport chain (ETC), and generates 2 ATP. As of now, these metabolic pathways have synthesized a net gain of only 4 ATP, 10 NADH, and 2 FADH\(_2\). The electron carriers are shuttled to ETC, in the inner mitochondrial membrane for oxidative phosphorylation. NADH and FADH\(_2\) are oxidized by the components of the ETC and their electrons are passed between the ETC complexes in a series of oxidation/reduction reactions. The final electron acceptor of the ETC is molecular oxygen, which reduces to H\(_2\)O. During the transfer of electrons through the ETC, H\(^+\) protons are pumped into the inner mitochondrial membrane space creating an electrochemical gradient. This electrochemical gradient drives the proton motive force used to synthesize ATP. Thus, the ETC generates 32-34 ATP per glucose molecule (3 ATP per NADH and 2 ATP per FADH\(_2\)).

Dietary fats and stored fats are both metabolized via lipolysis to release their glycerol backbone and their fatty acid side chains. The free fatty acids undergo \(\beta\)-oxidation to produce acetyl-CoA. The metabolic fate of acetyl-CoA is the same as the previously described steps of aerobic respiration from glucose. Each round of the \(\beta\)-oxidation cycle reduces the length of the free fatty acid’s aliphatic tail by two carbon atoms and produces one NADH and one FADH\(_2\). This process continues until the entire chain is cleaved into acetyl-CoA units. The ATP yield for
every β-oxidation cycle is 17 ATP (3 per NADH, 2 per FADH$_2$, and 12 for the acetyl-CoA/Krebs cycle). Thus, the total ATP yield will depend on chain length. Two ATP are required to activate the fatty acid; hence a net ATP yield for palmitic acid (C16:0) is 113 ATP.

1.5.2. Anabolism

Anabolic or biosynthetic pathways utilize the newly created energy to fuel the creation of necessary biomolecules. These include glycogenesis (glycogen synthesis), gluconeogenesis (glucose synthesis), protein synthesis, and the pentose phosphate pathway. The main biosynthetic pathways to focus on for this dissertation are the pentose phosphate pathway (PPP) and gluconeogenesis. As mentioned previously, the first step of glycolysis produces glucose 6-phosphate (G6P), which can be shuttled to the PPP, an anabolic pathway parallel to glycolysis that generates the reducing agent NADPH and synthesizes 5-carbon sugars. The PPP accounts for approximately 60% of NADPH production in the cells. It is important to note that NADPH is the reducing agent consumed during biosynthetic reactions, whereas NADH is a reducing agent generated in energy-yielding reactions. NADPH is used for fatty acid synthesis and helps fight oxidative stress by reducing the antioxidant glutathione via glutathione reductase. The ribose sugars generated from the PPP are utilized in the synthesis of nucleotides and nucleic acids.

Gluconeogenesis (GNG) is a series of eleven enzyme-catalyzed reactions to synthesize glucose from non-carbohydrate sources including pyruvate, amino acids (alanine and glutamine), lactate, and glycerol. The system is a reciprocal of glycolysis, which together creates a feedback mechanism to normalize and maintain blood glucose levels. While most steps in gluconeogenesis
are the exact reverse of glycolysis, there are three reactions that require different catalytic enzymes to make them more kinetically favorable.

In general, normal metabolism is hormone-mediated to alter energy production to meet energy need and to restore daily macronutrient balance, especially proteins, through the catabolic and anabolic pathways [45-50]. This balanced metabolic profile significantly changes in the presence of a wound.

1.6. Wound Healing Metabolism

The presence of any substantial wound increases metabolic energy demands from 30-50% above normal requirements to produce the necessary biomolecules for increased cellular proliferation and migration of the various cell types during wound healing [17, 51]. Cuthbertson famously described the metabolic response to injury as having an “ebb” phase, the period of traumatic shock or hypometabolism during the first few hours or days after injury and a “flow” or hypermetabolic phase (proliferation) that may last weeks to months depending on the complexity of the wound [17, 52, 53].

It has been well established that non-wounded epidermal cells, as well as wounded skin, are mainly dependent upon carbohydrate metabolism via glycolysis and the PPP rather than the Krebs cycle for their energy requirement [54-59]. In a hallmark study, Im et al. showed a 4-fold increase of glycolytic enzymes of wounded skin compared to normal skin [56]. The contribution of the Krebs cycle to glucose metabolism was reduced in wounded skin compared to normal skin (8% to 4%) demonstrating a metabolic shift to the PPP and other biosynthetic pathways, needed
for repairing the wound. This metabolic shift towards glycolysis was confirmed by the observations that the rate of nucleotide formation was higher in wounded skin compared to normal skin and a 7-fold increase in the PPP enzymatic activities. Furthermore, glucose-6-phosphate dehydrogenase (G6PDH) was increased 7-fold, corresponding to the increased contribution to the PPP [56]. Additionally, there was evidence that lactate production was tripled in day three post-wounding, further confirming the higher flux through glycolysis and subsequently anaerobic fermentation. Even though glycolysis is less efficient at creating ATP compared to flux through the TCA cycle, the rate of ATP formation was doubled after three days of wound healing. The ATP production was seen as a gradient with higher concentrations of ATP in the migrating epithelium compared to the marginal proliferating epithelium. The study showed a decrease in energy production per unit of glucose in wounded skin compared to normal skin, as would be expected in the switch to glycolytic metabolism compared to aerobic respirations. The inefficiency of glycolysis forming ATP compared to the ETC is overcome by the increased metabolic rate of wounded skin. It has been shown that elevated activities of glycolytic enzymes may contribute to increased cellular demand for energy during healing of acute wounds [51, 59, 60].

1.7. Nutrition and Wound Healing

Nutritional status is extremely critical for effective wound healing [61-81]. As mentioned earlier in this chapter, maintenance of a balanced anabolism: catabolism ratio is fundamental to optimizing the wound healing process. Since healing a wound is crucial to surviving, a wound requires high priority of the circulating nutrients in the blood stream. Increased macronutrient
intake is required to keep up with the catabolic losses and to allow wound-healing processes. The American Society for Parenteral and Enteral Nutrition and Wound Healing recommends approximately 30 to 35 kcal/kg/d for optimal wound healing, which is roughly 1.2-1.5 times more than a non-wounded patient would ingest [82]. These macronutrients are predominantly used as fuel; however, they also have direct effects essential for healing [64]. It should be noted that individual energy needs depend on age, gender, nutritional status, basal metabolic rate, body mass index (BMI), comorbid conditions, activity level, the stress of illness, severity and number of wounds, and wound size of that patient, which supports the heterogeneous classification of wounds [17, 83].

Macronutrients (fats, proteins, and carbohydrates) provide the energy for all body functions and are major building blocks for the reparative process. In a healing wound, the influx of inflammatory cells, as well as fibroblasts, use carbohydrates as an energy source. Additionally carbohydrates have been shown to have a variety of non-energy related roles (structural, lubricant, immunologic, hormonal, and enzymatic) [17]. Fats and their derived lipid components provide the building blocks for the cell membrane and certain inflammatory mediators, behave as signaling molecules, control tissue growth, wound remodeling, and collagen and extracellular matrix production [84-91]. Proteins are predominantly metabolized into amino acids and peptides for the purpose of protein synthesis; only 5% of protein metabolism is used for energy [83]. Protein derived amino acids provide the major structural building blocks of all proteins in the body, notably collagen. Amino acids are necessary for the cell membrane, enzymes, and cytokine production in the healing wound [64].

When dietary nutrients are not adequate, or if the catabolic state outweighs the anabolic state, the wound can proficiently use substrates available from the rest of the body for energy
production, resulting in what is called protein-energy malnutrition (PEM). The body catabolizes the muscle, skin, and bone to support the synthesis of proteins, inflammatory cells, and collagen needed to fight infection and repair the wound, causing a loss of lean body mass (LBM). As an individual loses more LBM, wound healing is more likely to be delayed. A loss of more than 15% LBM impairs wound healing, a 30% LBM loss stops wound healing, and a loss of 40% LBM typically results in death [64, 92-95]. As will be important later, a body in starvation (ketosis) has been shown to be muscle sparing, attenuating the catabolic effects [83]. LBM loss becomes vital in older adults as many suffer from pre-existing malnutrition and PEM.

1.8. Chronic Wounds

Chronic wounds represent an under-acknowledged socioeconomic epidemic, affecting 1.8 million new patients per year in the US. [96]. The care of these chronic wounds costs the US health care system at least $25 billion annually. This substantial cost is rapidly growing due to increased healthcare costs, an aging population, an increase in diabetes and obesity, and the expanding need for wound care for veterans [96-98]. As more of our population ages (>65 years), projected to be 20% of the population by 2030, there is a critical need to develop effective wound healing treatments to alleviate the considerable medical, social, and economic burden facing the country [99].
1.8.1. Classification of Chronic Wounds

Chronic wounds are defined as wounds that have failed to proceed through and orderly and timely reparative process to produce anatomic and functional integrity. All wound types have the potential to become chronic, and as such, chronic wounds are traditionally divided by etiology. A prolonged inflammatory stage, persistent infections, drug-resistant microbial biofilms, and the inability of cells to respond to reparative signals are pathophysiological features of all chronic wounds [8, 100, 101]. The Wound Healing Society’s Chronic Wound Care Guidelines divide chronic wound into four different phenotypes: vascular ulcers (venous and arterial), pressure ulcers, and diabetic ulcers.

The venous ulcer accounts for more than half of ulcer cases and 70-90% of ulcers found on the lower limbs [102]. Venous ulcers affect approximately 1.69% of older adults or 600,000 patients total in the US annually [96, 103]. They are associated with deep vein thrombosis, varicose veins, and venous hypertension. Physically, venous ulcers have skin that is shiny, smooth, with minimal to no hair, are superficial, shallow, irregularly shaped, and are accompanied by pain and edema. Less common than venous ulcers, arterial ulcers affect approximately 100,000 Americans annually [104]. Arterial ulcers occur from hypertension, atherosclerosis, and thrombosis, where the reduced blood supply leads to an ischemic state. Commonly, they are full thickness wounds with a punched out appearance and smooth edges. As compared to the venous ulcers that have prevalence on the lower limbs (between the knee and ankle), arterial ulcers usually occur more distal such as the tip of the toe and over distal bony prominences.
Pressure ulcers, also known as bedsores, are a result of prolonged pressure, friction and shear from body weight and usually occur at a bony prominence such as the sacrum or heel. Clinically, they present with redness, itching, blistering, warmth, swelling, and discoloration of the overlying skin. They are prevalent in any patient that is wheelchair bound, bedridden, or suffering from immobility or sensory neuropathies [105]. 2.5 million pressure ulcers are treated in the US per year [106]. The cost of treating one pressure ulcer is estimated to be as much as $70,000, totaling to an estimated $11 billion a year for pressure ulcer care [107, 108]. About 15% of patients in an acute care facility and up to 29% of patients in long-term care will experience a pressure ulcer [96]. According to the Centers for Medicare & Medicaid Services (CMS), there were a staggering 257, 412 preventable pressure ulcers in 2007 [109]. Additionally, pressure ulcers can be a major source of infection, leading to complications and even death. Development of a pressure ulcer increases mortality rate by 7.23% [110]. Over the last ten years nearly 60,000 deaths occurred annually from hospital-acquired pressure ulcers [96]. Even though many resources have been devoted to rectify these overwhelming statistics, it is still extremely challenging and costly to prevent pressure ulcers [111-114].

As of October 2014, 29.1 million people in the United States have diabetes mellitus of which 8.1 million people are undiagnosed [115]. Diabetic ulcers are a common complication in uncontrolled diabetes, resulting from impaired immune function, ischemia (due to poor blood circulation from high glucose content as well as vascular insufficiencies), and neuropathy. It is estimated that up to 25% of all diabetics will develop an ulcer in their lifetime [116]. In 2007, a single foot ulcer cost between $7,439 and $20,622, totaling to a $9 billion expense per year [117].
1.8.2. Current Standard of Care, Alternative Therapies, and Their Disadvantages

Identifying and treating the underlying etiology of chronic wounds such as venous insufficiency, arterial perfusion, diabetes, or continuous pressure as well as systemic factors such as age, nutritional status, immunosuppression, and infection is key to successful wound healing therapies. However, the establishment of effective wound healing therapies has been unsuccessful due to the magnitude of these underlying pathogenic factors and the heterogeneity across and within wound types [118]. Furthermore, it is important to emphasize that no two wounds are exactly the same; thus, a treatment that could work for one patient may not be effective for another.

Most current wound therapies being studied target a specific molecular or cellular mechanism where there are often multiple mitigating factors that prevent normal wound closure. This is likely one reason most wound therapies are minimally effective. Many growth factor supplements tested in animal models appeared to be promising therapeutic agents promoting wound healing [119]; however, human clinical trials have not been very successful [120, 121]. Additionally, there is a multitude of topical therapies (antimicrobials, dressings, silver-containing products) that are commercially available, with minimal data to support their effectiveness in promoting wound healing [122].

*In vitro* models have aided in the understanding of basic biology of wound healing; however, more complex models to simulate the wound environment (multiple cell types) in conjunction with comorbid conditions have not been developed. Many studies in wound repair have relied on rodent models; however, skin morphology and the mechanisms of repair are markedly different between rodents and humans [118, 123]. Additionally, some pre-clinical studies have used acute
wounds in young animals and not appropriately aged animals, demonstrating that there are no animal models that comprehensively mimic human chronic wounds [124].

1.8.2.1. TIME and Amputation

Standard clinical therapies for non-healing wounds use the wound bed preparation concept, which focuses on optimizing the condition of the wound bed to encourage normal healing. In 2002, the acronym TIME was developed as a practical guideline for wound management. TIME encompasses four main components of wound bed preparation: Tissue management (debridement), control of Infection and inflammation, manage Moisture, and the Edge of wound advancement [125, 126].

Debridement is the surgical removal of necrotic, damaged, or infected tissue, biofilms, exudate, and debris to improve the healing of the remaining healthy tissue [127-132]. The goal of debridement is to transition an excessively inflamed chronic wound to more of an acute profile, promoting resolution of inflammation [122, 133, 134]. Chronic wounds are well characterized as being stuck in the inflammatory phase with a marked increase in degrading proteases such as matrix metalloproteinases (MMPs) and elastases and persistence of inflammatory cells [126, 129, 135-137]. Resolution of prolonged inflammation renews tissue healing, lessens exudate, and reduces bioburden. It is important to distinguish between normal inflammation associated with physiological healing compared to excessive inflammation caused by underlying adverse etiologies and infection. Inflammation and infection are controlled using antimicrobials (disinfectants, antiseptics, and antibiotics) in a variety of applications including silver dressings, iodine dressings, PHMB dressings, honey, and surfactants [126]. Balanced moisture is essential
for the action of growth factors, cytokines, and cell migration. Excessive moisture damages surrounding skin and insufficient moisture inhibits cellular activities and leads to eschar (scab) formation [137]. Wound edge advancement assessment determines if wound contraction or epithelialization is progressing and evaluates the effectiveness of the wound treatment. An increasing range of treatment modalities has been proposed to improve wound edge advancement including electromagnetic therapy (EMT), laser therapy, ultrasound therapy, systemic oxygen therapy, and negative pressure wound therapy (NPWT) [126].

Amputation is the final and last course of action against chronic wounds (when they occur on an extremity). This is done to prevent the spread of infection to healthy tissue. Amputations are classified as either major (an amputation of the leg above or below the knee) or minor (amputation of toes or forefoot) [138]. Many diabetic patients eventually must undergo lower extremity amputations as a result of infection brought on by untreated foot ulcers. Diabetic foot ulcers are responsible for 20% of diabetes-related hospitalization and underlie up to 80% of non-traumatic amputations per year. It is estimated that 12% of individuals with a foot ulcer will require amputation. Mortality following amputation is 13-40% at one year, 35-65% at three years, and 39-80% at five years, which is worse than most malignancies. Once amputation occurs, 50% of patients will develop an ulcer in the contralateral limb within five years [117].

1.8.2.2. Adjunctive Therapies

Even with the standard care practices of wound healing, there is a multitude of treatment options and modalities. As previously mentioned, most of these treatments have limited research data to prove effectiveness and many have controversial clinical results. Adjunctive treatments
for wound healing include negative pressure therapy, hyperbaric oxygen therapy (HBOT),
dermal substitutes, growth factor supplementation therapy, and stem cell therapy [139, 140].

Negative Pressure Wound Therapy (NPWT), vacuum-assisted wound therapy or
commonly known as a wound VAC®, has become a popular adjunctive therapy[141-143].
Negative pressure is applied to the wound via foam or gauze dressing moistened with saline,
which is then covered with a transparent adhesive sheet attached to a vacuum pump. NPWT has
been shown to manage wound drainage, reduce edema, reduce the bio-burden of
microorganisms, increase wound perfusion, and loosen the exudate [126]. This therapy in
combination with debridement has been shown to promote granulation tissue formation,
contraction and epithelialization [135]. However, a systematic review and meta-analysis of 21
studies found no clear evidence that wounds heal either better or worse with NPWT compared to
standard of care [144]. Additionally, the mechanism of action for NPWT is poorly understood,
and few studies have focused on the efficacy of this therapy in older adults [118].

Hyperbaric oxygen therapy (HBOT), originally utilized for decompression illness, has
been an adjunctive therapy for wound healing for more than two decades. It involves placing a
patient in a sealed chamber where 100% oxygen is pressurized to 1.5-3 atmospheres absolute
(ATA) for 60-120 minutes over a course of multiple treatments [139]. There isn’t an optimized
protocol for treatment and the mechanism of action for HBOT to improve wound healing is
poorly understood. However, it is speculated that HBOT helps reduce edema via
vasoconstriction and fluid absorption, increases oxygen perfusion aiding collagen elongation and
deposition, and stimulates epithelial progenitor cells and stem cell release for vasculogenesis
[139]. Published systematic reviews have reported mix results regarding the efficacy of HBOT,
as observational studies have shown a significant advantage of HBOT for wound healing;
however, six randomized trials did not show a benefit [145]. There are some promising animal data suggesting that HBOT is effective for all ages; however, there are no studies specifically focused on the efficacy of HBOT in older adults [146, 147].

Both biosynthetic skin substitutes (synthetic) and cultured autologous-engineered skin (natural) are available to provide the protective barrier of skin when placed over a wound [148-154]. The synthetic substitutes are made of an acellular material, and the natural substitutes consist of cultures allogeneic or autologous cell suspensions or sheets used alone or with a dermal matrix. The purpose of engineered skin products is to expedite wound healing by serving as a scaffold for cellular ingrowth, stimulating the release of growth factors from the wound bed, and restoring the functional properties of the skin [154, 155]. The skin substitutes (SS) can be either permanent or temporary, depending on design and composition. It’s important to note that although each of skin substitutes has their advantages and have applications, none of them can fully simulate native skin. Both types of SS have been shown to be quite effective in increasing the speed of wound healing, decreasing recurrence, reducing the number of required surgical procedures, and reducing patient hospitalization time [148, 153, 155, 156]. Nevertheless, the commercially available skin substitutes have several limitations such as reduced vascularization, scarring, failure to integrate, poor mechanical integrity, and immune rejection. Furthermore, the cost associated with the use of the current skin substitutes is very high. For example, it is estimated that the cost for each 1% body surface areas covered by the commercially available Epicel ™ is more than $13,000 [157]. As it relates to this project, the effect of the patient age on skin substitutes is poorly characterized [118].

It is well characterized that growth factors play a substantial role in both healing and non-healing wounds. Wound healing products have been designed to accelerate repair by augmenting
or modulating these growth factors in hopes of mediating inflammation and promoting angiogenesis. Among all the possible growth factors, platelet-derived growth factor (PDGF) has been approved by the Food and Drug Administration (FDA) for the treatment of diabetic foot ulcers [4]. PDGF has been shown to stimulate granulation, increased odds of wound healing and decrease rates of amputations in diabetic patients [158-165]. Growth factors EGF and FGF have also been studied as topical wound healing therapies; however, the heterogeneity of wounds, applications, patient management protocols, and clinical trial outcomes has limited their use to outside of the USA [122].

Stem cells have the unique capability to differentiate into the variety of tissues in the body. Stem cells derived from various sources including bone marrow, adipose, peripheral and umbilical blood, in addition to vascular progenitor cells have been shown to migrate towards the wound site and contribute to the wound healing process [166-168]. These cells have been shown to increase angiogenesis, increase granular tissue formation, enhance wound healing, and reduce scarring [166, 168-172]. Even though stem cell research seems promising, the use of stem cells is hampered by immunogenicity, potential for malignancy, pluripotency maintenance, ethical consideration, and limited supply [118]. Additionally, harvesting bone marrow derived stem cells is painful and leads to donor site morbidity [173].

The field of wound care is ever expanding with advances in technology. However, there is still no superior substitute to allowing the body to heal systemically on its own accord in conjunction with reconstruction using patients’ own tissues and carefully thought-out surgical procedures. The therapy proposed in the next chapter of this dissertation focuses on exploiting the body’s systemic physiology to enhance wound healing.
1.9. Age Influence on Chronic Wounds

Chronic wounds disproportionally affect older adults. 72% of hospitalized patients with pressure ulcers were 65 and older. Most chronic wounds are associated with co-morbid conditions prevalent in older adults, including cardiovascular disease, unrelieved pressure, obesity, or diabetes mellitus [96]. However, Wicke and associates in 2009 demonstrated that age was an independent risk factor for delayed wound closure. By the age of 70, the frequency of wound closure was significantly lower than the younger counterparts [174]. It should be noted that the effect of aging on wound repair is primarily a temporal delay; a change in the quality of wound closure has not been reported [175]. The exact mechanisms responsible for this increased risk remain unknown. In this dissertation we focus on four physiological attributes that we feel lead to the prevalence of chronic wounds in older adults: unresolved inflammation, unmitigated ROS production, decreased angiogenesis/blood flow, and decreased metabolism.

1.9.1. Age-Related Changes in Skin Morphology

Though the skin is incredibly durable, like all other systems, it eventually succumbs to the inevitable effects of aging. The skin is also the most visible indicator of age. Clinically, drying, roughness, sagging, wrinkling, and alterations in pigmentation characterize aged skin. Physiological changes in the skin include both structural and biochemical changes. Morphologically, the skin is characterized by atrophy due to the flattening of the epidermis/dermis junction and a decrease of epidermal and dermal cellular content, collagen
disorganization, a decrease in microcirculation, and an increase in time for keratinocyte maturation [4, 176-179]. The thickness of the skin decreases about 6.4% per decade on average. Keratinocytes change shape as the skin ages, becoming shorter and fatter while corneocytes become bigger as a result of decreased epidermal turnover. The cellular content of the dermis (fibroblasts, mast cells, and macrophages) and protein content (collagen) are reduced with age. The collagen and elastin that remain in the dermis are less organized, resulting in a decreased elasticity of the skin [180]. Enzymatically active melanocytes decrease at a rate of 8-20% per decade, resulting in the irregular pigmentation of elderly skin. Alterations in the aging skin not only have an impact on wound healing but also make the skin more susceptible to injury. The flattening of the epidermis/dermis junction results in less resistance to shearing forces and increased vulnerability to insult as well as increased separation.

1.9.2. Age-Related Changes in the Wound Healing Cascade

Aging affects every phase of wound healing [176]. It has been demonstrated that aged wounds have decreased levels and response to growth factors, reduced microcirculation, diminished cell proliferation and migration, and diminished ECM secretion [181-183]. Aging is also associated with delays in macrophage and T cell infiltration, angiogenesis, and re-epithelialization [118]. It has been hypothesized that age-associated disadvantages in healing may arise from overexpression of matrix metalloproteinases, which normally aid in phagocytosis, angiogenesis, cell migration during epidermal restoration, and tissue remodeling. Protease expression and activity are elevated in older human adults, which leads to the perpetuation of the pro-inflammatory state and inhibits progression to the reparative phase [184].
Inflammation is a major contributing factor in wound chronicity and the aging phenotype. As previously mentioned, chronic wounds are characterized as being stuck in the inflammatory phase. Without resolution, the wound cannot advance to the reparative phase. This inability to resolve inflammation is believed to be the most significant delaying factor in the healing of chronic wounds [185]. It has been established that aged individuals have a consistent low-grade inflammation marked by elevated cytokines like TNF-α, IL-6, and CRP, even when the individual is healthy, or infection is absent [186]. This is in direct contrast to young individuals, who only produce elevated levels of cytokines in response to injury or infection. This low-grade inflammation has been implicated in the higher prevalence of co-morbidities such as cardiovascular disease and diabetes in older adults. It has been suggested that inflammation may be a common underlying cause of several age-related diseases or could be a common pathway by which multiple diseases lead to disability and adverse outcomes in older adults [186]. This already heightened inflammatory response is worsened in the presence of a wound. It has been shown that there are increased numbers of neutrophils with prolongation of the pro-inflammatory state as well as a delay in macrophage recruitment [185]. Additionally, a study by Mahbub and colleagues demonstrated that M1 macrophages fail to transition to the M2 phenotype, perpetuating the inflamed state in an aged population [187].

ROS are produced in the healing wound as a bactericidal tool; however, when the levels are not quenched by downstream antioxidants elevated ROS levels transcend the beneficial effect and cause mitochondrial dysfunction, DNA damage, lipid peroxidation, and even cell death [188, 189]. Additionally, ROS are produced as a normal by-product of oxidative phosphorylation; thus in a wound environment cells are hit with ROS from neutrophilic respiratory bursts as well as from enhanced energy metabolism necessary to heal the wound [23, 190, 191]. The energy
metabolism shift to anaerobic respiration during wound healing likely protects the mitochondria from over usage and damage related to ROS production [55]. Skin mitochondria in the human aged population demonstrated an increased incidence of mitochondrial DNA (mtDNA) mutations leading to mitochondrial dysfunction [192, 193]. Electron microscope images of aged mitochondria show disorganization and degeneration via absent cristae, vacuolation, and swelling [194]. Additionally, it has been determined that both superoxide anion and H$_2$O$_2$ are increased in aged dermal fibroblasts [195]. Several studies have shown that the aged phenotype has decreased levels of antioxidants to quench the increased ROS [196-199]. Treiber et al. demonstrated that the skin of manganese superoxide dismutase (MnSOD, SOD2) deficient mice was phenotypically similar to aged animals with characteristics such as atrophy of dermal connective tissue, muscle fibers, and subcutaneous fat, loosely packaged collagen fibrils, damaged mitochondria, and decreased cellular proliferation [200].

It has been demonstrated that angiogenesis is decreased with age because of reduced levels of the angiogenic factors: FGF, VEGF, and TGF-β, impaired vasodilation, proliferation and migration of angiogenic, microvascular endothelial cells. It is important to note that the coexistence of hypertension, diabetes, smoking, and hypercholesterolemia exacerbates the inherent effects of aging that are detrimental to angiogenesis [176, 201].

The metabolic challenge of a wound is exacerbated in elderly patients, who have a higher prevalence of malnutrition combined with reduced total energy expenditure, basal metabolic rate, and protein synthesis [17]. Without appropriate energy (ATP) levels, wound healing is significantly impaired. The ATP deficiency creates an imbalance of energy production versus utilization. To compensate, anaerobic respiration increases in the wound bed, which only produces 2 ATP per glucose molecule as compared to the potential 38 ATP made from normal
aerobic respiration [41]. Gupta et al. showed that key metabolic enzymes were decreased in aged rats [51]. It has been illustrated that mitochondria become larger and less numerous with age, mitochondrial respiratory chain (MRC) enzyme activities decrease as well as mitochondrial membrane potential [202]. Furthermore, there is a significant reduction in mitochondrial bioenergetic capacity with advancing age, which has been shown in numerous animal models and recently in a study with human volunteers [202, 203].

1.10. Closing Remarks

In summary, there is come controversy regarding whether chronological age alone affects wound healing. However, it is well documented that there is increased ROS production with age, leading to cellular damage, reduced blood flow leading to the lessening of nutrient exchange, decreased metabolic activity and ATP production at the wound bed, and unresolved chronic inflammation. It is easily deduced how these factors may lead to increased prevalence of chronic wounds in older adults. Most approaches to accelerate wound healing focus on delivery of growth factors by topical application. The discouraging results of focusing on single molecular targets are not surprising since wound healing is the product of a complex set of interactions between numerous factors. It is here that we aim to switch the approach to optimizing the body’s physiology to reach the wound bed systemically as well as targeting multiple facets of impaired wound healing with one therapy- exogenous ketone supplementation, which will be discussed further in the next chapter.
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CHAPTER 2: ORAL KETONE SUPPLEMENTATION AS A NOVEL THERAPEUTIC FOR DELAYED WOUND HEALING

2.1. Chapter Synopsis

In the previous chapter, I provided a thorough overview of chronic wounds and the non-efficacious therapies that are currently available, especially for the aged population. Here I provide a rationalization for the use of an oral exogenous ketone supplement as a novel therapy for wound healing. The ketogenic diet has been used since the 1920s for refractory epilepsy in children. Though it has been proven to be effective, it is hard to maintain and is limited in a clinical setting. Recently, it has been shown that ketone bodies themselves may confer therapeutic benefits, paving the way for an exogenous ketone supplement that does not require dietary restriction. Ketones have been shown to decrease inflammation, decrease ROS levels, increase blood flow, and increase ATP hydrolysis and increase metabolism. As previously described, these are the four underlying age-dependent features we will utilize to determine the efficacy of our treatment. For these reasons, we hypothesize that exogenous ketone supplementation will augment wound healing and we propose to test their effects in vivo using an ischemic wound model in young and aged Fischer 344 rats and in vitro using patient-derived primary human dermal fibroblasts isolated from discarded skin.

Portions of this chapter have previously been published in “Kesl SL, Poff AM, Ward NP,
2.2. Ketone Body Synthesis and Metabolism

Ketone bodies are naturally elevated to serve as alternative metabolic substrates for extra-hepatic tissues during the prolonged reduction of glucose availability, suppression of insulin, and depletion of liver glycogen, such as occurs during starvation, fasting, vigorous exercise, calorie restriction, or the ketogenic diet (KD). As blood glucose levels drop, the body goes through a metabolic shift from glucose-based metabolism towards fatty acid oxidation and hepatic ketogenesis to generate ATP. As a result, hepatic cells synthesize necessary glucose through gluconeogenesis (GNG) and β-oxidation metabolizes fats (dietary and stored) to form Acetyl CoA. During GNG, the oxaloacetate stores are depleted, causing the Acetyl CoA from fatty acid oxidation to accumulate and not enter the Krebs cycle. The excess Acetyl CoA produces the ketone bodies beta-hydroxybutyrate (βHB) and acetoacetate (AcAc) via a 3-step enzymatic process known as ketogenesis. From the liver, these substrates are transported
systemically to extra-hepatic tissues (i.e. muscle, brain, and skin) where Acetyl CoA is reconstructed and used to generate ATP via the Krebs cycle and the ETC [1, 2]. Additionally, AcAc can be decarboxylated to produce the third ketone body, acetone. Although acetone has been considered mainly to be a metabolic by-product that is eliminated via the lungs and urine, it has recently been shown to play an important role in the anticonvulsant properties of the ketogenic diet [3-7].

2.3. Review of Ketogenic Diet and Therapeutic Uses

The classical ketogenic diet (KD) consists of a 4:1 ratio of fat to protein and carbohydrate combined, with 80-90% of total calories derived from fat [8]. For most patients this equates to eating less than 50 grams of carbohydrates and around 100 grams of protein a day [9]. Actual carbohydrate and protein intake must be individually optimized to maintain therapeutic ketone production. The macronutrient ratio of the KD induces the metabolic shift towards hepatic ketogenesis, elevating AcAc and βHB in the blood. Compared to other low carb diets like the Atkins diet, the ketogenic diet requires only adequate protein. Excess protein intake can fuel GNG, resulting in glucose synthesis that disables ketogenesis. It is important to emphasize that not all low carbohydrate diets are ketogenic. The ketogenic diet is really a high-fat diet, and not a high-protein diet.

Emerging evidence supports the therapeutic potential of the ketogenic diet (KD) for a variety of disease states, leading investigators to research methods of harnessing the benefits of nutritional ketosis without the dietary restrictions. The KD has been used as an effective non-pharmacological therapy for pediatric intractable seizures since the 1920s [10-12]. In a study of
150 epileptic children who averaged 400 seizures per month and were on multiple anti-epileptic medications, 60% reported a significant decrease in seizure frequency [12]. In addition to epilepsy, the ketogenic diet has elicited significant therapeutic effects for weight loss and type-2 diabetes (T2D) [13]. Several studies have shown significant weight loss on a high fat, low carbohydrate diet without significant elevations of serum cholesterol [14-21]. Another study demonstrated the safety and benefits of long-term application of the KD in T2D patients. Patients exhibited significant weight loss, reduction of blood glucose, and improvement of lipid markers after eating a well-formulated KD for 56 weeks [22]. Recently, researchers have begun to investigate the use of the KD as a treatment for acne, polycystic ovary syndrome (PCOS), cancer, amyotrophic lateral sclerosis (ALS), traumatic brain injury (TBI) and Alzheimer’s disease (AD) with promising preliminary results [23-35].

2.3.1. Potential Concerns and Side Effects

Although the KD has clear therapeutic potential, several factors limit the efficacy and clinical utility of the KD for metabolic therapy. Patient compliance to the KD can be low due to the severe dietary restriction, the diet being perceived as unpalatable, and intolerance to high-fat ingestion. Maintaining ketosis can be difficult as consumption of even a small quantity of carbohydrates or excess protein can rapidly inhibit ketogenesis [36, 37]. Furthermore, enhanced ketone production and tissue utilization can take several weeks (keto-adaptation), and patients may experience mild hypoglycemic symptoms during this transitional period [38]. One common concern regarding the KD is its purported potential to increase the risk of atherosclerosis by elevating blood cholesterol and triglyceride levels [39, 40]. This topic remains controversial as
some, but not all studies have demonstrated that the KD elevates blood levels of cholesterol and triglycerides [41-46]. Kwitervich and colleagues demonstrated an increase in low-density lipoprotein (LDL) and a decrease in high-density lipoprotein (HDL) in epileptic children fed the classical KD for two years [8]. In this study, total cholesterol increased by ~130%, but then stabilized at the elevated level over the 2-year period. A similar study demonstrated that the lipid profile returned to baseline in children who remained on the KD for six years [47]. Children usually stay on the diet for approximately two years then return to a diet of normal fat and carbohydrate ingestion [48]. In contrast, the majority of recent studies have suggested that the KD can lead to significant benefits in biomarkers of metabolic health, including blood lipid profiles [49-56]. In these studies, the KD positively altered blood lipids, decreasing total triglycerides and cholesterol while increasing the ratio of HDL to LDL [52-61]. Although, the KD is well established in children, the diet has only recently been accepted as a strategy to control seizures in adults. In 2014, Schoeler et al reported on the feasibility of the KD for adults, concluding that 39% of individuals achieved > 50% reduction in seizure frequency, similar to results seen in pediatric studies. Patients experienced similar gastrointestinal adverse events that have been previously described in pediatric patients, but they did not lead to discontinuation of the diet in any patient [62].

2.4. Exogenous Ketone Supplementation

Considering both the broad therapeutic potential and limitations of the KD, an oral ketone supplement that could rapidly induce and sustain therapeutic ketosis without the need for dietary restriction would be an attractive and practical alternative. Recent studies suggest that many of
the benefits of the KD are due to the effects of ketone body metabolism. Veech et al. have summarized the potential therapeutic uses for ketone bodies, which will be discussed later in this chapter [3, 63]. Furthermore, there is potential for an additive therapeutic effect between a standard KD and an oral ketone supplement for patients consuming a low carbohydrate or ketogenic diet. Additionally, in some scenarios, a ketone supplemented ketogenic diet may be more effective than KD or ketone supplementation alone. Several natural and synthetic ketone supplements capable of inducing nutritional ketosis have been identified. There are numerous sources of ketones and ketogenic precursors being developed and tested, including medium chain triglycerides, diols, salts, and esters that have been shown to elevate blood ketone levels without dietary restriction. The investigation of natural and synthetic ketogenic precursors to establish nutritional ketosis without the need for dietary restriction has revealed that each formulation has distinct properties in terms of extent and duration of ketosis as well as metabolic effects. Most of the developed ketone supplements are currently under investigation for safety and efficacy in a number of disease states. For this dissertation, we focus on five ketogenic agents including 1, 3-butanediol (BD), medium chain triglyceride oil (MCT), sodium/potassium-βHB mineral salt (BMS), combination of BMS+MCT, and 1,3-butanediol acetoacetate diester/ ketone ester (KE).

2.4.1 1,3-Butanediol

1,3-Butanediol (BD) is a FDA approved organic alcohol used as a food flavoring solvent, an intermediate in the manufacture of certain polyester plasticizers, and a humectant for cosmetics [64, 65]. When ingested orally, BD is metabolized by the liver via alcohol dehydrogenase (ADH) to β-hydroxybutyraldehyde, which is rapidly oxidized to βHB by aldehyde dehydrogenase [66].
BD contributes approximately 6 kcal/g of energy and can produce dose-dependent millimolar concentrations of ketones in the blood [67-69]. Extensive toxicology studies have concluded that BD is safe with very little adverse health effects in humans or animals [70-73]. BD has been investigated as a therapy to reduce brain damage following cerebral ischemia. BD attenuated hypoxic damage via a cerebral protective effect mediated by brain ketone metabolism [74]. Additionally, ketosis induced via exogenous BD supplementation has been shown to increase survival time of mice exposed to hypoxia in a stroke model [75]. BD supplementation has been shown to significantly prolong the survival time in mice with metastatic cancer by 51% [26]. Recently, BD has been investigated therapeutically as a backbone of ketone mono- and di-esters, which are discussed later in this chapter.

2.4.2. MCT Oil

Medium chain triglycerides (MCTs) contain a glycerol backbone esterified to medium-chain fatty acids (MCFAs), which are fatty acids with hydrocarbon side chains 6 to 12 carbons in length. MCFAs include caproic acid (C6:0, hexanoic acid), caprylic acid (C8:0, octanoic acid), capric acid (C10:0, decanoic acid), and lauric acid (C12:0, dodecanoic acid). MCTs are naturally found in coconut oil (15%), palm kernel oil (7.9%), cheese (7.3%), milk (6.9%), butter (6.8%), and yogurt (6.6%) [76, 77]. Compared to long-chain fatty acids (LCFAs), MCFAs have a lower melting point, smaller molecule size, and are less calorically dense (8.3 calories per gram versus 9.2). These distinct physiochemical properties allow MCTs to be absorbed directly into the bloodstream through the portal vein without the need for bile or pancreatic enzymes for degradation. Additionally, MCTs do not require carnitine to enter the mitochondria, but rather
quickly cross the double mitochondrial matrix where they are metabolized to acetyl co-A, and subsequently, to ketone bodies. Thus, they are easily and rapidly digested, transferred to the liver, and used for energy rather than stored as fat. In comparison, LCFA metabolism is much slower requiring re-esterification in the small intestine, transport by chylomicrons via the lymphatic and vascular systems, and oxidation in the liver for energy or storage. MCTs are metabolized as rapidly as glucose but have roughly twice the energy density [78]. In the early 1980s, Dr. Vigen K. Babayan of the Nutrition Laboratory at Harvard University developed a process to produce MCTs in large quantities [79]. These commercialized MCTs are acquired through lipid fractionation from natural fats such as coconut oil and milk and are predominantly comprised of C:8 and C:10 MCTs [80, 81].

Since the 1970s, MCTs have been used in a modification of the classical KD as an alternative fat source [82]. The ketogenic properties of MCTs allow patients to eat less total fat in their diet and include more carbohydrate and protein without sacrificing their nutritional ketosis. Although MCTs have the potential to be an efficient and beneficial ketogenic fat, they are currently limited in clinical use due to gastrointestinal (GI) side effects stimulated by the large dose needed to induce ketonemia (greater than 40 g/day) [78]. The original modified KD with MCT allowed 60% of its energy to be derived from MCTs; however, this amount caused GI distress in some children [83-86]. For this reason, an additional modified KD with MCT was developed using only 30% of its energy from MCTs; however, it induced much lower levels of ketosis [87, 88].
2.4.3. βHB Salt

Originally, researchers attempted to administer βHB or AcAc in their free acid forms; however, this was shown to be too expensive and ineffective at producing sustained ketosis. Subsequently, it was suggested to buffer the free acid from of βHB with sodium salts, but this causes potentially harmful sodium overload and mineral imbalance at therapeutic levels of ketosis, and is largely ineffective at preventing seizures in animal models [89]. A study showed that oral administration of Na+/βHB in doses from 80-900mg/kg/day elevated blood ketone levels to 0.19-0.36 mM in children with acyl CoA dehydrogenase deficiency [90]. However, to achieve the same level of ketosis a 70 kg man would need to ingest between 5.6 to 63 g/day, costing up to $200 a day. Considering the potential effects of such a large sodium load, the costs of the administration of Na+/βHB salts to achieve ketosis made this approach unrealistic [63].

Recently, a βHB mineral salt was developed in collaboration with Savind Inc. These ketone salts are balanced with minerals to prevent sodium overload and can include arginine, potassium, calcium, magnesium, lithium, lysine, histidine, ornithine, creatine, agmatine (guanidine), or citrulline. Maintaining an optimal sodium-mineral ratio should help offset any potential adverse effects of sodium on blood pressure. It is speculated that this formulation will be especially beneficial for elderly patients most susceptible to sodium-induced hypertension [91]. The mineral salt has to be delivered as a concentrated liquid because potassium βHB is too hygroscopic to isolate as a powder (it picks up water from the atmosphere and turns into a paste at an astonishing rate, whether by itself or mixed with sodium βHB). Many people experience the “low carb flu” during keto-adaptation (2-3 week phase characterized by enhancement of ketone production and tissue utilization from being on a ketogenic diet) caused by glucose withdrawal in the brain and a depletion of minerals, especially sodium and potassium, in the
plasma [38]. These symptoms can be attenuated or reversed with sufficient supplementation of sodium, potassium, calcium, and magnesium.

Several mineral combinations are currently being tested for safety and efficacy. In a recent case study, a 100 kg male subject was administered a 4% Na+/K+ βHB salt solution (containing 11 grams of sodium and 7.1 grams βHB) and his plasma levels of βHB were significantly elevated 30-60 minutes after administration. After receiving the same dose for three days, the patient had sustained elevated blood ketone levels from 15-120 minutes after administration. Similar results were seen in a 70kg male who fasted three days prior to administration of the ketone salt supplement [92]. In a 15-week study, Sprague-Dawley rats were administered Na+/Ca2+-β-hydroxybutyrate mineral salt (S-BHB), 20% by weight (~25 g/kg/day) in their food fed ad libitum. The S-BHB supplemented rats exhibited sustained elevated blood ketone levels (1mM βHB) at 1, 4, 8, 10, and 13 weeks of chronic feeding which did not affect blood glucose levels compared to controls (unpublished data).

2.4.4. Combination BMS+MCT

Considering the variety of ketogenic precursors available, researchers are investigating unique combinations of the individual supplements in hopes of optimizing their benefits. A combination of βHB salts and MCT oil has been administered in 1:1 to 1:2 mixtures. This mixture allows for a lower dosing of the components compared to administering the individual compounds, thus reducing potential for side effects (sodium-induced hypertension, GI side effects, etc) and resulting in distinct synergistic blood ketone profile [92].
In a case study, a 100 kg male was administered a combination of a 4% Na⁺/K⁺ βHB salt solution (containing 11 grams of sodium and 7.1 grams βHB) + 20 mL MCT oil. This combination demonstrated higher elevation of blood ketone levels than either βHB salts or MCT oil alone, starting at 15 minutes post consumption and lasting for 4 hours. Similar results were observed in a 70-kg male who fasted 3 days prior to administration; however elevated blood ketone levels were observed sooner after supplementation and were sustained for a considerable longer time after administration (8 hours). This effect was accompanied by a reduction in blood glucose, and a lower starting blood glucose concentration on each subsequent day of supplementation [92]. In a 15-week study, Sprague-Dawley rats were administered a 1:1 mixture of Na⁺/Ca⁺2-β-hydroxybutyrate mineral salt + Medium Chain Triglyceride Oil (S/MCT) (20% by weight (~25 g/kg/day) in their food fed ad libitum. The combination-supplemented rats had significantly sustained and elevated blood ketone levels as weeks 3, 4, 8, 10, and 13 without significantly affecting blood glucose levels during the study (unpublished data).

2.4.5. Ketone Esters

Researchers have developed and investigated several synthetic ketone mono- and diesters to produce nutritional ketosis without dietary restriction. In these compounds, gastric esterases rapidly liberate ketones as a free acid from a backbone molecule, which varies depending upon the specific formulation, but is often R,S-1,3-butanediol. As previously discussed, 1,3-BD is subsequently metabolized by the liver to produce βHB [4]. Thus, the ketone esters currently available are unique amongst the aforementioned ketone supplements in that many contain, and thus directly elevate, AcAc rather than only βHB.
In the late 1970s, Birkhahn et al. were the first to synthesize a monoester of glycerol and AcAc (monoacetoacetin) for parenteral nutrition. These studies demonstrated that monoacetoacetin induced hyperketonemia comparable to fasted rats at a dose of 50 g/kg per day [93-95]. In attempts to increase the caloric density of monoacetoacetin, they synthesized both a monoester and triester of glycerol and βHB. These esters are hydrolyzed to release free βHB, thus elevating blood ketones. Later, Desrochers and colleagues synthesized mono- and di-esters of 1,3-BD with either AcAc or βHB [96]. These and other esters developed by or in collaboration with Henri Brunengraber and Richard Veech have been shown to induce various degrees of hyperketonemia (2-7mM) in rats, mice, dogs, pigs, and humans [97-102]. Clarke and colleagues demonstrated the safety of these ketone esters in rats and humans [103, 104]. Recently, a R,S-1,3-butanediol acetoacetate diester (KE) was developed in collaboration with Savind Inc. A single dose of the KE administered via intragastric gavage elevated both AcAc and βHB blood levels to >3mM in rats [4]. In a 15-week study, the KE was administered to Sprague-Dawley rats in a low dose (5%, 10 g/kg/day) (LKE) and a high dose (20%, 25 g/kg/day) (HKE) in food fed ad libitum. Both doses significantly elevated blood ketone levels without affecting blood glucose levels. Serum clinical chemistry of both LKE and HKE did not reveal any changes in any major markers for kidney and liver function compared to standard diet fed rats (unpublished data).

2.4.6. Potential Concerns and Side Effects: Ketoacidosis

Ketoacidosis is one major concern for the use of oral ketone supplements without dietary restriction. Ketoacidosis is normally only seen in patients with diabetes mellitus. In this circumstance, the lack of insulin prevents glucose delivery to the tissues to be used as energy.
High glucose levels spill over into the urine, taking water and solutes (sodium and potassium) with it in a process known as osmotic diuresis. The body senses that it is in starvation mode and enhances b-oxidation of fatty acids for ketogenesis. The resulting ketone bodies are used as fuel. Additionally, insulin acts as a negative feedback loop to ketogenesis. Any rise in insulin and/or glucose normally inhibits ketogenesis immediately or excess ketones are expelled through ketonuria. Without insulin, the body is overwhelmed by ketone bodies. Since the ketone bodies are acidic, the uncontrolled elevation of ketone bodies (20-25 mM) drops the blood pH and can lead to organ failure, cerebral edema, and even death. If not properly administered, oral ketone supplementation does have the potential to induce ketoacidosis; however, optimal dosing circumvents this danger. Oral ketone supplementation will be optimized to elevate blood ketone levels to the 2-7 mM range, which is far below the elevation seen in ketoacidosis.

2.5. Potential Physiology Factors Affected to Enhance Wound Healing

It has been well established that optimized nutritional support aids wound healing. [105, 106]. Demling et al. stated that in order to optimize healing, a substrate that is more dependent on intake rather than on the bodily breakdown of protein needs to be available [107]. In the traditional classification, ketone bodies are known as a carrier of energy from the liver to extra-hepatic tissue. However they can also be considered as a nutritional supplement and an anabolic agent [108]. Indeed, Nair et al. concluded that βHB promotes protein synthesis in human beings [109]. Recently, it has been shown that βHB possess a variety of signaling functions that may regulate a broad range of cellular functions. [110-113]. It has been shown that βHB can regulate cellular processes via histone deacetylase (HDAC) inhibition, binding to cell surface receptors,
and altering the levels of other regulatory metabolites including acetyl-CoA, succinyl-CoA, and NAD+ [110]. These unique cellular and physiological properties support the assertion that ketone bodies themselves confer many of the benefits associated with the KD. We hypothesize that ketone bodies may augment wound healing via the suppression of inflammation, inhibition of oxidative stress, enhancement of blood flow, and enhancement of metabolic efficiency. Additionally, ketones may aid diabetic foot ulcer healing via modulation of blood glucose levels and enhancement of insulin sensitivity.

2.5.1. Ketones and Inflammation

As mentioned in the previous chapter, chronic wounds are stuck in the inflammatory phase. A therapy that promotes resolution of inflammation may push the chronic wound to a more acute wound phenotype, therefore progressing to closure. Several studies have determined that the KD reduces circulating inflammatory markers in animals and in humans [114-117]. Additionally, Ketone bodies themselves have been shown to decrease inflammatory biomarkers including TNF-α, IL-6, IL-8, MCP-1, E-selectin, I-CAM, and PAI-1 [17, 118]. In an unpublished study from the D’Agostino laboratory, rats were fed one of three exogenous ketone supplements LKE, HKE, S-BHB, or S/MCT mixed into standard rodent chow at 5- 20% by weight for 15 weeks. Inflammatory profiling was performed on serum collected from the animals at the end of the chronic feeding study and revealed decreases in several pro-inflammatory cytokines including IL-1β, IL-6, IFN-γ, MCP-1, and RANTES.

Recently, Dixit and colleagues demonstrated in both in vitro and in vivo that βHB inhibits the NLRP3 inflammasome, which controls activation of caspase-1 and release of the pro-
inflammatory cytokines IL-1β and IL-18 from M1 macrophages. The authors concluded that elevating blood ketones could be a therapeutic option for patients with NLRP3-mediated chronic inflammatory diseases [119]. It has been established that elevated levels of IL-1β are present in wounds of diabetic humans and mice, consistent with increased inflammasome activity. Since M1 macrophages are activated by IL-1β, the NLRP3 is thought to perpetuate this pro-inflammatory phenotype, leading to impaired wound healing [120]. Two studies have shown that blocking the activation of the inflammasome decreased pro-inflammatory cytokine levels and accelerated wound healing in diabetic mice [120, 121]. This evidence supports the hypothesis that oral ketone supplementation could produce similar effects on the inflammasome.

2.5.2. Ketones and ROS

Veech and others have determined some of the molecular mechanisms by which ketone bodies suppress oxidative stress [122-124]. In non-wounded skin, the major source of free radicals is the half-reduced semiquinone of co-enzyme Q in the electron transport chain of the mitochondria. At physiological levels, ketone bodies increase the oxidation of co-enzyme Q, decreasing mitochondrial oxygen radical generation, thereby reducing the amount of ROS produced [3, 63, 124]. Additionally, ketone metabolism induces reduction of the mitochondrial NAD⁺ and cytoplasmic NADP electron carriers. As previously mentioned in Chapter 1, NADH is important for energy producing pathways and NADPH is necessary for the regeneration of the endogenous antioxidant reduced glutathione (GSH) [125].

Eric Verdin and colleagues have recently demonstrated βHB is an endogenous and specific inhibitor of class 1 histone deacetylases (HDACs) [126]. Elevated βHB levels by fasting,
calorie restriction, and exogenous administration increased global histone acetylation in mouse tissues and induced the transcription of genes encoding oxidative resistance factors FOXO3A and MT2 via selective depletion of HDAC 1 and HDAC2. Furthermore, the authors demonstrated in vivo that exogenous ketone supplementation could prevent oxidative stress. Mice were pretreated with βHB via a subcutaneous pump for 24 hours prior to receiving an injection of paraquat, which induces the production and accumulation of ROS. Protein carbonylation was suppressed by 54%, lipid peroxidation was completely suppressed, and endogenous antioxidants mitochondrial superoxide dismutase (MnSOD, SOD2) and catalase (CAT) were elevated in the renal tissue of βHB pre-treated mice.

Regulated ROS levels are critical for optimal wound healing. Normal oxidative phosphorylation, transient hypoxia, and increased neutrophil and macrophage respiratory bursts release ROS during wound healing. The increased level of ROS transcends the beneficial effect and causes additional tissue damage and healing delay. This unquenched ROS production is prevalent in the aged population. Recently, Moor et al demonstrated age-dependent deficiencies in the glutathione and SOD2 antioxidant pathways. In an ischemic wound model, wounds from aged rats had lower SOD2 protein and activity, decreased ratio of reduced/oxidized glutathione, and decreased glutathione peroxidase activity [127]. These age-exaggerated insufficiencies lead to excessive inflammation and impaired wound healing.

One of the hallmarks of aging is mitochondrial dysfunction [128]. Electron microscope images of aged mitochondria show disorganization and degeneration via absent cristae, vacuolation, and swelling. Treatment with lipoic acid and carnitine stimulates repair of mitochondrial membranes, returns functionality to the mitochondria, and demonstrates a young phenotype [129]. Bough et al showed increased mitochondrial biogenesis in rats maintained on a
KD for 4-6 weeks [130, 131]. Interestingly, exogenous ketone supplementation with a ketone ester has been shown to induce mitochondrial biogenesis [132]. Mice in this study were fed a diet from which approximately 30% of calories were derived from the D-β-hydroxybutyrate-R-1,3-butanediol monoester for one month. The mitochondrial content and expression of electron transport chain proteins were significantly increased in the intrascapular brown adipose tissue as compared to control mice, although calorie intake was matched between the two groups. These experiments support the hypothesis that ketone supplementation could have therapeutic benefits for an aged population.

2.5.3. Ketones and Blood Flow and Angiogenesis

Restoration of blood flow via angiogenesis is critical for healing a wound, as well as the integration of skin substitutes. Venous, arterial and diabetic blood flow insufficiencies are major underlying contributors to chronic wound development. Additionally, most older patients have hypertension or other comorbidities that affect blood flow. In a study by Hasselbalch and colleagues, 8 volunteers (4 male, 4 female, 24±4 years of age, normal weight) were given an infusion of Na⁺-βHB via antecubital vein at a rate of 4-5 mg/kg/min corresponding to an infusion of ~350mL/hr for 3-3.5 hours. Global cerebral blood flow was measured via the Kety-Schmidt technique; ketone supplementation showed a 39% increase in cerebral blood flow [133].

As noted in Chapter 1 of this dissertation, VEGF is the most potent angiogenic factor for its effects on multiple components of the angiogenesis cascade: endothelial cell mitogenesis, chemotaxis, and induction of vascular permeability [134, 135]. In addition to VEGF, endothelin-1 (ET-1) is another cytokine produced in the response to hypoxia and is a crucial promoter of
cell proliferation, migration and chemotaxis, and angiogenesis in wound healing [136-138]. A study by Isales and colleagues demonstrated that supplementation of mouse brain microvascular endothelial cells with βHB and AcAc both independently increased ET-1 and had an added affect when applied together. βHB but not AcAc increased VEGF levels [139]. Though this area needs further exploration, this may be one mechanism that oral ketone supplementation may augment wound healing.

2.5.4. Ketones and Metabolism

In the 1940s Henry Lardy demonstrated that βHB and AcAc had distinct energetic efficiencies compared to 16 major carbohydrate, lipid, and intermediary metabolites in their ability to increase bull sperm mobility while simultaneously decreasing oxygen consumption [140, 141]. Nearly 50 years later, Richard Veech and colleagues confirmed that ketone bodies increased metabolic efficiency and elucidated the molecular mechanisms in the working perfused rat heart [124, 142]. They demonstrated that supplementation of glucose-containing perfusate (10 mM glucose) with 5 mM ketones (4 mM βHB, 1 mM AcAc) increased cardiac hydraulic work by approximately 25% while simultaneously reducing oxygen consumption [124]. Their study demonstrated the ketone-mediated increase in metabolic efficiency was facilitated by a reduction of the mitochondrial NAD couple and an oxidation of the coenzyme Q couple increasing the energy span between the sites (mentioned previously to decrease ROS production). This results in an increase in energy released by electrons in the ETC, causing more protons to be pumped into the inner mitochondrial space. The electrochemical gradient is enhanced, hyperpolarizing the cell, and increasing the $\Delta G_0$ (free enthalpy) of ATP hydrolysis; thus, increasing metabolic
efficiency. Additionally, ketone bodies were shown to cause a 16-fold elevation in acetyl-CoA content and increased Krebs cycle intermediates. Furthermore, thermodynamic tables for heat of combustion, calculated with bomb calorimeter experiments, show that βHB produces more energy than glucose per carbon molecule [143].

Decreased availability of ATP negatively impacts nearly every aspect of the healing process [144]. Decreased nutrient and oxygen exchange during wound healing limits ATP production to fuel wound healing processes. Additionally, the skin predominantly uses anaerobic respiration, which decreases the potential ATP production by 90% compared to aerobic respiration [145-147]. Chiang and colleagues developed a new intracellular ATP delivery technique in which highly fusogenic lipid vesicles (ATP-vesicles) are used to encapsulate magnesium-ATP (Mg-ATP). When the vesicles come into contact with the cell membrane, they fuse together and deliver the contents into the cytosol. Using eleven controlled-pairs of adult nude mice, they demonstrated accelerated wound healing via enhanced development of granulation tissue, more rapid re-epithelialization, and elevated VEGF levels [31, 63].

2.5.5. Ketones Suppress Blood Glucose via Increasing Insulin Sensitivity

Failure to heal in diabetic lower limbs is associated with hyperglycemia, insulin resistance, tissue hypoxia, chronic inflammation, oxidative stress, impairment of the immune system, and metabolic dysfunction [148, 149]. A study by Rubinstein et al. showed that once diabetes was a controlled, diabetic foot ulcers in 11 of 15 patients healed in 4 to 13 weeks [150]. In addition, diabetic patients with glucose under 200 mg/dl showed a decrease in surgical site infections after foot and ankle surgery [151]. Recent studies suggest that many of the benefits of
the KD are due to the effects of ketone body metabolism. Interestingly, in studies on T2D patients, improved glycemic control, improved lipid markers, and retraction of insulin and other medications occurred before weight loss became significant, suggesting physiological effects of ketone metabolism [3, 63]. Exogenous ketone supplements may provide therapeutic benefits for the underlying hyperglycemia and insulin resistance as reports have demonstrated that oral ketone administration lowers blood glucose by increasing insulin sensitivity. This could be critical for older adults as all have demonstrable insulin resistance, even if not diabetic. Richard Veech has suggested this hypoglycemic effect is the result of ketones activating pyruvate dehydrogenase (PDH), which enhances insulin-mediated glucose uptake and the production of acetyl-CoA. In addition, the body regulates ketone production via ketonuria and ketone-induced insulin release, which shuts off hepatic ketogenesis. The insulin from this process may increase glucose disposal which, when coupled with PDH activation, could drive down blood glucose levels. The administration of 5 mM ketone has been shown to increase acetyl-CoA production 16-fold in the glucose-perfused isolated rat heart. Additionally, in this model, ketones and insulin increased cardiac hydraulic efficiency to a similar degree, approximately 25-35% [124].

Male rats were fed a standard diet with 30% of calories replaced with the R-3-hydroxybutyrate-R-1,3-butanediol monoester for 14 days. The ketone ester-supplemented diet induced nutritional ketosis (3.5mM βHB), and both plasma glucose and insulin were decreased by approximately 50% [132]. Glucose was decreased from 5 mM to 2.8 mM, and insulin was decreased from 0.54 ng/mL to 0.26 ng/mL. In a similar study by the same group, mice receiving a KE diet exhibited a 73% increase in the Quantitative Insulin-Sensitivity Check Index (QUICKI), a surrogate marker of insulin sensitivity, compared to control, calorie-matched mice [132]. Fasting plasma glucose levels were not altered in these mice, but fasting plasma insulin
levels were reduced by approximately 85% in the KE-fed mice compared to controls, demonstrating that exogenous ketones enhance insulin sensitivity [152].

The histone deacetylase inhibitor (HDACI) activity of βHB could also be beneficial in T2D by altering the direct regulation of HDAC-dependent glucose metabolism and by inducing resistance to oxidative stress. HDACs regulate the expression of genes encoding many metabolic enzymes, and HDAC3 knockout animals exhibit reduced glucose and insulin. SAHA, a class I HDAC inhibitor, has been shown to improve insulin sensitivity and increase oxidative metabolism and metabolic rate in a mouse model of diabetes [153]. Butyrate, a short-chain fatty acid that is structurally similar to β-hydroxybutyrate and also acts as a HDACI lowers blood glucose and insulin levels and improves glucose tolerance and respiratory efficiency [154]. The vascular dysfunction in T2D is thought to be caused by oxidative stress [155]. HDAC inhibition prevents renal damage in mouse models of diabetic nephropathy through modulation of redox mechanisms [156]. Therefore, βHB suppression of oxidative stress through HDAC inhibition may help restore insulin sensitivity and manage complications of diabetes.

2.6. Ischemic Wound Healing Model

As mentioned previously, a chronic wound is by definition one that entirely fails to heal. In this respect, there is no standardized animal model that reflects the human chronic wound environment. However, animal models have been developed that attempt to mimic these conditions for the purpose of furthering our understanding of the complexity of chronic wounds. The rat species, often employed due to its wide availability, size and docile nature is used for wound healing studies as it is large enough to provide a suitable skin area for incisional and
excisional wounding, imaging and tissue collection [157]. Yet, it should be noted that the skin of a rat and a human are different morphologically, as rats are loose-skinned animals. This distinct anatomical characteristic enhances wound contraction over re-epithelialization in rat integument [157]. Additionally, the presence of a subcutaneous panniculus carnosus muscle in rats, contributes to healing by both contraction and collagen formation [158, 159]. These very important morphological distinctions were considered in the optimization of the following rat ischemic skin wound model used for this dissertation. Additionally, specific modifications were implemented to decrease wound contraction and reduce the influence of the panniculus carnosus muscle [160].

In the rat ischemic wound model, a dorsal bi-pedicle 10.5 x 3 cm flap is surgically created on day 0. A silicone sheet is placed under the panniculus carnosus fascia and above the paraspinous muscles, limiting revascularization from the underlying tissue, which increases the duration of flap ischemia. Two 6mm punches are created within the flap, extending just above the fascia, creating ischemic wounds. Additionally, two 6 mm punches are created lateral to the flap, where there isn’t silicone sheet, providing control non-ischemic wounds within the same animal.

One of the main factors in the development of a chronic wound is localized tissue ischemia (reduced blood flow) contributing to the inability to clear inflammation [160]. In 2005, Dr. Lisa Gould developed and validated this model as a modification of the ischemic wound model originally described by Schwartz et al. and subsequently used in modified form by Chen et al. [161, 162]. The wound model was modified to test the induction of angiogenesis in the wound bed.
Wound healing in rats has often been the subject of debate due to their ability to heal infected wounds and high rate of inter-animal variability [160]. One of the original goals of the model during its development was to decrease this variation. Modifications to the width of the flap, reducing the number of wounds with specific placement (centered on the flap with consistent cranio-caudal location) and introduction of a silicone sheet has accomplished this goal. Wound healing by contraction has also been reduced and healing by epithelialization, as in humans, is the measured outcome. Adaptation of the model to a different strain of rat, ie the F344, has also proven successful and reproduces the degree of ischemia observed using Sprague Dawley rats.

To achieve consistency with this model while performing multiple surgeries, it was found that it is important to create the ischemic wounds prior to elevation of the flap for silicone sheet placement [160]. Additionally, not punching through the panniculus carnosus fascia is critical to provide a viable wound bed to remain over the silicone. The silicone acts not only to prevent vascular regrowth but also as a “splint” that reduces wound contraction. The application of the adhesive and dressings to prevent infection and maintain a moist environment for wound healing is also important. Product choice can be what is preferred or used in the researcher’s animal facility. However, it is not uncommon for some of the animals to be able to remove their dressings, no matter what type of adhesive/dressing combination is used.

The bi-pedicled flap should remain viable throughout a time course of healing which is approximately 28 days, depending on rat strain and other co-morbidities present. Rarely, abscesses can form in the flap (particularly near sutures) and seromas may form under the flap. Fluid can be drained and antibiotics administered if necessary. However, if the flap loses viability and becomes necrotic it is recommended that that animal no longer be used. Wound
excision for biochemical analysis does introduce variability due to (1) some normal tissue must be retained for support (2) the choice of tissue homogenization and preparation for isolation of RNA, DNA or protein and (3) inherent inter-animal variability [127, 160, 163]. One could consider this last point a limitation to the model. It was found that reducing the size of the flap (<2.0 cm) or flap trauma can cause necrosis, indicating that minor variations in technical or environmental factors such as temperature or stress levels, may also lead to biochemically detectable variation between wound samples from one rat to another [160].

In summary, this model, with a longitudinal, bi-pedicle flap ranging from 2.0-3.0 cm in width and a strategically placed silicone sheet, is a reliable model of prolonged tissue ischemia. Once the user is adept at using the techniques to create a consistent ischemic wound, they should be able to adapt it to additional ages and species of rodents (mice included). The excisional wounds can be treated topically, or systemic treatments utilized to further explore the mechanism(s) involved in chronic wound formation, exacerbated inflammatory responses, aberrant angiogenesis and delayed wound closure.

2.7. Use of Primary Human Dermal Fibroblasts in vitro

Though immortal cells lines are more abundant and more cost efficient to maintain, in this dissertation, we utilized primary human dermal fibroblasts isolated from discarded skin. Compared to immortal cell lines, primary cell lines maintain a physiology that is more closely identified to in vivo physiology. The caveat is that they must be used at early passage, generally passage 2-8. Additionally, due to the heterogeneity of patients, the in vitro work was repeated in at least two different patient-derived cell lines thereby reducing individual confounding. Also,
fibroblasts behave differently depending on the location from which they are isolated; therefore, skin for these studies was collected from similar anatomic locations. The complex extracellular environment, paracrine, and autocrine interactions and range of cell types involved in wound repair limits in vitro models to confirm what we see in *in vivo* models. Additionally, since there is such a gamut of players and interactions, by focusing on just one cell type we may miss some of the potential mechanism for our therapy.

### 2.8. Central Hypothesis and Project Goals

There is a dire need to discover novel and effective treatments for chronic wounds. There are often multiple mitigating factors that prevent normal wound closure leading to minimally effective wound therapies as few multifactorial treatments are available.

Exogenous ketone supplements are being developed as an alternative or adjuvant method of inducing therapeutic ketosis aside from the classic ketogenic diet. It seems clear that these novel compounds have the potential to offer benefits for both healthy and diseased individuals alike. It is likely that most, if not all, of the conditions which are known to benefit from the KD would receive some benefit from exogenous ketone supplementation. Importantly, ketone supplementation provides a tool for achieving therapeutic ketosis in patients who are unable, unwilling, or uninterested in consuming a low carbohydrate or ketogenic diet. It may also help circumvent some of the difficulties associated with KD therapy, as it allows for rapid induction of ketosis in a dose-dependent fashion, which can be sustained with prolonged consumption. Simultaneously, it could provide patients with the opportunity to reap the benefits of ketosis without the practical and social difficulties of a highly restrictive diet.
Therefore, we hypothesize that exogenous ketone supplementation will improve metabolic and physiological attributes to promote accelerated healing in the impaired wound environment associated with aging. To date, the majority of ketone-based metabolic enhancement strategies have focused on enhancing brain and heart metabolism; thus, this dissertation proposes a novel therapy, which will be evaluated in a cutaneous wound-healing model [164, 165]. Although this dissertation focuses on the underlying features of the aging phenotype, similar molecular deficits have been described in diabetes and other disorders [166], suggesting that administration of ketones as an alternative fuel may have broader applications. The studies completed in this dissertation provide an important step in the potential discovery of effective and novel treatments for chronic wounds.

2.9. Closing Remarks

In this chapter I have provided scientific rationale for the potential utility of oral ketone supplementation against age impaired wound healing. Approaching the healing wound from a metabolic perspective provides an innovative opportunity to stimulate beneficial physiological and cellular mechanisms that may be lacking or attenuated in the aged population. Chapters 3-5 will consist of the results and a detailed discussion of this dissertation work. A summary of the findings, closing remarks, and major implications of this data will be discussed in Chapter 6.
2.10. References to Chapter 2


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CHAPTER 3: ESTABLISHING THERAPEUTIC KETOSIS (METABOLIC THERAPY) WITH EXOGENOUS KETONE SUPPLEMENTATION

3.1. Chapter Synopsis

In this chapter, we present data demonstrating the effects of five oral ketone supplements on blood ketones, glucose, triglyceride, and lipoprotein levels in Sprague-Dawley rats. Though each ketogenic precursor had a distinctive effect on each of the parameters, overall oral ketone supplementation rapidly elevated blood ketone levels and suppressed blood glucose levels without significantly affecting heart health biomarkers. Additionally we sent serum and hippocampal samples from KE (5 g/kg) and BMS+MCT (10 g/kg) supplemented rats to Metabolon Inc. to determine the effects that oral ketone supplementation would have on the metabolome. Ketone supplements increased Kreb’s cycle intermediates, antioxidants, and adenosine, which supports our hypothesis that oral ketone supplementation, will enhance wound healing.


A copy of this article may be found in Appendix C. See Appendix B for copyright permissions. Materials and methods
presented in this chapter can be found in Appendix A.

3.2. Effects of Exogenous Ketone Supplementation on Blood Ketones, Glucose, Triglyceride, and Lipoprotein Levels in Sprague-Dawley Rats

Nutritional ketosis induced with the KD has proven effective for the metabolic management of seizures and potentially other disorders [1-26]. We hypothesized that oral administration of ketone supplements could safely produce sustained nutritional ketosis (>0.5 mM) without carbohydrate restriction. Thus, we tested the effects of 28-day administration of five ketone supplements on blood glucose, ketones, and lipids in male Sprague-Dawley rats. The supplements included: 1,3-butanediol (BD), a sodium/potassium β-hydroxybutyrate (βHB) mineral salt (BMS), medium chain triglyceride oil (MCT), BMS+MCT 1:1 mixture, and 1,3 butanediol acetoacetate diester (KE). Rats received a daily 5-10g/kg dose of their respective ketone supplement via intragastric gavage. Weekly whole blood samples were taken for analysis of glucose and βHB at baseline and 0.5, 1, 4, 8, and 12 hrs post-gavage, or until βHB returned to baseline. In this section, we present evidence that chronic administration of ketone supplements can induce a state of nutritional ketosis without the need for dietary carbohydrate restriction with little or no effect on lipid biomarkers. The notion that we can produce the therapeutic effects of the KD with exogenous ketone supplementation is supported by our previous study which demonstrated that acutely administered KE supplementation delays central nervous system (CNS) oxygen toxicity seizures without the need for dietary restriction [27]. We propose that exogenous ketone supplementation could provide an alternative method of attaining the therapeutic benefits of nutritional ketosis, and as a means to further augment the therapeutic potential of the KD.
3.2.1 Ketone supplementation causes little to no change in triglycerides and lipoproteins

One common concern regarding the KD is its purported potential to increase the risk of atherosclerosis by elevating blood cholesterol and triglyceride levels [28, 29]. This topic remains controversial as some, but not all studies have demonstrated that the KD elevates blood levels of cholesterol and triglycerides [30-35]. Kwitervich and colleagues demonstrated an increase in low-density lipoprotein (LDL) and a decrease in high-density lipoprotein (HDL) in epileptic children fed the classical KD for two years [36]. In that study, total cholesterol increased by ~130%, and stabilized at the elevated level over the 2-year period. A similar study demonstrated that the lipid profile returned to baseline in children who remained on the KD for six years [37]. Children typically remain on the diet for approximately two years then return to a diet of common fat and carbohydrate ingestion [38]. The implications of these findings are unclear, since the influence of cholesterol on cardiovascular health is controversial and macronutrient sources of the diet vary per study. In contrast, more recent studies suggest that the KD can actually lead to significant benefits in biomarkers of metabolic health, including blood lipid profiles [39-46]. In these studies, the KD positively altered blood lipids, decreasing total triglycerides and cholesterol while increasing the ratio of HDL to LDL [42-51]. Although, the KD is well established in children, it has only recently been utilized as a strategy to control seizures in adults. In 2014, Schoeler and colleagues reported on the feasibility of the KD for adults, concluding that 39% of individuals achieved > 50% reduction in seizure frequency, similar to the results reported in pediatric studies. Patients experienced similar gastrointestinal adverse events to those previously described in pediatric patients, but they did not lead to discontinuation of the diet in any patient [52].
With oral ketone supplementation, we observed a significant elevation in blood βHB without dietary restriction and with little change in lipid biomarkers (Figure 3.1). Over the 4 week study, MCT-supplemented rats demonstrated decreased HDL compared to controls. No significant changes were observed in any of the triglycerides or lipoproteins (HDL, LDL) with any of the remaining exogenously applied ketone supplements. It should be noted that the rats used for this study had not yet reached full adult body size [53]. Their normal growth rate and maturation was likely responsible for the changes in triglyceride and lipoprotein levels observed in the control animals over the 4 week study (baseline data not shown, no significant differences) [54, 55]. Future studies are needed to investigate the effect of ketone supplementation on fully mature and aged animals. Overall, our study suggests that oral ketone supplementation has little effect on the triglyceride or lipoprotein profile after 4 weeks. However, it is currently unknown if ketone supplementation would affect lipid biomarkers after a longer duration of consumption. Further studies are needed to determine the effects of ketone supplements on blood triglyceride and lipoproteins after chronic administration and as a means to further enhance the hyperketonemia and improve the lipid profile of the clinically implemented (4:1) KD.

LDL is the lipoprotein particle that is most often associated with atherosclerosis. LDL particles exist in different sizes: large molecules (Pattern A) or small molecules (Pattern B). Recent studies have investigated the importance of LDL-particle type and size rather than total concentration as being the source for cardiovascular risk [29]. Patients whose LDL particles are predominantly small and dense (Pattern B) have a greater risk of cardiovascular disease (CVD). It is thought that small, dense LDL particles are more able to penetrate the endothelium and cause in damage and inflammation [56-59]. Volek et al. reported that the KD increased the pattern and volume of LDL particles, which is considered to reduce cardiovascular risk [47].
Though we did not show a significant effect on LDL levels for ketone supplements, future chronic feeding studies will investigate the effects of ketone supplementation on lipidomic profile and LDL particle type and size.

### 3.2.2. Therapeutic levels of hyperketonemia suppress blood glucose levels

We demonstrated that therapeutic ketosis could be induced without dietary (calorie or carbohydrate) restriction and that this acute elevation in blood ketones was significantly correlated with a reduction in blood glucose (Figure 3.2-3.4). The BMS ketone supplement did not significantly induce blood hyperketonemia or reduce glucose in the rats. The KE supplemented rats trended towards reduced glucose levels; however, the lower dose of this agent did not lower glucose significantly, as reported previously in acute response of mice (60). MCTs have previously been shown to elicit a slight hypoglycemic effect by enhancing glucose utilization in both diabetic and non-diabetic patients [60-62]. Kashiwaya et al. demonstrated that both blood glucose and blood insulin decreased by approximately 50% in rats fed a diet where 30% of calories from starch were replaced with ketone esters for 14 days, suggesting that ketone supplementation increases insulin sensitivity or reduces hepatic glucose output [63]. This ketone-induced hypoglycemic effect has been previously reported in humans with IV infusions of ketone bodies [64, 65]. Recently, Mikkelsen et al. showed that a small increase in βHB concentration decreases glucose production by 14% in post-absorptive healthy males [66]. However, this has not been previously reported with any of the oral exogenous ketone supplements we studied. Ketones are an efficient and sufficient energy substrate for the brain, and will therefore prevent side effects of hypoglycemia when blood levels are elevated and the
patient is keto-adapted. Owen et al. most famously demonstrated this in 1967 wherein keto-adapted patients (starvation induced therapeutic ketosis) were given 20 IU of insulin. The blood glucose of fasted patients dropped to 1-2mM, but they exhibited no hypoglycemic symptoms due to brain utilization of ketones for energy [67]. Therefore, ketones maintain brain metabolism and are neuroprotective during severe hypoglycemia. The rats in the MCT group had a correlation of blood ketone and glucose levels at week 4, whereas the combination of BMS+MCT produced a significant hypoglycemic correlation both at baseline and at week 4. No hypoglycemic symptoms were observed in the rats during this study. Insulin levels were not measured in this study; however, future ketone supplementation studies should measure the effects of exogenous ketones on insulin sensitivity with a glucose tolerance test. An increase in insulin sensitivity in combination with our observed hypoglycemic effect has potential therapy implications for glycemic control in T2D [68]. Furthermore, it should be noted that the KE metabolizes to both AcAc and βHB in 1:1 ratio [27]. The ketone monitor used in this study only measures βHB as levels of AcAc are more difficult to measure due to spontaneous decarboxylation to acetone; therefore, the total ketone levels (βHB +AcAc) measured were likely higher, specifically for the KE (14). Interestingly, the 10 g/kg dose produced a delayed blood βHB peak for ketone supplements MCT and BMS+MCT. The higher dose of the ketogenic supplements elevated blood levels more substantially, and thus reached their maximum blood concentration later due to prolonged metabolic clearance. It must be noted that the dosage used in this study does not translate to human patients, since the metabolic rate of rats is considerably higher. Future studies will be needed to determine optimal dosing for human patients.
3.2.3. Effects of ketone supplementation on organ weight and body weight percentage

Ketone supplementation did not affect the size of the brain, lungs, kidneys or heart of rats. As previously mentioned, the rats were still growing during the experimental time frame; therefore, organ weights were normalized to body weight to determine if organ weight changed independently to growth. There could be several reasons why ketones influenced liver and spleen weight. The ratio of liver to body weight was significantly higher in the MCT supplemented animals (Figure 3.5). MCTs are readily absorbed in the intestinal lumen and transported directly to the liver via hepatic portal circulation. When given a large bolus, such as in this study, the amount of MCTs in the liver will likely exceed the β-oxidation rate, causing the MCTs to be deposited in the liver as fat droplets [69]. The accumulated MCT droplets in the liver could explain the higher liver weight to body weight percentage observed with MCT supplemented rats. Future toxicology and histological studies will be needed to determine the cause of the observed hepatomegaly. It should be emphasized that the dose in this study is not optimized in humans. We speculate that an optimized human dose would be lower due to the higher metabolic rate of the rats and may not cause hepatomegaly or potential fat accumulation. Nutritional ketosis achieved with the KD has been shown to decrease inflammatory markers such as TNF-α, IL-6, IL-8, MCP-1, E-selectin, I-CAM, and PAI-1 [8, 70], which may account for the observed decrease in spleen weight. As previously mentioned, Veech and colleagues demonstrated that exogenous supplementation of 5mM βHB resulted in a 28% increase in hydraulic work in the working perfused rat heart and a significant decrease in oxygen consumption [71-73]. Ketone bodies have been shown to increase cerebral blood flow and perfusion [74]. Also, ketone bodies have been shown to increase ATP synthesis and enhance the efficiency of ATP production [14, 68, 73]. It is possible that sustained ketosis results in enhanced cardiac efficiency and O₂
consumption. Even though the size of the heart did not change for any of the ketone supplements, further analysis of tissues harvested from the ketone-supplemented rats will be needed to determine any morphological changes and to understand changes in organ size. It should be noted that the Harlan standard rodent chow 2018 is nutritionally complete and formulated with high-quality ingredients to optimize gestation, lactation, growth, and overall health of the animals. The same cannot be said for the standard American diet (SAD). Therefore, we plan to investigate the effects of ketone supplements administered with the SAD to determine if similar effects will be seen when the micronutrient deficiencies and macronutrient profile mimics what most Americans consume.

MCT oil has recently been used to induce nutritional ketosis although it produces dose-dependent gastrointestinal (GI) side effects in humans that limit the potential for its use to significantly elevate ketones (>0.5 mM). Despite these limitations, Azzam and colleagues published a case report in which a 43-year-old man had a significant decrease in seizure frequency after supplementing his diet with 4 tablespoons of MCT oil twice daily [75]. An attempt to increase his dosage to 5 tablespoons twice daily was halted by severe GI intolerance. Henderson et al. observed that 20% of patients reported GI side effects with a 20g dose of ketogenic agent AC-1202 in a double blind trial in mild to moderate Alzheimer’s patients [76]. We visually observed similar gastrointestinal side effects (loose stools) in the rats treated with MCT oil in our study. Rats were closely monitored to avoid dehydration, and gastric motility returned to normal between 12-24 hrs. Interestingly, the BMS+MCT supplement elevated βHB similarly to MCT oil alone, without causing the adverse gastrointestinal effects seen in MCT-supplemented rats. However, this could be due to the fact in a 10 g/kg dose of BMS+MCT, only 5 g/kg is MCT alone, which is less than the 10 g/kg dose that elicits the GI side effects. This
suggests that this novel combination may provide a more useful therapeutic option than MCT oil alone, which is limited in its ability to elevate ketones in humans.

Exogenously delivered ketone supplements significantly altered rat weight gain for the duration of the study (Figure 3.6). However, rats did not lose weight and maintained a healthy range for their age. Rats have been shown to effectively balance their caloric intake to prevent weight loss/gain [77-79]. Due to the caloric density of the exogenous ketone supplements (Table 3.1) it is possible for the rats to eat less of the standard rodent chow and therefore fewer carbohydrates while maintaining their caloric intake. Food intake was not measured for this study. However, if there was a significant carbohydrate restriction there would be a significant change in basal blood ketone and blood glucose levels. As the hallmark to the KD, carbohydrate restriction increases blood ketone levels and reduces blood glucose levels. Neither an increase in basal blood ketone levels nor a decrease in basal blood glucose levels was observed in this study (Figure 3.7). Additionally, if there were an overall blood glucose decrease due to a change in food intake, this would not explain the rapid reduction (within 30 minutes) in blood glucose correlated with an elevation of blood ketone levels after an intragastric bolus of ketone supplement (Figures 3.2-3.4).

Several studies have investigated the safety and efficacy of ketone supplements for disease states such as AD and Parkinson’s disease, and well as for parenteral nutrition [68, 80-86]. Our research demonstrates that several forms of dietary ketone supplementation can effectively elevate blood ketone levels and achieve therapeutic nutritional ketosis without the need for dietary carbohydrate restriction. We also demonstrated that ketosis achieved with exogenous ketone supplementation can reduce blood glucose, and this is inversely associated with the blood ketone levels. Although preliminary results are encouraging, further studies are needed to
determine if oral ketone supplementation can produce the same therapeutic benefits as the classic KD in the broad-spectrum of disease states that are currently under investigation. Ketone supplementation could be used as an alternative method for inducing ketosis in patients who have previously had difficulty implementing the KD because of palatability issues, gall bladder removal, liver abnormalities, or intolerance to fat. Additional experiments should be conducted to see if ketone supplementation could be used in conjunction with the KD to assist and ease the transition to nutritional ketosis and enhance the speed of keto-adaptation. In this study we have demonstrated the ability of several ketone supplements to elevate blood ketone levels, providing multiple options to induce therapeutic ketosis based on patient need. Though additional studies are needed to determine the therapeutic potential of ketone supplementation, many patients that previously were unable to benefit from the KD may now have an alternate method of achieving therapeutic ketosis. Ketone supplementation may also represent a means to further augment ketonemia in those responsive to therapeutic ketosis, especially in those individuals where maintaining low glucose is important.
Figure 3.1 Effects of ketone supplementation on triglycerides and lipoproteins: Ketone supplementation causes little change in triglycerides and lipoproteins over a 4-week study. Graphs show concentrations at 4-weeks of total cholesterol (A), Triglycerides (B), HDL (C), and LDL (D). MCT supplemented rats had significantly reduced concentration of HDL blood levels compared to control (p<0.001)(B). One-Way ANOVA with Tukey’s post hoc test, results considered significant if p<0.05. Error bars represent mean (SD).
Figure 3.2 Effects of ketone supplementation on blood βHB. (A, B) Blood βHB levels at times 0, 0.5, 1, 4, 8, and 12 hours post intragastric gavage for ketone supplements tested. (A) BMS+MCT and MCT supplementation rapidly elevated and sustained significant βHB elevation compared to controls for the duration of the 4-week dose escalation study. BMS did not significantly elevate βHB at any time point tested compared to controls. (B) BD and KE supplements, maintained at 5 g/kg, significantly elevated βHB levels for the duration of the 4-week study. Two-Way ANOVA with Tukey's post hoc test, results considered significant if p<0.05. Error bars represent mean (SD).
Figure 3.3 Effects of ketone supplementation on blood glucose. (A, B) Blood glucose levels at times 0, 0.5, 1, 4, 8, and 12 hours (for 10 dose) post intragastric gavage for ketone supplements tested. (A) Ketone supplements BMS+MCT and MCT significantly reduced blood glucose levels compared to controls for the duration of the 4-week study. BMS significantly lowered blood glucose only at 8 hrs/week 1 and 12hrs/week (B) KE, maintained at 5 g/kg, significantly reduced blood glucose compared to controls from week 1-4. BD did not significantly affect blood glucose levels at any time point during the 4-week study. Two-Way ANOVA with Tukey's post hoc test, results considered significant if p<0.05. Error bars represent mean (SD)
Figure 3.4 Relationship between blood ketone and glucose levels: (A) BMS+MCT (5 g/kg) supplemented rats demonstrated a significant inverse relationship between elevated blood ketone levels and decreased blood ketone levels ($r^2=0.4314$, $p=0.0203$). (B) At week 4, BMS+MCT (10 g/kg) and MCT (10 g/kg) showed a significant correlation between blood ketone levels and blood glucose levels ($r^2=0.8619$, $p<0.0001$; $r^2=0.6365$, $p=0.0057$). Linear regression analysis, results considered significant if $p<0.05$. 
Figure 3.5 Effects of ketone supplementation on organ weight. Data is represented as a percentage of organ weight to body weight. (A, B, D, F) Ketone supplements did not significantly affect the weight of the brain, lungs, kidneys or heart. (C) Liver weight was significantly increased as compared to body weight in response to administered MCT ketone supplement compared to control at the end of the study (day 29) (p<0.001). (E) Rats supplemented with BMS+MCT, MC and BD had significantly smaller spleen percentage as compared to controls (p<0.05, p<0.001, p<0.05). Two-Way ANOVA with Tukey's post-hoc test; results considered significant if p<0.05. Error bars represent mean (SD).
**Figure 3.6 Effects of ketone supplementation on body weight:** Rats administered ketone supplements gained less weight over the 4-week period; however, did not lose weight and maintained healthy range for age. KE supplemented rats gained significantly less weight during the entire 4-week study compared to controls. BMS+MCT, BMS, and BD supplemented rats gained significantly less weight than controls over weeks 2-4. MCT supplemented rats gained significantly less weight than controls over weeks 3-4. Two-Way ANOVA with Tukey's post hoc test, results considered significant if p<0.05. Error bars represent mean (SD).
Figure 3.7 Effects of ketone supplementation on basal blood ketone and basal blood glucose levels: Rats administered ketone supplements did not have a significant change in basal blood ketone levels (A) or basal blood glucose levels (B) for the four week study. Two-Way ANOVA with Tukey’s post-hoc test, results considered significant if $p<0.05$. Error bars represent mean (SD).
3.3. Effect of Sustaining Dietary Ketosis Through Exogenous Ketone Supplementation on the Serum and Hippocampal Metabolome of Sprague-Dawley rats

In the previous section, we reported that oral administration of ketone supplements produced nutritional ketosis (>0.5 mM) without carbohydrate restriction and the effects of a 28-day administration of five ketone supplements on blood glucose, ketones, and lipids in male Sprague-Dawley rats. We hypothesized that the 28-day administration would affect metabolomic markers. Serum (~300 µL) and hippocampal tissues for ketone supplements KE and BMS+MCT were collected on day 28 at 4 hours post-intragastric gavage (peak ketone elevation). Metabolon analyzed samples by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC-MS). Both ketone supplements significantly increased serum Krebs cycle intermediates: citrate, fumarate and malate. KE supplement significantly increased alpha-ketoglutarate and BMS+MCT supplement significantly increased succinate in the rat serum. Additionally, both supplements affected medium chain fatty acids, antioxidants, and adenosine in both serum and hippocampal tissue. Although both forms of ketone supplementation increased brain and blood ketone levels, the global metabolic profiles were different.

3.3.1 Oral administration of ketone supplements elevates blood ketone levels, increases Krebs cycle intermediates, MCFAs, antioxidants, and adenosine in serum and hippocampal tissue and implications for wound healing

Global metabolomic profiling of serum from KE and BMS+MCT supplemented rats exhibited a total of 142 out of 388 metabolites that were significantly changed from control for
KE-treated rats and 119 out of 388 metabolites were significantly altered compared to control for BMS+MCT treated rats. Additionally, 12 of 290 metabolites were significantly changed in the hippocampal tissue for KE-treated rats and 36 of 290 metabolites were significantly altered for BMS+MCT treated rats (Table 3.1). These metabolic changes provide preliminary insight to possible mechanisms by which ketones could enhance wound healing as well as potential mechanisms to target other disease states.

Metabolomics analysis confirmed elevated ketone bodies βHB as well as AcAc in the serum and elevated βHB in the hippocampus (Figure 3.8). βHB is a more stable molecule since it cannot be decarboxylated to acetone; therefore, it is the predominant ketone body within the bloodstream and likely a reason only βHB was elevated in the hippocampus. It should be noted that KE metabolizes to both βHB and AcAc as compared to BMS+MCT that only metabolizes to βHB. However, both supplements elevated AcAc, KE showed a 9-fold increase and interestingly, BMS+MCT demonstrated a 15-fold increase. It should be noted that KE was at a 5 g/kg dose and BMS+MCT was given at a 10 g/kg dose. Both ketone supplements demonstrated a 2:1 ratio of βHB:AcAc (KE 15.78:8.84, BMS+MCT 35.33:15.39) in the serum. Thus, total blood ketones seen in our previous study were likely higher in the KE-treated and BMS+MCT-treated animals. The commercially available blood ketone meters used in previous study offer the most cost feasible method of measuring blood ketones, but are only able to measure βHB, not AcAc. Additionally, BMS+MCT treated animals had significantly lower serum glucose (0.87, p<0.05). This observation supports the hypoglycemic effect demonstrated in the previous study as well as previous studies by Veech and colleagues [87, 88]. As discussed, this hypoglycemic effect has been attributed to the enhancement insulin sensitivity, which may help diabetic foot ulcers specifically. As discussed, MCFAs are one of the main components of MCT oil namely
caprylic acid (C8:0) and Capric acid (10:0); these medium chain fatty acids were significantly elevated in the serum and hippocampal tissue of BMS+MCT supplemented rats (Figure 3.9). This demonstrates that the MCT oil is being metabolized into its MCFAs, which are available in the bloodstream and tissues to be used for energy. Interestingly, KE supplemented rats also had significantly elevated capric acid in the serum and hippocampal tissue.

Krebs cycle intermediates citrate, fumarate and malate were significantly elevated in the serum of both KE-treated and BMS+MCT-treated animals (Figure 3.10). Additionally, KE significantly elevated the intermediate α-ketoglutarate and BMS+MCT significantly elevated succinate. These elevations were not seen in the hippocampal tissue. As discussed, non-wounded skin predominantly uses anaerobic respiration with only about 30% going to the Krebs cycle; this is exaggerated during wound healing when hypoxia due to tissue damage shifts the metabolic profile to only using 4% Krebs cycle [89-92]. However, ketones have been shown to increase blood flow; thus, increased blood flow would lead to potential increased oxygenation and the restoration of the non-wounded skin metabolic profile. Even with a 30% Krebs cycle flux, an increase in Acetyl CoA via ketolysis would produce a net increase in ATP production. Indeed, Veech and colleagues demonstrated a 16-fold increase in acetyl CoA with a ketone ester supplementation [88]. Acetyl co-A was significantly elevated via BMS+MCT supplementations but not KE in the hippocampal tissue, supporting this finding. Levels of pyruvate and lactate were unchanged in both ketone supplements in both the serum and hippocampal tissue demonstrating that there wasn’t an increased flux of glycolysis and anaerobic respiration. Even though there initially wouldn’t be sufficient oxygen for the wound bed to use the intermediates in the ETC, Krebs cycle intermediates have other anabolic fates that would be important to wound healing. Citrate is a precursor to fatty acid synthesis; α-ketoglutarate has been shown to scavenge
H\textsubscript{2}O\textsubscript{2} in cell cultures and is an amino acid synthesis precursor [93]. Krebs cycle intermediates were shown to be decreased in aged rats; this supplementation may restore these levels to those of a young person or at least elevate them [94]. Significant amino acid synthesis is supported by significant serum elevations in the amino acids glycine and serine. Additionally, KE-treated rats had a significant increase in PPP intermediates ribose-5-phosphate and ribulose/xylulose-5-phosphate demonstrating an increase flux through the pathway and potentially nucleic acid synthesis needed for wound healing. From this data, we can speculate that if the ketones were being used as energy a higher portion of the glucose can be shunted to the PPP instead of being metabolized for energy.

Surprisingly, oxidative stress (oxidized glutathione, GSSG (serum)) and antioxidant capacity (reduced glutathione (hippo), carnosine (serum), and anserine (serum)) were elevated, suggesting a heightened state of oxidative stress and antioxidant defense with ketone supplementation. As discussed by Moor and colleagues, age alone affects the levels of SOD2 and glutathione antioxidant profile [95]. Without appropriate oxidative stress, neutrophil respiratory bursts cannot effectively clear bacteria from the wound bed; however, without appropriate antioxidant capacity, the reactive oxygen species become damaging to neighboring tissue. This ketogenic elevation of both oxidative stress and antioxidant defense may help clear bacteria as well as quench ROS to promote wound healing in an aged phenotype. Additionally, carnosine (\(\beta\)-alanyl-L-histidine) and anserine (\(\beta\)-alanyl-N-methylhistidine) were elevated by KE and BMS+MCT supplementation (Figure 3.11). Carnosine is a natural dipeptide widely and abundantly distributed in excitable tissue. Although its physiologic role is not completely understood, many beneficial actions have been attributed to carnosine, such as being an antioxidant, antiglycating and ion-chelating agent, and a free-radical scavenger. Several studies
have shown that supplementation with carnosine accelerates wound healing [96-100]. Anserine is a dipeptide containing beta alanine and histidine and has been shown to exhibit equal antioxidant activity to carnosine by reducing the primary molecular products of lipid peroxidation [101-103]. A study by Altavilla et al. demonstrated that reduction of lipid peroxidation restores impaired VEGF expression leading to accelerated wound healing and angiogenesis in a diabetic wound model [104]. This supports a possible mechanism of action for ketone supplementation to augment wound healing. However, further experiments need to be performed to optimize dose as too high of an oxidative state, which can be seen in the KE supplementation can potentially be damaging, especially in an aged patient.

Commonly known for its role in energy transfer via phosphorylation, adenosine also plays a role in the regulation of blood flow as a potent vasodilator. Adenosine was significantly elevated in the serum via KE supplementation and trended towards significance for BMS+MCT; however, BMS+MCT supplemented rats showed a significant elevation of adenosine in their hippocampal tissue (KE not significant). Even though BMS+MCT did not show a significant elevation in the blood stream, this suggests that the BMS+MCT supplemented rat tissues were more efficient at using it; therefore a larger portion had already been sequestered in the hippocampal tissue. High levels of AMP could reflect a metabolic insufficiency for the KE supplement, which may be due to the high dose; further studies optimizing the dose of ketone supplements are needed. Masino and colleagues have demonstrated that increased adenosine of the A1 subtype plays a key role in the anticonvulsant success of ketogenic strategies [105-108]. Additionally, several studies have shown that topical adenosine application accelerates wound healing via adenosine A2A receptors and promoting angiogenesis [109-117].
A recent study by Sood et al, was the first to document a global metabolomic profile of diabetic and non-diabetic wounds, 7 days post injury. In non-diabetic mice, 88 of the 129 detected metabolites had a significant response to injury, 85 up regulated and 3 down-regulated. In diabetic wounds, 81 metabolites had a significant response to injury with 76 up regulated and 5 down regulated. Interestingly, they found 62 unique metabolites that differed between the non-diabetic and diabetic wound phenotype [118]. From their study, glycine was dysregulated in diabetic wounds and both ketone supplements significantly increased glycine in the serum (1.3, 1.27 fold change). A recent metabolic profiling of cancer cells has correlated glycine with increased cell proliferation; however, it remains to be determined whether glycine is essential for cell proliferation during wound healing [119]. Two recent studies suggest that Kynurenine, a kynurenate precursor, may play a role in both anti-inflammatory activity and fibroblast proliferation during wound repair; additionally, it has been shown to be dysregulated in diabetic wounds [120, 121]. KE supplemented rats showed an increase in kynurenate but didn’t reach significance (2.61 fold) (0.05<p<0.10). Additionally, metabolite OH-phenylpyruvate was shown to be dysregulated and KE supplementation significantly enhanced the levels 3.59 fold in the serum. Little is known about the role during wound healing. Further experiments need to be conducted to determine the meaning of this observation.

The metabolomics profiling data presented here help us to understand the metabolic consequences of KE and BMS+MCT administration and offers insights into potential mechanisms of action by which ketone supplementation may augment wound healing as well as affect other disease states for future studies.
Table 3.1 Global metabolism profile of serum and hippocampal tissue following chronic administration of KE and BMS+MCT: Rats received a daily 5-g/kg dose of water (control) (n=11), KE (n=11), or BMS+MCT (n=12) via intragastric gavage for 29 days. On day 29, blood serum (~300 µL) and hippocampal tissues were collected 4-hours post-intragastric gavage (peak ketone elevation) and global metabolomics profiling was performed at Metabolon Inc. using gas and liquid chromatography and tandem mass spectrometry. Tx1=KE and Tx2=BMS+MCT. Results were considered significant when p<0.05 and direction is indicated by red or green arrows. 388 known metabolites were identified in the serum and 290 were identified in the hippocampus. KE significantly increased 106 metabolites and significantly decreased 36 in the serum compared to control; increased 10 and decreased 2 compared to control in the hippocampus (p<0.05, Welch’s two sample t-test). BMS+MCT significantly increased 57 metabolites and significantly decreased 62 metabolites compared to control in the serum; increased 28 and decreased 8 compared to control in the hippocampus (p<0.05). An additional 24 metabolites trended towards significance compared to control for KE and 58 for BMS+MCT (0.05<p<0.10).

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Figure 3.8 Ketone supplementation elevates blood ketone levels in serum and hippocampal tissues: KE and BMS supplemented rats had elevated blood ketone levels in their serum. KE elevated $\beta$HB 15.76 fold and AcAc 8.84 fold. BMS+MCT elevated $\beta$HB 35.33 fold and AcAc 15.39 fold. KE elevated $\beta$HB 2.7 fold in hippocampus and BMS+MCT elevated $\beta$HB 3.39 fold.

Figure 3.9 Ketone supplementation increases medium chain fatty acids in serum and hippocampal tissue: KE significantly increased medium chain fatty acid (MFCA) Caprate in the serum (3.15 fold) and in the hippocampus (1.92 fold) (p<0.05). BMS+MCT significantly increased Caprate in the serum (3.98 fold) and in the hippocampus (3.16 fold) (p<0.05). BMS+MCT significantly increased Caprylate in the serum (13.19 fold) and in the hippocampus (3.24 fold) (p<0.05).
**Figure 3.10 Ketone supplementation increases Krebs cycle intermediates:** KE and BMS+MCT supplemented rats had significantly elevated Krebs cycle intermediates in their serum. Both, KE and BMS+MCT increased citrate (1.99 fold, 2.34 fold) (A), fumarate (1.92, 2.45 fold) (B), and malate (2.03, 2.68 fold) (C). Additionally KE increased alpha-ketoglutarate (1.98 fold) (D) and BMS+MCT increased succinate (1.66 fold) (E). Krebs cycle precursor Acetyl CoA was significantly elevated by BMS+MCT in the hippocampus (1.37 fold) (F). Krebs cycle intermediates were unchanged in hippocampal tissues.
Figure 3.11 Ketone supplementation increases antioxidants: KE and BMS+MCT significantly increased antioxidants carnosine (3.23, 1.79 fold) and anserine (5.66, 3.70 fold) in the serum (p<0.05). Carnosine and anserine were unchanged in the hippocampus.

Figure 3.12 Ketone supplementation increases Adenosine: KE significantly increased adenosine levels in the serum (9.18 fold) (p<0.05). BMS+MCT significantly increased adenosine levels in the hippocampus (10.95 fold).
3.4. References for Chapter 3


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CHAPTER 4: ENHANCING WOUND HEALING WITH EXOGENOUS KETONE SUPPLEMENTATION

4.1. Chapter Synopsis

In the previous chapter, we determined that exogenous ketone supplementation significantly elevated blood ketone levels and reduced blood glucose levels in juvenile Sprague-Dawley rats. In this chapter, we present data demonstrating that both BD and BMS+MCT ketone supplements elicit age-dependent rapid elevation of blood ketone levels and reduction of blood glucose levels in young (8 month) and aged (20 month) Fischer 344 rats similar to those determined in Chapter 3. Additionally, we present data demonstrating the effects of food-integrated oral ketone supplementation on an ischemic wound-healing model in young and aged Fischer 344 rats. In the ischemic wound model, the flap placement on the dorsum prevents the use of oral gavage; therefore, the ketone supplements were mixed into the food and fed ad libitum. Nonetheless, this allowed continuous delivery of the ketone supplements in smaller doses as the rats fed throughout the day. Compared to a bolus administration, multiple daily doses or inclusion in the food may provide more sustained benefits. Additionally, we chose one synthetic (BD) and one natural (BMS+MCT) ketone supplement for the following studies. Though KE would seem to be a logical choice to continue with, the Fischer 344 rats refused to eat the food mixed with this supplement; therefore, BD was used.
4.2. Dietary Ketone Supplementation Increases Blood Flow and Wound Closure in an Ischemic Wound Model in Young and Aged Fischer Rats

It has been well established that the ketogenic diet (KD) induces “keto-adaptation”, a physiologic state characterized by a shift away from glucose metabolism and towards fat and ketone body metabolism. [1-3]. As discussed in previous chapters, there are many physiological changes associated with sustained ketosis that may contribute to its multifaceted therapeutic potential for many disease states including chronic wounds. Thus, we previously measured blood glucose, ketones, lipids, and other biochemical metabolites in response to chronic oral ketone administration to show physiological equivalence to KD [4]. Increasing evidence shows that limited energy and nutrient exchange is associated with age-related impairment of wound healing. We hypothesized that oral ketone supplementation without dietary restriction would enhance wound closure in young and aged Fischer rats by improving blood flow and supplying an alternative energy substrate. In our preliminary studies, we measured the magnitude and duration of ketosis following administration of a single 6.5g/kg dose of ketone precursors: 1,3-Butanediol (BD), Na+/K+ βHB salt and medium chain triglyceride (MCT) oil 1:1 mixture (BMS+MCT), or water in young and aged Fischer 344 rats (n=6). Substances were administered through an intragastric gavage, and whole blood samples
(10 µl) were acquired for analysis of glucose and βHB at 0, 0.5, 1, 1.5, 2, 4, 8, 12, and 24 hours following administration. Following the creation of ischemic wounds, the ketogenic supplements were added to a standard diet fed *ad libitum* for 28 days. Laser Doppler imaging of the ischemic peri-wound tissue every seven days demonstrated significantly increased blood flow in young rats (n=10) fed BD at day 14 and 28 (p<0.001) and BMS+MCT at day 7, 14, and 28 (p<0.01). In aged rats, blood flow was significantly increased in BD-fed at day 14 and BMS+MCT-fed at days 7 and 14 (p<0.05). Wound size was significantly smaller in young rats fed BD and BMS+MCT compared to control at 11 and 14 days following wound creation (p<0.05). In aged rats, BD-fed wounds were significantly smaller at days 11 and 14 (p<0.05) and in BMS+MCT-fed at days 11, 14, and 28 (p<0.05). Wound healing improved by three days in aged BD-fed, seven days in young BMS+MCT-fed, and ten days in aged BMS+MCT-fed compared to the healing time line of the control animals.

4.2.1 Aged rats metabolize exogenous ketone supplements differently than young rats

In Chapter 3, we studied if oral ketone administration could elicit similar physiological effects as the KD by determining how blood glucose, ketones, and lipids and other biochemical metabolites are affected by chronic ketone administration [4]. Here we present evidence that chronic administration of ketone supplements can induce a state of nutritional ketosis without the need for dietary carbohydrate restriction, enhance blood flow, and augment wound closure in young and aged Fischer 344 rats.

Both young and aged rats exhibited elevated ketones within 30 minutes of the bolus administration for both BD and BMS+MCT supplements and were sustained for 12 hours (p<0.05). However, BD supplemented aged rats demonstrated a peak elevation of blood βHB
levels at 8 hours, which was delayed compared to their younger counterparts who had peaked blood βHB levels at 4 hours post intragastric gavage. Additionally, aged animals supplemented with either of the ketone supplements demonstrated a ~1.5x higher elevation of blood BHB levels for the same dose compared to the young animals (Figures 4.1A, B). An additional set of young and aged animals were tested with a 10 g/kg dose of their respective ketone supplements and a similar pattern emerged, young and aged animals’ blood βHB levels both peaked at 12 hours post gavage (data not shown). Aged animals administered BD appeared sedated, had increased blood BHB up to 8 mM, and hypoglycemia (<50 mg/dL). We speculated that these sedative effects might be because BD is metabolized as an alcohol; thus, we decided that an intragastric gavage at 10 g/kg was not optimal for aged animals. Young animals were able to tolerate the dose.

The metabolic challenge of a wound is exacerbated in elderly patients [5]. Without sufficient energy (ATP) levels, wound healing is significantly impaired. As discussed, the ATP deficiency creates an imbalance of energy production versus utilization (the catabolism to anabolism ratio) leading to loss of lean body mass (LBM) [6, 7]. Additionally, Gupta and colleagues demonstrated that metabolic enzymes hexokinase, citrate synthase, phosphofructokinase, and lactate dehydrogenase were decreased in aged rats. Additionally, the metabolic enzyme glucose-6-phosphate dehydrogenase exhibited elevated activity and the beginnings stages followed by a significant decreased activity in a later phase compared to controls [8]. These age-dependent metabolic changes could explain the difference between the young and aged rats’ response to acute ketone supplementation. However, ketones have been shown to increase Kreb’s cycle intermediates, mitochondrial biogenesis, and enhance overall
metabolic efficiency; thus, this is one potential mechanism of how the prolonged ketone administration could have enhanced wound healing.

4.2.2. Hypoglycemic effect of hyperketonemia attenuated with age

In Chapter 3, we demonstrated that at baseline and 4 weeks, 4 hours after intragastric gavage with BMS+MCT (5 g/kg), the elevation of blood ketones was inversely correlated with the reduction in blood glucose ($r^2=0.4314$, $p=0.0203$, $r^2=0.8619$, $p<0.0001$). This correlation was not observed at any time point for BD supplemented rats [4]. Veech and colleagues have demonstrated that administration of similar ketone supplement simultaneously decreased blood glucose and blood insulin by approximately 50%. Our data show the same correlation between elevated blood ketone levels and reduced blood glucose levels with exogenous BMS+MCT supplementation in 8-month-old Fischer rats, supporting the work of Veech and confirming our previous work (Figure 4.2). However, this hypoglycemic effect is not apparent in the 20-month animals (Figure 4.2). As discussed, metabolic changes that occur during aging may play a role in diminishing this relationship. Furthermore, insulin resistance has been shown to increase with age [9]. Veech and colleagues have determined that ketone-induced hypoglycemia occurs via increasing insulin sensitivity [10-12]. In the older adult, with pre-existing has insulin resistance, the same ketone-induced effect on insulin sensitivity may not occur or it may not be enough to cause hypoglycemia. It should be noted that even though there wasn’t a significant correlation between blood ketone levels and blood glucose levels in aged ketone supplemented rats, there was suppression of glucose based on the linear regression analysis (data not shown). This
observation supports the speculation that an age-dependent metabolic change diminishes the significant correlation but doesn’t completely neutralize the relationship.

Additionally, hyperketonemia was observed throughout the 28-day wound healing study in the BD supplemented young and aged rats, but was not noted in the BMS+MCT supplemented rats (Figure 4.5A). In Chapter 3, we demonstrated that ketone supplementation caused a sustained reduction in glucose over the time course of the intragastric gavage study; however, this sustained reduction of glucose was not observed over the course of the wound healing study (Figure 4.5B). In a follow-up chronic feeding study (15 weeks), food-integrated ketone supplementation resulted in elevated blood ketone levels without affecting the blood glucose levels throughout the study, which supports our findings in this study (data not shown, unpublished data).

4.2.3. Ketone supplementation increases blood flow in both young and aged rats

Restoration of blood flow via angiogenesis is critical for healing a wound and to support revascularization of grafts including tissue engineered skin substitutes. Venous, arterial, and diabetic blood flow insufficiencies are major underlying contributors to chronic wound development. Additionally, older patients have a greater prevalence of hypertension, diabetes, and smoking, which exacerbate the age-dependent angiogenic insufficiencies [13, 14]. In this study, oral ketone supplementation significantly increased blood flow in young and aged rats supplemented with BD or BMS+MCT (Figure 5.3B). In the raw Laser Doppler data, darker colors are indicative of low blood flow/ischemia and lighter colors demonstrate increased blood flow. The increased blood flow in the ketone-supplemented rats is clearly visible in the raw data
Figure 5.3A). This wound-healing model was created to induce ischemia and thus delay wound healing [15, 16]. Further studies need to be conducted to measure oxygen concentrations during wound healing to establish that increased blood flow reverses flap ischemia leading to accelerated wound healing. In a study by Hasselbalch and colleagues, exogenous ketone supplementation exhibited a 39% increase in cerebral blood flow [17]. Additionally, VEGF, a potent angiogenic factor, has shown to be elevated with exogenous ketone supplementation [18-20]. As discussed in Chapter 3, ketone supplementation has been determined to increase the potent vasodilator, adenosine. These two mechanisms may explain how ketone supplementation increased blood flow in the ischemic wound model.

4.2.4 Ketone supplementation accelerates wound closure in young and aged rats

As the population continues to age, there is a critical need to develop effective wound healing therapies. In this study, we have demonstrated that exogenous ketone supplementation enhanced wound closure by ten days (36% faster) in aged rats supplemented with BMS+MCT and three days (10% faster) in aged rats supplemented with BD compared to control aged rats (Figure 4.4). Additionally, young rats supplemented with BMS+MCT healed three days earlier (14% faster) compared to standard diet fed young rats (Figure 4.4). Future studies are designed to determine the wound healing effect of topical ketone administration alone and in combination with the oral exogenous ketone therapy. As is, this therapy could allow patients to benefit from nutritional ketosis without dietary restriction.

Studies by Nevin and colleagues investigated the influence of a topical application of virgin coconut oil (VCO) on the healing of dermal wounds in young rats [21-23]. Their results
demonstrated a significant beneficial effect of VCO administration on intracellular and extracellular matrix components compared to controls including increased cross-linking collagen molecules indicating greater wound tensile strength and increased total DNA of the granulation tissue. Additionally, VCO therapy induced greater antioxidant capacity (superoxide dismutase 2, glutathione reductase, glutathione peroxidase) during wound healing, which led to a decrease in lipid peroxides (MDA). They concluded that the wound healing property of VCO might be due to its minor biologically active components and antimicrobial fatty acids. Previous studies have demonstrated that food integrated coconut oil was able to eliminate bacterial infection and stimulate the immune response [24]. Coconut oil is a natural source of medium chain triglycerides (MCTs), which have been shown to modulate cellular proliferation, cell signaling, and growth factor activities as well as elicit ketogenesis as seen in this study and our previous study [4, 25-27].

The BMS contains potassium and other minerals to prevent sodium overload. Maintaining an optimal sodium-mineral ratio should help offset any potential adverse effects of sodium on blood pressure. For example, multiple studies have shown that potassium provides an antihypertensive effect and protects cardiovascular damage in salt-sensitive hypertension [28, 29]. It is speculated that this formulation will be especially beneficial for elderly patients most susceptible to sodium-induced hypertension [11]. The dose of the salt solution is easily adjusted to benefit the patients’ needs. In this study a 10% ketone salt solution is mixed in a 1:1 ratio with MCT oil, which allows for reduced dosing of each component compared to administering the compounds individually. This reduces the potential for side effects (sodium-induced hypertension, gastric side effects, etc.) and results in distinct synergistic blood ketone profile [30]. A noteworthy observation from our previous study revealed that in rats, MCT alone
was more effective than BMS+MCT or BMS alone at inducing ketosis. However, preliminary human data suggests that the BMS+MCT mixture is most effective at inducing ketosis and MCT the least. This suggests that there is inter-species variability in the metabolic response to ketone supplements, which will need to be further characterized to fully understand effects in humans. In the rats, the BMS+MCT supplement elevated blood ketones similar to that of MCT alone; however, the gastric side effects were not observed suggesting a potential method for avoiding this unwanted adverse effect [31].

4.2.5. Food-integrated ketone supplementation did not elicit weight loss in young and aged rats

The KD is hypothesized to induce weight loss by reducing appetite through the satiety effect of ketone bodies, reducing lipogenesis and increasing lipolysis, and enhancing metabolic efficiency with fat and ketone metabolism [32]. Recently, preliminary studies have shown that exogenous ketone supplementation can also induce weight loss. The administrations of both βHB and BD have been shown to decrease food intake in rats and pigmy goats [33-37]. Similarly, it is suggested that MCTs increase satiety, resulting in reduced food intake and weight loss as a consequence of their rapid oxidation into ketone bodies [38-40]. MCTs may further counteract fat deposition in adipocytes by increasing thermogenesis [41]. Several studies in animals and humans have revealed increased energy expenditure and lipid oxidation with MCTs compared to LCTs [42-51]. Ketone esters have also been shown to affect weight in mice, rats, and humans [20, 52-55]. In Chapter 3, all five ketogenic supplements tested via intragastric oral gavage (BD, KE, MCT, BMS, BMS+MCT) inhibited weight gain compared to
control animals [4]. Similarly, a 15-week chronic feeding study in which KE, BMS, or BMS+MCT replaced approximately 20% of the diet by weight, fed *ad libitum*, led to reduced weight gain compared to control animals (data not shown, unpublished data).

Even though weight loss has been noted in a variety of studies, significant weight loss was not observed in either young or aged rats of this study (*Figure 4.6A*). To improve post-anesthetic appetite, thereby limiting the initial weight loss that is standard with an invasive surgery, the rats were fasted for 18 hours before surgery. Weight loss was noted during the first week after surgery, but did not reach significance in any group, and was similar across all groups demonstrating that it wasn’t a ketone-mediated effect. Weight was maintained in all groups for the remainder of the study. This initial fasting may be a reason that the initial weight loss during the first week post-surgery did not reach significance. In the previously discussed studies, rats were Sprague Dawley and were either juvenile or still in a growth phase, whereas in this study the rats were mature or elderly Fischer 344s. Age and strain variability may account for the lack of weight loss in this study compared to previously discussed experiments.

Particularly in elderly patients where malnutrition may already be present, it is critical to prevent the wound from parasitizing substrates from the rest of the body for energy production, resulting in what is called protein-energy malnutrition (PEM). The body catabolizes the muscle, skin, and bone to support the synthesis of proteins, inflammatory cells, and collagen needed to fight infection and repair the wound, causing a loss of lean body mass (LBM). As an individual loses more LBM, wound healing is more likely to be delayed. A loss of more than 15% LBM impairs wound healing, a 30% LBM loss stops wound healing, and a loss of 40% LBM typically results in death [56-60]. A body in ketosis has been shown to be muscle sparing as it is adapted to using the readily available fat stores, attenuating the catabolic effects [7]. The
weight maintenance seen in this wound healing study may reflect this protein-sparing effect. Moreover, if ketosis enhances insulin sensitivity, glucose uptake by the insulin-sensitive skeletal muscle should be increased. Together, these effects would help support muscle tissue health and function, suggesting another possible mechanism of ketone supplementation in diminishing LBM and PEM.

4.3 Closing Remarks

It has been debated if chronological age alone affects wound healing. However, with the aged population showing decreased blood flow leading to the decline of nutrient exchange, decreased metabolic activity and ATP production at the wound bed, it is clear how these factors may result in an exacerbation of chronic wounds in older adults. The discouraging results from studies focusing on single molecular targets are not surprising since wound healing is the outcome of a complex set of interactions between numerous factors. This is likely one reason most wound therapies are minimally effective. Our approach utilizes endogenous physiology to reach the wound bed systemically with one therapy, exogenous ketone supplementation, that has multiple downstream effects. Even though there is much work still to be done, this approach shows promising results as a potential wound therapy.
Figure 4.1 Effects of ketone supplementation on blood ketone and blood glucose levels: (A, B) Blood βHB and blood glucose levels at times 0, 0.5, 1, 4, 8, 12, and 24 hours post (6.5 g/kg) intragastric gavage for ketone supplements tested. BMS+MCT and BD supplementation rapidly elevated and sustained significant βHB elevation (p< 0.05) (A) and significantly reduced glucose (p < 0.05) (B) compared to controls in both young (8M) and aged (20M) rats. Two-Way ANOVA with Tukey’s post-hoc test, results considered significant if p<0.05. Error bars represent mean (SD).

Figure 4.2 Relationship between blood ketone and blood glucose levels attenuated with age: At four hours post intragastric gavage, BMS+MCT (6.5 g/kg) supplemented rats demonstrated a significant correlations between elevated blood ketone levels and decreased blood glucose levels in young (8M) rats ($r^2=0.4775$, p=0.0393); however, correlation was not present in aged (20M) rats ($r^2=0.0088$, p=0.8431). Linear regression analysis, results considered significant if p<0.05. Error bars represent mean (SD).
Figure 4.3 Ketone Supplementation increases blood flow in both young and aged rats:
Laser Doppler was used to measure blood flow weekly in the ischemic flap. Raw data from aged rats fed SD, BD and BMS ketone supplements at day 14 demonstrates the difference in blood flow seen by the laser doppler (A). Laser Doppler imaging of the ischemic peri-wound tissue every 7 days for 28 days demonstrated significantly increased blood flow in young rats (n=10 per group) with the BD at day 14 (p<0.0001) and 28 (p=0.0002) and the BMS+MCT at day 7 (p=0.0007), 14(p<0.0001), and 28 (p=0.0036) compared to control. There was an increase in blood flow in the aged rat flaps (n=10 per group) treated with the BD at day 14 (p=0.0039) and BMS+MCT at days 7 (p=0.0305) and 14 (p=0.0008) compared to control. Two-Way ANOVA with Tukey’s post-hoc test, results considered significant if p<0.05. Error bars represent mean (SD).
Figure 4.4 Ketone supplementation enhances wound closure time in young and aged rats:
Visualized using Kaplan-Meier survival plot. Wound healing (closure) was determined to be significantly different in the young (n=10 per group) Fischer rats in BD at day 11 (p=0.0493) and in BMS+MCT at day 11 (p=0.0022) and 14 (p=0.0349). In the aged Fischer rats (n=10 per group), the BD was significantly different at day 11 (p=0.0230) and 14 (p=0.0233) and BMS+MCT was significantly different at day 11 (p=0.0115), 14 (p=0.0016) and 28 (p=0.0010). Two-Way ANOVA with Tukey’s post-hoc test, results considered significant if p<0.05.
Figure 4.5 Ketone supplementation elevates blood ketones levels but does not affect blood glucose levels: (A) Hyperketonemia was sustained for the duration of the wound healing progression in BD supplemented young and aged animals. (B) Ketone supplementation did not significantly reduce blood glucose levels at any point of the healing period. Two-Way ANOVA with Tukey’s post-hoc test, results considered significant if p<0.05. Error bars represent mean (SD).
Figure 4.6 Body and Spleen Weight: (A) Ketone supplementation did not result in significant weight loss through the study in young and aged animals. (B) Though aged animals’ spleen size tended towards splenomegaly, results did not reach significance. Two-Way ANOVA with Tukey’s post-hoc test, results considered significant if p<0.05. Error bars represent mean (SD).
4.3. References for Chapter 4


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CHAPTER 5: POTENTIAL MECHANISMS FOR EXOGENOUS KETONE SUPPLEMENTATION TO ENHANCE WOUND HEALING

5.1. Chapter Synopsis

In this chapter, we present data defining some potential mechanisms by which exogenous ketone supplementation augments ischemic wound healing. We focus on four main age dependent factors: inflammation, ROS production, angiogenesis, and metabolism. Since ketone supplementation enhanced wound healing, we hypothesized that there would be measurable physiological changes in wound healing as early three days post-wounding. Periwound tissue was harvested at day three and day seven post-wounding for mechanistic analysis. Additionally mechanistic studies were conducted using primary human dermal fibroblast (HDFs) to confirm ex vivo observations. From these studies we conclude that exogenous ketone supplementation decreases ROS production, increases migration and proliferation, and decreases lactate production. Ketone supplementation did not affect inflammatory markers or antioxidants SOD2 and NQO1. Though there were similarities in effects of ketone supplements, BD and BMS+MCT elicited distinctive mechanistic profiles. We propose further studies at later time points of wound healing to determine if exogenous ketone supplementation will affect wound healing at a later phase than we originally hypothesized.
5.2. Potential Mechanisms of Action for Exogenous Ketone Enhancement of Ischemic Wound Healing in Young and Aged Fisher Rats

In the previous chapter, we reported that oral ketone supplementation without dietary restriction enhanced wound closure and increased blood flow in young and aged Fisher 344 rats. We hypothesized that exogenous ketone supplementation promoted wound healing via enhancement of physiological factors such as increasing proliferation, advancing migration, reducing ROS production, and resolving inflammation. Experiments in vitro with young and aged primary human dermal fibroblasts supplemented with 5mM βHB for 72 hours ahead of an oxidative stimulus (100μM tert-butyl-hydrogen peroxide) resulted in significantly decreased cellular ROS production, enhanced cell migration, and augmented cellular proliferation (p<0.05). Comparing peri-wound tissue lysates isolated from rats fed 1,3 Butanediol (BD), βHB NA+/K+ salt mixed with MCT oil in a 1:1 ratio (BMS+MCT), or standard diet (SD) we show that ketone administration during wound healing induced changes in cytokines, including a significant elevation in epidermal growth factor (EGF) at day 7 of wound healing compared to control (p<0.05). BMS+MCT supplementation in aged rats significantly decreased tumor necrosis factor- alpha (TNF-α) levels on day 7 compared to control (p<0.05). Furthermore, ketone supplementation significantly reduced leptin levels in ischemic wound tissue lysates in both young and aged rats. Changes in pro-inflammatory (IRF-5 labeled, M1) to anti-inflammatory (CD206 labeled, M2) macrophage ratio and blood vessel density were demonstrated by immunohistochemistry. We conclude that ketone supplementation in vivo and in vitro modifies systemic physiology to enhance wound closure.
5.2.1 Effects of ketone supplementation on inflammation

Inflammation is a key regulatory step in the progression of wound healing. Chronic wounds are stuck in the inflammatory phase characterized by a proteolytic environment in which pro-inflammatory cells, cytokines, and chemokines inhibit the normal progression of wound healing [1]. Pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α are upregulated during the inflammatory phase of wound healing [2]. IL-6 is produced by neutrophils and monocytes and has been shown to be important in initiating the healing response, has mitogenic and proliferative effects on keratinocytes, and is chemoattractive to neutrophils [3-8]. TNF-α alone has been shown to enhance wound healing in a concentration-dependent manner. Low levels of TNF-α can promote wound healing indirectly by stimulating inflammation and macrophage-produced growth factors. However, at higher levels, TNF-α has a detrimental effect on wound healing by suppressing ECM synthesis and TIMPs while increasing MMPs, inhibiting re-epithelialization. Additionally, levels of TNF-α and IL-1β are elevated in chronic wounds and have been shown to work synergistically and perpetuate each other’s expression to amplify the pro-inflammatory environment [9-11].

Exogenous ketone supplementation did not affect pro-inflammatory markers IL-6 or IL-1β in young or aged periwound tissue lysates on day three or day seven of wound healing (Figure 5.2). BMS+MCT supplementation significantly reduced TNF-α levels in periwound tissue lysates of aged rats after seven days of wound healing compared to aged controls. Significant changes were not observed in selected anti-inflammatory cytokines including IL-4, IL-10, or IL-13 (Figure 5.2). In an unpublished study from our laboratory, rats were fed one of three exogenous ketone supplements (1, 3-butanediol diacetate ester (KE), Na+/Ca⁺²-β-hydroxybutyrate mineral salt (BHB-S), or a 1:1 mixture of Na+/Ca⁺²-β-hydroxybutyrate mineral
salt: Medium Chain Triglyceride Oil (S/MCT) mixed into standard rodent chow at 5-20% by weight for 15 weeks. Inflammatory profiling was performed on serum collected from the rats at the end of the chronic feeding study and revealed decreases in several pro-inflammatory and anti-inflammatory cytokines including IL-1β, IL-6, IFN-γ, MCP-1, RANTES, IL-4, IL-10, IL-13. These findings suggest that ketone supplementation may suppress total inflammation and not affect pro-inflammatory or anti-inflammatory markers independently; however, further experiments need to be conducted to confirm this observation. Because ketone supplementation hastened wound healing, we hypothesized that we would see early resolution of inflammation. Further studies need to be conducted on subsequent days of wound healing to see if ketone supplementation affects inflammatory markers after day 7. Epidermal growth factor (EGF) is secreted by platelets, macrophages and fibroblasts to accelerate epithelialization and increase the tensile strength of wounds [12-15]. Topical EGF administration has shown promising results for venous ulcers and diabetic ulcers; however, the heterogeneity of wounds, modes of administration, patient management protocols, and varied clinical trial outcomes has limited clinical application to outside of the USA [16]. Exogenous ketone supplementation with BD elevated EGF levels in day seven periwound tissue lysates of young rats (Figure 5.3B).

Leptin is a hormone that works in opposition of ghrelin to regulate energy homeostasis by inhibiting hunger and establishing satiety. The KD has been demonstrated to slow weight gain by altering leptin levels. Juvenile rats fed a KD for two weeks had slower weight gain, had higher leptin levels, and lower insulin levels compared to those fed an SD [17]. We demonstrated a similar slowing of weight gain in juvenile Sprague-Dawley rats in Chapter 3, consistent with this observation. In the same study, they demonstrated that calorie restriction did not elicit the same leptin elevation as the KD. However, Kolacynski and colleagues investigated the responses of
leptin following a short term fasting, demonstrating that leptin levels declined following a 12 hours fast. Re-feeding prompted leptin levels to rise and return to normal values within 24 hours [18]. They demonstrated a reverse relationship between levels of βHB and leptin. Small amounts of glucose added to inhibit hepatic ketogenesis was enough to prevent the fall of leptin. However, exogenous ketone administration did not reduce leptin, causing the team to conclude that the relationship was due to a physiological response to ketogenesis and not ketone bodies themselves.

An increasing number of studies have determined that leptin has a broad spectrum of physiological effects in the wound-healing environment. Leptin has been shown to directly cause T cells and monocytes to release pro-inflammatory cytokines, to produce angiogenesis in endothelial cells, and to induce neo-vascularization in corneal cells. Leptin production has been documented in a variety of non-adipose cells including placental trophoblasts, mammary epithelial cells, gastric fundic mucosa, and ovarian follicle cells [19-29]. In a study by Murad and colleagues, it was demonstrated that cells within an incisional dermal wound produced a burst of leptin shortly after surgery [30]. The study concluded that leptin might have a multifunctional role during wound healing and serves a critical functional role as an autocrine/paracrine regulator of normal wound healing.

As discussed previously, leptin is produced locally in the wound bed. In our experiment leptin was only measured in the wound bed tissue lysates, not in circulating blood serum. BD and BMS+MCT significantly reduced leptin levels in ischemic wound tissue lysates in young animals compared to controls on day three post-wounding but this observation was no longer apparent after seven days of wound healing (Figure 5.3C). In aged animals, leptin levels were decreased in both ketone-supplemented groups on both day three and day seven post-wounding
(Figure 5.3C). This decrease in leptin levels in the ischemic wound bed may play a role to the enhanced wound healing via exogenous ketone supplementation. The circulating blood serum levels may give more insight to the data. If these measurements were in systemic blood serum, it may indicate that the rats were not eating the food-integrated ketone supplements, as leptin levels were elevated in the SD. Additionally, this would suggest that the elevations in blood ketone levels would be due to caloric restriction and not ingestion of the supplement. Further experiments monitoring food intake would be required to decipher this observation. Further experiments need to be conducted to determine the full impact of decreasing leptin levels with ketone supplementation.

As discussed in Chapter 1, macrophages in the wound bed can exhibit two distinct functional phenotypes: M1 (classically activated, pro-inflammatory) and M2 (alternatively activated, anti-inflammatory). During the early inflammatory phase, macrophages that are activated by lipopolysaccharide (LPS) or inflammatory cytokines like interferon gamma (IFN-γ) continue the job of neutrophils by phagocytizing bacteria and damaged tissue. Additionally, the M1 macrophages release pro-inflammatory cytokines such as TNF-α and IL-6 [31]. During the late inflammatory phase, initiated by the phagocytosis of apoptotic cells, M1 macrophages phenotypically convert to M2 macrophages [32]. M2 macrophages, activated by interleukin-4 (IL-4) and interleukin-13 (IL-13), play a critical role in the resolution of inflammation. Additionally, they facilitate transition to the proliferative phase by promoting angiogenesis, tissue remodeling, and repair [31, 33-40]. In this study, we did not see an effect of exogenous ketone supplementation on IFN-γ, IL-4, or IL-13 as previously hypothesized (Figure 5.2). Additionally, exogenous ketone supplementation did not elicit a significant change in M1 to M2 phenotype in day seven aged ischemic wounds (Figure 5.4). This observation supports the
inflammatory array data and further supports that the aged ischemic wounds were still in a pro-inflammatory environment seven days post-wounding. Heterogeneity seen in the wound healing in this model is consistent with patient heterogeneity seen in the clinical setting and accounts for the lack of statistical significance in the immunohistochemical analysis (Figure 5.1). In general, ketone supplemented wounds demonstrated more epithelial advancement, granulation tissue formation, and fewer labeled macrophages (Figure 5.1, Figure 5.4). Further studies need to be conducted to confirm these histological observations.

5.2.2 Effects of ketone supplementation on ROS production

As discussed in previous chapters ROS production is an important bactericidal component in acute wound healing. However, in a chronic wound environment, ROS production isn’t properly mitigated, leading to mitochondrial dysfunction, DNA damage, lipid peroxidation, and even cell death, which delays wound healing [41, 42]. Ketones have been shown to decrease ROS production through a variety of physiological effects [43-46]. Additionally, ketones have been shown to decrease oxidative stress by increasing the downstream antioxidant glutathione via the Nrf2-ARE pathway [47]. Nrf2 is the primary transcription factor that responds to oxidative stress. Under normal conditions, Nrf2 is targeted for proteasomal degradation by the E3 ubiquitin ligase, Keap1. Oxidants and electrophiles disrupt the Nrf2/Keap1 binding allowing Nrf2 to translocate to the nucleus, bind DNA at the antioxidant or electrophilic response element (ARE), and transcribe downstream antioxidants including SOD2, NAD (P) H: quinone oxidoreductase (NQO1) and enzymes for glutathione synthesis [48-50]. Additionally, Nrf2 has been shown to play a role in wound healing as disruption of Nrf2 has shown to impair the
angiogenic capacity of endothelial cells [51]. It has been shown that Nrf2 and antioxidant capacity are decreased with age, leading to increased oxidative stress [52, 53]. Recently, Moor and colleagues demonstrated age-dependent deficiencies in the glutathione and SOD2 antioxidant pathways. In an ischemic wound model, wounds from aged rats had lower SOD2 protein expression and activity, decreased ratio of reduced/oxidized glutathione, and decreased glutathione peroxidase activity [54]. These age-exaggerated insufficiencies lead to excessive inflammation and impaired wound healing. New data indicate that nutrient intake, energy metabolism, and ROS are linked at the nuclear level via the Nrf2/ARE pathway [55]. The KD has been shown to increase glutathione synthesis via the Nrf2/ARE pathway [56-59]. Additionally, the age-dependent deficit in Nrf2 has been reversed with lipoic acid supplementation [60]. Lipoic acid is derived from the MCFA caprylic acid (a main component of MCT oil); thus we hypothesized that exogenous ketone supplementation would result in similar effects.

In this study, we were unable to measure Nrf2 levels via Western blot due to inefficient antibodies; the downstream antioxidants SOD2 and NQO1, measured by Western blot analysis, were not significantly altered by exogenous ketone supplementation in either primary human dermal fibroblasts (HDFs) or ischemic wounds (Figure 5.6). Western blot sensitivity may not be able to detect the subtle changes in these antioxidants during the first few days of wound healing; thus, further experiments need to be conducted to determine antioxidant levels. However, DHE and Mitosox Red labeled ROS production in primary human dermal fibroblasts was significantly reduced with 5mM βHB supplementation (Figure 5.5). Metabolomic studies demonstrated that ketone supplementation increased antioxidants carnosine and anserine. Further studies are
needed to investigate other antioxidant pathways and determine how ketone supplementation is suppressing ROS production in a wound-healing environment.

5.2.3. Effects of ketone supplementation on angiogenesis

As a continuation of Chapter 4, we sought to delineate if the observed increase in blood flow occurred via enhanced angiogenesis or vasodilatation. Immunohistochemical analysis of CD31 blood vessel density demonstrated a trend of increased blood vessels in the ischemic wounds of aged rats supplemented with BD and BMS+MCT seven days post-wounding; however, this observation did not reach significance (Figure 5.7). As discussed, VEGF is a potent angiogenic factor. Periwound tissue lysates were analyzed for VEGF levels, revealing a trend of elevation of VEGF on day seven of wound compared to day three in both young and aged animals though this did not reach significance. Ketone supplementation did not affect levels of VEGF in this study, (Figure 5.2) but in the unpublished chronic feeding study, in which rats were ketone supplemented via food-integration for 15-weeks, low dose KE significantly increased serum (systemic) VEGF levels. As discussed in the metabolomics data in Chapter 3, exogenous ketone supplementation leads to an increase in the potent vasodilator adenosine. These observations suggest that the increased blood flow may be due to increased vasodilation and not angiogenesis. Further studies need to be conducted to determine the impact of vasodilation on ketone supplemented wound healing and to determine the exact mechanisms of the observed increase in blood flow.
5.2.4. Effects of ketone supplementation on metabolism

Proliferation and migration represent crucial processes by which epidermal and dermal layers restore anatomical integrity to the wound site. During the formation of granulation tissue in the dermis, platelets, monocytes, and other cellular blood constituents release various peptide growth factors, stimulating fibroblasts to migrate into the wound site where they proliferate to reconstitute the various connective tissue components [61]. We investigated if ketone supplementation enhanced proliferation and migration of primary human dermal fibroblasts; indeed, 5mM βHB supplementation elicited a significant increase in migration and proliferation in both young and aged HDFs (Figure 5.8 and Figure 5.9). Further experiments need to be conducted using keratinocytes of the epidermal layer to examine if ketone supplementation also increases proliferation and migration in those cells. These further studies will help determine if ketone supplementation affects all layers of the wound healing process or the dermal layer independently. Additionally, the enhanced proliferation and migration may explain the enhanced granulation tissue detected in the histological analysis (Figure 5.1, Figure 5.4).

Proliferating and migrating cells require more nutrients and energy production to perform their functions; thus, an increase in these physiological factors may be a reflection of a higher metabolic state. As discussed in Chapter 1, one of the fates of pyruvate is lactate production and is indicative of anaerobic respiration. The hypoxic wound environment shifts to higher anaerobic respiration and thus higher lactate production. Throughout the last decade, wound-healing studies have revealed that high lactate levels (10-15 mM) are characteristic in healing, stimulate collagen deposition, and enhance angiogenesis [62-66]. Subsequent studies demonstrated that lactate has several sources in wounds, remains elevated even in the presence of oxygen, and intense neutrophil respiratory bursts drive lactate and ROS accumulation through aerobic
glycolysis (Warburg effect) [64, 67-71]. These studies have concluded that lactate is produced in both aerobic and anaerobic environments during tissue repair and the presence of both lactate and oxygen is required for optimal wound healing. However, it is the quantity of lactate that is critically significant [72]. Systemic lactate concentration rarely exceeds 10 mM due to tight metabolic control; accumulation exceeding this level is associated with a poor wound-healing prognosis [73]. When lactate levels reach ~20 mM it becomes deleterious to the wound bed as high lactate levels reduce cell viability, diminish cell motility, and decrease polymerized cytoskeletal actin, which impedes angiogenesis, fibroplasia, and wound contraction [74]. Aging further complicates this environment as cells of older patients have shown decreased sensitivity to growth factor and cytokine stimulation, diminished antioxidant capacity, and reduced cell motility and proliferation, causing their tissues to be very susceptible to hypoxic and oxidant stress and worsening patient survival outcome [75-78]. In this study, we demonstrated the 5 mM βHB supplementation in young and aged HDFs significantly reduced lactate production (Figure 5.10). Further research needs to be conducted to investigate lactate levels in the wound bed with exogenous ketone supplementation and explore lactate production in keratinocytes of the epidermal layer to determine the full impact of ketone supplementation on lactate production during wound healing.

5.3. Closing Remarks

In summary, we have just begun to elicit some of the mechanisms by which exogenous ketone supplementation impacts ischemic wound healing. As ketone supplementation represents a multifaceted tool, it is likely that multiple mechanisms are acting simultaneously to provide a
therapeutic effect and unlikely that a single, clearly delineated mechanism of action could be elucidated. However, understanding these mechanistic attributes will help to optimize exogenous ketone supplementation as a wound healing therapy.

Figure 5.1 Heterogeneity of Aged Ischemic Wound Healing: Histological hematoxylin and eosin staining of aged ischemic wounds on day seven post-wounding demonstrated heterogeneity in healing within rats supplemented with SD, BD, and BMS+MCT.
Figure 5.2 Effects of ketone supplementation on pro-inflammatory and anti-inflammatory cytokines: Multiplex immunoassay on peri-wound tissue lysates isolated from SD, BD, and BMS+MCT fed rats on day three and day seven post-wounding. BMS+MCT supplementation in aged rats significantly decreased tumor necrosis factor- alpha (TNF-α) levels on day 7 of wound healing compared to controls (p<0.05) (A). No other pro-inflammatory (IL-1β, IL-6) or anti-inflammatory (IL-4, IL-10, IL-13) cytokines were significantly changed (A, B). Two-way ANOVA with Tukey’s post-hoc analysis, results were considered significant when p<0.05. Error Bars Represent mean (SD).
Figure 5.3 Effects of ketone supplementation on growth factors and the hormone leptin:
Peri-wound tissue lysates isolated from SD, BD, and BMS+MCT fed rats on day three and day seven post-wounding. Eve Technologies analyzed samples with a 27-biomarker multiplex immunoassay. Neither ketone supplement affected vascular endothelial growth factor (VEGF) levels in young nor aged animals (A). BD ketone supplementation elicited a significant elevation in epidermal growth factor (EGF) at day 7 of wound healing in young rats compared to control (p<0.05)(B). Ketone supplementation with BMS+MCT and BD significantly reduced leptin levels in both young and aged rats (C). Two-way ANOVA with Tukey’s post-hoc analysis, results were considered significant when p<0.05. Error Bars Represent mean (SD).
Figure 5.4 Ketone supplementation does not affect M1 to M2 ratio of day 7 ischemic wounds in aged rats: Paraffin embedded wound tissues harvested from SD, BD, and BMS+MCT supplemented rats were stained with H&E for morphological analysis or labeled with IRF-5 (M1, pro-inflammatory macrophage, red) or CD206 (M2, anti-inflammatory macrophage, red) and CD68 (all macrophages, green) and DAPI (blue). Immunofluorescent scans demonstrated that there was not a significant transition from M1 to M2 macrophage phenotype with exogenous ketone supplementation, suggesting a pro-inflammatory environment at day seven of wound healing.
Figure 5.5 Ketone supplementation deceases ROS production: Primary human dermal fibroblasts from young and aged donors (24 and 88 years) were incubated in the presence or absence of 5mM βHB for 72 hours. The cells were stressed with 100μM tert-butyl-hydrogen peroxide for 3 hrs. ROS production was measured via dihydroethidium (DHE) and Mitosox Red fluorescence (A, B). DHE fluorescence: Aged HDFs produced 25% more ROS compared to their young counterparts (p<0.05). βHB treatment decreased ROS production in young HDFs by 40% and aged HDFs by 41% (p<0.05) (A). Mitosox Red fluorescence: Aged HDFs produced 27% more ROS than young HDFs (p<0.05). βHB treatment decreased ROS production in young HDFs by 29% and by 51% in aged HDFs compared to control (p<0.05) (B). One-way ANOVA with Tukey’s post-hoc analysis, results were considered significant when p<0.05. Error Bars Represent mean (SD).
Figure 5.6 Ketone supplementation does not affect antioxidants SOD2 and NQO1 in vitro and ex vivo: Primary human dermal fibroblasts from young and aged donors (24 and 88 years) were incubated in the presence or absence of 5mM βHB for 72 hours. The cells were stressed with 100uM tert-butyl-hydrogen peroxide for 3 hrs. Western blot protein expression analysis of antioxidants SOD2 and NQO1 demonstrated no change fibroblast expression of these antioxidants with ketone supplementation (A). Periwound tissue lysates were isolated on day three and day seven post wounding. Western blot protein analysis showed no change in SOD2 or NQO1 expression with BD or BMS+MCT supplementation (B). Two-way ANOVA with Tukey’s post-hoc analysis, results were considered significant when p<0.05. Error Bars Represent mean (SD).
Figure 5.7 Ketone supplementation does not significantly increase blood vessel density in day 7 aged ischemic wounds: Quantification of histological staining with CD31 demonstrated that exogenous ketone supplementation did not significantly increase blood vessel density at this time point. One-way ANOVA, Tukey’s post-hoc analysis, results were considered significant when $p<0.05$. Error Bars Represent mean (SD).
Figure 5.8 Ketone supplementation increases migration in young and aged HDFs: Young and aged HDFs were grown to confluence in 0.2% FBS 5mM glucose DMEM media, cells were treated with or without the presence of 5mM βHB for 72 Hrs, cells were scratched with 200uL yellow pipette tip. After 18 hours, migrated cells were counted using Dapi staining. Migration was increased in both young and aged HDFs compared to the controls (p=0.0260 and p=0.0004) (A, B). One-way ANOVA, Tukey’s post-hoc analysis, results were considered significant when p<0.05. Error Bars Represent mean (SD).
Figure 5.9 Ketone supplementation increases proliferation in young and aged HDFs: Young and aged HDFs were seeded with 75,000 cells in 10% FBS, 5mM Glucose DMEM media with or without the presence of 5mM βHB. Total cell number was counted at 24, 48, 72, 96, and 120 hours post seeding. Young and aged HDFs treated with 5mM βHB had significantly more cells at 72 and 96 hours (p<0.05). Two-way ANOVA with Tukey’s post-hoc analysis, results were considered significant when p<0.05. Error Bars Represent mean (SD).
Figure 5.10 Ketone supplementation decreases lactate production in young and aged HDFs: Young and aged primary human dermal fibroblasts were seeded with 250,000 cells in 10% FBS, 5mM glucose DMEM media with or without the presence of 5mM βHB. Extracellular lactate was measured in cellular media at 24, 48 and 72 hours using Nova Biomedical Lactate Plus blood lactate measuring meter. Young and aged HDFs treated with 5mM βHB produced significantly less extracellular lactate at 72 hours (p<0.05). Two-way ANOVA with Tukey’s post hoc analysis, results were considered significant when p<0.05. Error Bars Represent mean (SD).
5.4. References for Chapter 5:


CHAPTER 6: IMPLICATIONS FOR WOUND HEALING

6.1. Chapter Synopsis

In this chapter, we describe the major findings of this work based on the detailed discussion of the data in previous chapters. This chapter will discuss the major implications for wound healing as well as future directions for the project.

6.2. Implications for Wound Healing

The major goals of this dissertation work were to establish a method of sustaining nutritional ketosis via exogenous ketone administration, determine the healing capabilities of exogenous ketone supplementation in an aged rat population, and to acquire data of potential mechanisms of action. As mentioned earlier, there is a dire need for a therapy that can accelerate wound healing and efficiently restore normal tissue functionality. Chronic wounds, as discussed in Chapter 1, impact several million people annually and is a billion dollar growing market. The therapy presented within this dissertation exhibits great potential to accelerate cutaneous wound healing closure and to be used as a multifaceted tool to stimulate wound repair and regeneration in poor healing tissues especially chronic wounds in the aged population.

The KD has proven effective for the metabolic management of seizures and potentially numerous other disorders, but is limited clinically [1-26]. Exogenous ketone supplements are
primarily being developed as an alternative or adjuvant method of inducing therapeutic ketosis to free patients from the restrictiveness of the KD. In Chapter 3, we were able to clearly demonstrate that nutritional ketosis can be established with exogenous ketone supplementation without negatively affecting blood triglyceride and lipoprotein profiles. Importantly, exogenous ketone supplementation provides a tool for achieving ketosis in patients that are unable, unwilling, or uninterested in consuming a low carbohydrate or ketogenic diet. It may also help circumvent some of the difficulties associated with KD therapy initiation and maintenance, as it allows for rapid induction of ketosis in a dose-dependent fashion, eases the transition to nutritional ketosis, enhances the speed of keto-adaptation, and easily sustains ketosis with prolonged consumption. Simultaneously, it could provide patients with the opportunity to reap the benefits of ketosis without the practical and social difficulties of a highly restrictive diet. Additionally, we demonstrated the ability of several ketone supplements to elevate blood ketone levels, providing multiple options to induce therapeutic ketosis based on patient need. Many patients that previously were unable to benefit from the KD may now have an alternative method of achieving therapeutic ketosis. Ketone supplementation may also represent a means to further augment ketonemia in those that respond to the ketogenic diet, especially in those individuals where maintaining low glucose and insulin through carbohydrate restriction is important.

In Chapter 3, we demonstrated the exogenous ketone supplementation produced a hypoglycemic effect, which in Chapter 4, was no longer apparent in the aged rats. Further experiments need to be performed to determine what role age has explicitly on the observed hypoglycemic effect. Additionally, further experiments need to be conducted to determine the role that insulin sensitivity has in the age-dependent diminished hypoglycemic effect. As Chapter 3’s hypoglycemic effect was the result of an intragastric bolus and Chapter 4 transitioned to
food-integrated administration, future studies need to be conducted to determine the effects of administration on the observed hypoglycemic effect.

It should be noted that in these studies, rats were administered high doses due to their higher basal metabolic rate (5.6x faster than humans). Future studies are needed to optimize doses for humans. Preliminary data from the D’Agostino laboratory demonstrates that a 100 kg male can obtain elevated ketone levels at a 0.4 g/kg dose compared to the 5 g/kg and 10 g/kg doses that were administered to the rats. Follow up studies are currently being conducted to determine the safety and toxicity profile of the ketone supplements KE and BMS as BD is already FDA approved and MCT is a naturally occurring food source. Clarke and colleagues have already demonstrated the safety of a similar ketone ester administration in humans; thus, we don’t anticipate difficulty with the feasibility of using ketone supplements in humans [27]. However, one obstacle to overcome in the synthetic ketone supplements tested in this project is their palatability. Both BD and KE are not pleasing to taste and will need to be optimized for flavor without sacrificing their functionality. Additionally, future studies are needed to determine the best route of administration for ketone supplements in patients, it may be more suitable to have the supplements available in a variety of applications including parenteral nutrition, integrated into food that the patients could ingest, meal replacement drinks, and oral pills.

As described in Chapter 2, exogenous ketone supplementation has the potential to simultaneously target several underlying etiologies associated with age-impaired wound healing, including unrelieved inflammation, unquenched ROS production, insufficient blood flow, and dysfunctional metabolism. This is very different from most current wound therapies being studied, which target a specific molecular or cellular mechanism. Because of the complexity of wound healing, and the fact that there are often multiple mitigating factors that prevent normal
wound closure, most wound therapies are minimally effective. We hypothesize that using exogenous ketone supplementation, as a component of standard care would target the multifaceted etiologies underling most chronic wounds in the aged population. In Chapter 4, we demonstrated that both BD and BMS+MCT ketone supplement therapies were effective in enhancing wound healing in aged animals and the BMS+MCT also enhanced wound healing in young animals. Further studies are needed to confirm the optimal dosing protocol to maximize wound-healing efficacy.

Additionally, we elucidated some potential mechanisms of actions by which this novel proposed therapy was able to significantly enhance wound healing. We demonstrated that exogenous ketone supplementation altered the serum and hippocampal metabolome including increasing Kreb’s cycle intermediates, antioxidants carnosine and anserine, and the potent vasodilator adenosine. Further metabolomic studies need to be conducted to determine the effects of age on ketone supplementation changes to the metabolome. Furthermore, young and aged metabolomic studies of the wound healing environment with and without ketone supplementation would offer great insights to the wound healing process. Mechanistically, we demonstrated that ketone supplementation increases fibroblast proliferation and migration, reduces ROS production, reduces lactate production, and increases blood flow. However, we did not demonstrate a significant effect of ketone supplementation on antioxidants SOD2 or NQO1, further studies need to be conducted to determine if the administration of ketone supplements elicit the same physiological changes in other antioxidants. Further investigation into exploring potential mechanisms of action are needed to fully understand the impact exogenous ketone supplementation has on wound healing, specifically in an aged population. However, the complexity of wound healing and the multifaceted targets of ketone bodies may constrain
elucidating a single, clearly delineated mechanism of action.

Moreover, futures studies will explore a topical application of ketone bodies. Even though there was a significant increase in blood flow, this may not efficient for some patients; thus, a direct application of the ketones or a combination of both therapies may be more beneficial. Base on the results included in this dissertation we propose that exogenous ketone supplementation could offer a novel therapeutic option for chronic wounds, especially in the aging population.

6.3. References for Chapter 6


APPENDIX A: MATERIALS AND METHODS

A.1 Ethics Statement

All animal studies were approved by and performed within strict adherence to the University of South Florida and James A. Haley Veterans Hospital’s Institutional Animal Care and Use Committee (IACUC) protocols R00006 and V4251.

A.2 Materials and Methods for Experiments included in Chapter 3: Establishing Therapeutic Ketosis (Metabolic Therapy with Exogenous Ketone Supplementation)

A.2.1 Synthesis and Formulation of Ketone Supplements

KE was synthesized as previously described [1]. BMS is a novel agent (sodium/potassium- βHB mineral salt) supplied as a 50% solution containing approximately 375mg/g of pure βHB and 125 mg/g of sodium/potassium. Both KE and BMS were developed and synthesized in collaboration with Savind Inc. Pharmaceutical grade MCT oil (~65% caprylic triglyceride; 45% capric triglyceride) was purchased from Now Foods (Bloomingdale, IL). BMS was formulated in a 1:1 ratio with MCT at the University of South Florida (USF), yielding a final
mixture of 25% water, 25% pure βHB mineral salt and 50% MCT. BD was purchased from Sigma-Aldrich (Prod # B84785, Milwaukee, WI).

A.2.2. Daily Gavage to Induce Dietary Ketosis

Animal procedures were performed in accordance with the University of South Florida Institutional Animal Care and Use Committee (IACUC) guidelines (Protocol #0006R). Juvenile male Sprague-Dawley rats (275-325 g, Harlan Laboratories) were randomly assigned to one of six study groups: control (water, n=11), BD (n=11), KE (n=11), MCT (n=10), BMS (n=11), or BMS+MCT (n=12). Caloric density of standard rodent chow and dose of ketone supplements are listed in Table 1. On days 1-14, rats received a 5 g/kg body weight dose of their respective treatments via intragastric gavage. Dosage was increased to 10 g/kg body weight for the second half of the study (days 15-28) for all groups except BD and KE to prevent excessive hyperketonemia (ketoacidosis). Each daily dose of BMS would equal ~1000-1500 mg of βHB, depending on the weight of the animal. Intragastric gavage was performed at the same time daily, and animals had ad libitum access to standard rodent chow 2018 (Harlan Teklad) for the duration of the study. The macronutrient ratio the standard rodent chow was 62.2%, 23.8% and 14% of carbohydrates, protein and fat respectively.
Table A.1. Caloric density of standard rodent chow and dose of ketone supplements

<table>
<thead>
<tr>
<th>Macronutrient Information</th>
<th>Standard Diet</th>
<th>Water</th>
<th>BMS+MCT</th>
<th>BMS</th>
<th>MCT</th>
<th>KE</th>
<th>BD</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Cal from Fat</td>
<td>18.0</td>
<td>0.0</td>
<td>N/A</td>
<td>50.0</td>
<td>100.0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>% Cal from Protein</td>
<td>24.0</td>
<td>0.0</td>
<td>N/A</td>
<td>N/A</td>
<td>0.0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>% Cal from Carbohydrates</td>
<td>58.0</td>
<td>0.0</td>
<td>N/A</td>
<td>N/A</td>
<td>0.0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Total Caloric Density (Kcal/g)</strong></td>
<td>3.1</td>
<td>0.0</td>
<td>5.1</td>
<td>1.9</td>
<td>8.3</td>
<td>5.6</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Dose 0-14 Days (g/kg)  
*ad libitum* | N/A  | 5.0 | 5.0 | 5.0 | 5.0 |

Dose 15-28 Days (g/kg)  
*ad libitum* | N/A  | 10.0| 10.0| 10.0| 5.0 |

A.2.3. Measurement and analysis of blood glucose, ketones, and lipids

Every 7 days, animals were briefly fasted (4 hrs, water available) prior to intragastric gavage to standardize levels of blood metabolites prior to glucose and βHB measurements at baseline. Baseline (time 0) was immediately prior to gavage. Whole blood samples (10 µL) were taken from the saphenous vein for analysis of glucose and βHB levels with the commercially available glucose and ketone monitoring system Precision Xtra™ (Abbott Laboratories, Abbott Park, IL). Blood glucose and βHB were measured at 0, 0.5, 1, 4, 8, and 12 hrs after test substance administration, or until βHB returned to baseline levels. Food was returned to animals after blood analysis at time 0 and gavage. At baseline and week 4, whole blood samples (10 µL) were taken from the saphenous vein immediately prior to gavage (time 0) for analysis of total cholesterol, high-density lipoprotein (HDL), and triglycerides with the commercially available CardioChek™ blood lipid analyzer (Polymer Technology Systems, Inc., Indianapolis, IN). Low-density lipoprotein (LDL) cholesterol was calculated from the three measured lipid levels using the Friedewald equation: (LDL Cholesterol = Total Cholesterol - HDL - (Triglycerides / 5))[2,
Animals were weighed once per week to track changes in body weight associated with hyperketonemia.

**A.2.4. Organ Weight and Collection**

On day 29, rats were sacrificed 4-8 hrs after intragastric gavage which correlated to the time range where the most significantly elevated blood βHB levels were observed. Brain, lungs, liver, kidneys, spleen and heart were harvested, weighed (AWS-1000 1kg portable digital scale (AWS, Charleston, SC)), and flash-frozen in liquid nitrogen or preserved in 4% paraformaldehyde for future analysis.

**A.2.5. Statistics**

All data are presented as the mean ± standard deviation (SD). Data analysis was performed using GraphPad PRISM™ version 6.0a and IBM SPSS Statistics 22.0. Results were considered significant when p<0.05. Triglyceride and lipoprotein profile data were analyzed using One-Way ANOVA. Blood ketone and blood glucose were compared to control at the applicable time points using a Two-Way ANOVA. Correlation between blood βHB and glucose levels in ketone supplemented rats was compared to controls using ANCOVA analysis. Organ and body weights were analyzed using One-Way ANOVA. Basal blood ketone and blood glucose levels were analyzed using Two-Way ANOVA. All mean comparisons were carried out using Tukey’s multiple comparisons post-hoc test.
A.2.6. Metabolomics Profiling of KE and BMS+MCT Treated Rats

On day 29, approximately 500 uL of whole blood was collected from the saphenous vein of control, KE, and BMS+MCT-treated animals 4 hours post-intragastric gavage. Serum was separated by centrifugation using Microtainer© Tubes with Serum Separator (Becton Dickinson). Serum was transferred to screw-to cryovials and flash frozen in liquid nitrogen. Frozen serum samples were shipped to Metabolon, Inc. for global metabolomics profiling using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC-MS). The concentration of 388 named biochemicals of known identity was analyzed in the serum samples. Welch’s two-sample t-test was used to determine which metabolites differed significantly in concentration between ketone supplemented animals and controls. Results were considered significant when p<0.05.

A.3. Materials and Methods for Experiments included in Chapter 4: Enhancing Wound Healing With Metabolic Therapy

A.3.1. Synthesis and Formulation of Ketone Precursors

BMS is a novel agent (sodium/potassium- βHB mineral salt) supplied as a 50% solution containing approximately 375mg/g of pure βHB and 125 mg/g of sodium/potassium. Both KE and BMS were developed and synthesized in collaboration with Savind Inc. Pharmaceutical
grade MCT oil (~65% caprylic triglyceride) was purchased from Now Foods (Bloomingdale, IL). BMS was mixed in a 1:1 ratio with MCT at the University of South Florida (USF), yielding 25% water, 25% pure \( \beta \)HB mineral salt and 50% MCT. BD was purchased from Sigma-Aldrich (Prod # B84785, Milwaukee, WI).

**A.3.2 Animal Gavage to Induce Dietary Ketosis**

Animal procedures were performed in accordance with the University of South Florida Institutional Animal Care and Use Committee (IACUC) guidelines (Protocol #V4251) and abided by all requirements of the Animal Welfare Act and the Guide for Care and Use of Laboratory Animals. Healthy 8 month (young) and 20 month (aged) male Fisher 344 rats (Harlan Laboratories and NIA) were randomly assigned to one of three groups: Control (water, \( n \geq 6 \)), BD (\( n \geq 6 \)), or BMS+MCT (\( n \geq 6 \)). Rats received one 6.5 g/kg dose of their respective treatments via intragastric gavage. Animals had *ad libitum* access to standard rodent chow 2018 (Harlan Teklad) for duration of the study. Whole blood samples (10 \( \mu \)L) were taken from the saphenous vein for analysis of glucose and \( \beta \)HB levels with the commercially available glucose and ketone monitoring system Precision Xtra™ (Abbott Laboratories, Abbott Park, IL). The Precision Xtra™ ketone monitoring system only measures \( \beta \)HB blood levels; therefore, total blood ketone levels would be higher than measured. Blood glucose and \( \beta \)HB were measured at 0, 0.5, 1, 4, 8, 12, and 24 hrs after test substance administration.
A.3.3. Surgical Procedure

Healthy 8 month (young) and 20 month (aged) Fisher 344 rats (National Institutes of Aging, Bethesda, MD) were utilized to create and ischemic wound model adapted from our previous work [4-6]. In all Fisher 344 rats, a dorsal bi-pedicle 10.5x3 cm flap was surgically created on day 0 (time of wounding), in which a silicone sheet was placed under the panniculus carnosus fascia and above the paraspinoius muscles, limiting revascularization and causing the flap to become ischemic. Two 6mm punch biopsies created full thickness wounds extending just above the fascia within the flap (ischemic wounds) and two 6mm punches were created laterally to ischemic flap giving the control (non-ischemic) wounds in the same animal.

A.3.4. Diet Administration

The day before flap creation, animals are fasted overnight (~18hrs) to enable initiation of the diet immediately upon waking from surgery. On the day of ischemic flap creation (day 0), young and aged rats were randomly assigned to one of three study groups: Control standard diet (SD), BD or BMS+MCT. Control rats were fed a combination of standard rodent chow (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories), water, and peanut butter mixed into a pudding and fed ad libitum. Rats in BD group received a pudding diet of standard rodent chow, peanut butter, water, 1% saccharine, and 20% 1,3-Butanediol by weight ad libitum. Rats in the BMS+MCT group received a pudding diet of standard rodent chow, peanut butter, water, 1% saccharine, and 10% MCT oil by weight and 10% βHB mineral salt solution (10% βHB) by weight ad libitum. Diets were continuously replaced to maintain freshness and to allow rat to feed ad libitum.
A.3.5. βHB, Glucose, and Weight Measurements

Every 7 days, whole blood samples (10 µL) were taken from the saphenous vein for analysis of glucose and βHB levels with the commercially available glucose and ketone monitoring system Precision Xtra™ (Abbott Laboratories, Abbott Park, IL) to confirm that the dietary ingestion of BD and BMS+MCT induced continuous ketosis in the blood. Rats were weighed 2x weekly with scales (Mittler Toledo SB16001) available in the Morsani College of Medicine vivarium. Blood and weight measurements were taken at the same time of day each week to limit variation based on normal metabolism fluctuations.

A.3.6. Analysis of Blood Flow and Wound Size

Every 7 days, while under anesthesia, Laser Doppler scanning was used to measure blood flow over the course of the study. On days 3, 7, 10, 14, 21, 25, and 28 animals were anesthetized, both ischemic and non-ischemic wounds were digitally photographed, and wound sizes were determined (Bamboo Create v5, Wacom Co, Ltd, and Image J 64 Version 10.2).

A.3.7. Extraction of Tissue

On day 28 of the study, the rats were euthanized by carbon dioxide (CO2) asphyxiation where periwound tissue (tissue surrounding the wound) including the wound, spleen and liver were surgically harvested. Portions of the spleen, liver and wounds were flash frozen by liquid
nitrogen, formalin-fixed, or preserved in optimal cutting temperature (OCT) freezing media for future analysis.

A.3.8. Statistics

All data are presented as the mean ± standard deviation (SD). Data analysis was performed using GraphPad PRISM™ version 6.0a. Results were considered significant when p<0.05. Blood ketone and blood glucose were compared to controls at the applicable time points using a Two-Way ANOVA. Correlation between blood βHB and glucose levels was determined by linear regression analysis. Blood flow and wound healing progression were analyzed compared to controls at the applicable time points using Two-Way ANOVA. Organ and body weights were analyzed using One-Way ANOVA. All mean comparisons were carried out using Tukey’s multiple comparisons post-hoc test.


A.4.1. Cytokine Array

After 3 days and 7 days post-wounding, young and aged Fischer rats were humanely euthanized and per-wound tissue was harvested and preserved in liquid nitrogen. 500 mg of periwound tissue was measured and razor blade minced on a petri dish in 400 µL of RIPA Lysis
Buffer (Sigma Chemical Co, St. Louis, MO) with 10 µL / mL of Halt protease and phosphatase inhibitor cocktail (Pierce, Rockford, IL). Minced tissue with liquid was placed into lysing matrix-A tubes (MP Biomedical, Santa Ana, CA). An additional 400 µL of RIPA Lysis Buffer was used to wash petri dish and added to the tube. Tissues were homogenized for 20 seconds using FastPrep®-24 Instrument (MP Biomedical, Santa Ana, CA). Tubes were centrifuged at 16,000xg for 10 minutes, supernatants were transferred to a clean 1.5 mL conical tube, and lysates were sonicated at low power setting and centrifuged at 16,000xg for 10 minutes. The supernatant of the lysates was frozen at -80° for future use. Protein concentration of the lysate supernatant was determined using Pierce BCA protein Assay Kit (Rockford, IL) reading absorbance at 540 nanometers. 50µg of samples were sent to Eve Technologies Inc. (Calgary, Alberta, Canada), where they were analyzed on Bio-Plex 200 Systems (Bio-Rad Laboratories, Inc., Hercules, CA) via Rat Cytokine Array/ Chemokine Array 27-Plex/ Discovery Assay, a multiplex assay that measures twenty-seven inflammatory biomarkers in a single microwell (Millipore MILLIPLEX®, Billerica, MA).

A.4.2. Macrophage Profile Analysis with Immunofluorescence of CD68, IRF-5, CD206

After 3 days and 7 seven days post-wounding, young and aged Fischer 344 rats were humanely euthanized and peri-wound tissue, livers, and spleen were harvested and preserved in 10% neutral, paraformaldehyde or phosphate buffered formalin. The peri-wound tissue was paraffin-embedded, cut into 5-micron sections, and mounted onto microscope slides at the USF Morsani College of Medicine Histology Core. One set of slides was stained with hematoxylin and eosin and mounted with cover slips for morphological analysis. Another set of slides was co-
probed with either interferon regularity factor-5 (IRF-5) to label M1 pro-inflammatory macrophages or CD206 to label M2 anti-inflammatory macrophages and CD68 to label all macrophages. These slides were rehydrated through a series of xylene and ethanol washes, and antigen was retrieved via standard heat-mediated citrate buffer method. Slides were incubated in 0.3% H$_2$O$_2$ in dH$_2$O to inhibit endogenous peroxide activity. Immunofluorescent analysis of the slides was performed using the VECTASTAIN Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA). Slides were blocked for 60 minutes in normal anti-rabbit blocking serum, and then incubated in anti-IRF-5 antibody (1:100, ab175317, Abcam) or ant-CD206 antibody (1:100, ab64693, Abcam) over night at 4 degrees in a humidified container. Slides were then incubated in ALEXA Fluor 568 donkey anti-rabbit secondary antibody (Life Technologies, Eugene, OR) in normal blocking serum for 30 mints at room temperature in the dark, followed by VECTASTAIN ABC reagent containing avidin and biotinylated horseradish peroxidase solution for 30 minutes at room temperature in the dark. Slides were blocked for 60 minutes in normal anti-mouse blocking serum in the dark, and then incubated in anti-CD68 antibody (1:25, ab955, Abcam) over night at 4 degrees in a dark humidified container. Slides were then incubated in ALEXA Fluor 488 donkey anti-mouse secondary antibody (Life Technologies, Eugene, OR) in normal anti-mouse blocking serum for 30 mints at room temperature in the dark, followed by VECTASTAIN ABC reagent containing avidin and biotinylated horseradish peroxidase solution for 30 minutes at room temperature. Slides were wet mounted with VECTASHIELD mounting media with DAPI (Vector Laboratories Inc., Burlingame, CA) and sealed with clear nail polish.
A.4.3. Cellular ROS production

*DHE*

Cells were grown on 6-well poly-d lysine coated plates in DMEM +Glutamax 4.5g/L glucose media with 10% FBS and 1% penicillin and streptomycin until 50-60% confluent, appropriate wells were grown with or without the addition of 5 mM βHB to the media for 72 hours. After 72 hours, they were then serum starved for 18 hrs with DMEM phenol red free 4.5 g/L glucose + L-Glutamine media with 0.2% FBS and 1% penicillin and streptomycin. At the end of the 18 hours, cells were subjected to either 100uM tBOOH (Sigma Chemical Co, St. Louis, MO) in serum starve media for three hours or media only. At the end of three hours, the media was taken off and the cells were treated with 5 μM Dihydroethidium (DHE) in DMSO (Life Technologies, Eugene, OR) dissolved in serum starve media for 30min. The cells were put into Hank’s BSS and fluorescence was read on the plate reader at [528, 617] excitation and emission. 3 wells in each grouping did not receive the DHE and the average RFUs of these wells were used as a blank to normalize the readings. The control values have an N=3 (total N=9) and the 100 μM has an N=6 (total N=18). Graph and statistics were created using GraphPad PRISM™ for Mac OSX version 6.0a, statistical significance was determined using a one-way ANOVA with Tukey’s post-hoc analysis.

*Mitosox Red*

Cells were grown on 6-well poly-d lysine coated plates in DMEM +Glutamax 4.5g/L glucose media with 10% FBS and 1% penicillin and streptomycin until 50-60% confluent,
appropriate wells were grown with or without the addition of 5 mM βHB to the media for 72 hours. After 72 hours, they were then serum starved for 18 hrs with DMEM phenol red free 4.5 g/L glucose + L-Glutamine media with 0.2% FBS and 1% penicillin and streptomycin. Cells were subjected to 100μM tBOOH (Sigma Chemical Co, St. Louis, MO) in serum starve media for three hours or media only. At the end of three hours, the media was taken off and the cells were treated with 1 μM Mitosox Red (Life Technologies, Eugene, OR) in Hank’s Balanced Salt Solution (with magnesium and calcium) for 10 min. The cells were put into fresh Hank’s BSS and fluorescence was read on the plate reader at [510, 580] excitation and emission. 3 wells in each grouping did not receive the Mitosox Red and the average RFUs of these wells were used as a blank to normalize the readings. The control values have an N=3 (total N=12) and the 100 uM has an N=6 (total N=24). Graph and statistics were created using GraphPad PRISM™ for Mac OSX version 6.0a, statistical significance was determined using a one-way ANOVA with Tukey’s post-hoc analysis.

A.4.4. Western Blot Analysis for in vitro and ex vivo SOD2 and NQO1

In Vitro

Cells were grown on 100 mm poly-d lysine coated petri dishes in DMEM +Glutamax 4.5 g/L glucose media with 10% FBS and 1% penicillin and streptomycin until cells were around 50-60% confluent. Cells were changed to DMEM phenol red free 5mM glucose + L-Glutamine media with 10% FBS and 1% penicillin and streptomycin and appropriate cells were treated with 5mM βHB for 72 hours. Media was replaced every 24 hours. After 72 hours, appropriate cells were subjected to 100μM tBOOH (Sigma Chemical Co, St. Louis, MO) or media only. At the
end of three hours, the media was taken off, cells were washed with 1mL 1x cold PBS, then incubated with 300 uL per well of Triton X-100 Lysis Buffer [containing 50mM Tris-HCl pH 7.4, 150 mM NaCl, 5mM EDTA and 1% Triton-X 100], with 10 uL/mL of Halt protease and phosphatase inhibitor cocktail (Pierce, Rockford, IL) for 30 minutes at 4° Celsius. The cells were scrape-harvested, and three wells were pooled for each sample. The lysates were sonicated at low power setting and centrifuged at 16,000xg for 10 minutes. The supernatant of the lysates was frozen at -80° for future use. Protein concentration of the lysate supernatant was determined using Pierce BCA protein Assay Kit (Rockford, IL) reading absorbance at 540 nanometers. 20 µg of samples were separated on a 4-20% SDS gel and transferred to nitrocellulose membrane. Membrane was blocked with 5% Milk in TBS-T (50 mM Tris HCl, 150 mM NaCl and 0.1% Tween 20, pH 7.5) and then probed with anti-SOD2 antibody (1:2500, ab13533, Abcam), anti-NQO1 antibody (1:500, ab34173, Abcam) followed by anti Pan-Actin antibody (1:3000, Cell Signaling, Boston, MA) as a loading control and Anti-Rabbit IgG, HRP-linked (1:3000, Cell Signaling, Boston, MA) as secondary antibody. Membrane was developed using Pierce ECL Western Blotting Substrate (Rockford, IL). Quantitative densitometry was determined using Image J software version 1.45s and graph and statistics were determined using GraphPad PRISM™ for Mac OSX version 6.0a. Data in graph is represented as an N=3 for each group.

Ex vivo

After 3 days and 7 days post-wounding, young and aged Fischer rats were humanely euthanized and per-wound tissue was harvested and preserved in liquid nitrogen. 500 mg of periwound tissue was measured and razor blade minced on a petri dish in 400 µL of RIPA Lysis
Buffer (Sigma Chemical Co, St. Louis, MO) with 10 µL / mL of Halt protease and phosphatase inhibitor cocktail (Pierce, Rockford, IL). Minced tissue with liquid was placed into lysing matrix-A tubes (MP Biomedical, Santa Ana, CA). An additional 400 µL of RIPA Lysis Buffer was used to wash petri dish and added to the tube. Tissues were homogenized for 20 seconds using FastPrep®-24 Instrument (MP Biomedical, Santa Ana, CA). Tubes were centrifuged at 16,000xg for 10 minutes, supernatants were transferred to a clean 1.5 mL conical tube, and lysates were sonicated at low power setting and centrifuged at 16,000xg for 10 minutes. The supernatant of the lysates was frozen at -80° for future use. Protein concentration of the lysate supernatant was determined using Pierce BCA protein Assay Kit (Rockford, IL) reading absorbance at 540 nanometers. 20 µg of samples were separated on a 4-20% SDS gel and transferred to nitrocellulose membrane. Membrane was blocked with 5% Milk in TBS-T (50 mM Tris HCl, 150 mM NaCl and 0.1% Tween 20, pH 7.5) and then probed with anti-SOD2 antibody (1:2500, ab13533, Abcam), anti-NQO1 antibody (1:500, ab34173, Abcam) followed by anti Pan-Actin antibody (1:3000, Cell Signaling, Boston, MA) as a loading control and Anti-Rabbit IgG, HRP-linked (1:3000, Cell Signaling, Boston, MA) as secondary antibody. Membrane was developed using Pierce ECL Western Blotting Substrate (Rockford, IL). Quantitative densitometry was determined using Image J software version 1.45s and graph and statistics were determined using GraphPad PRISM™ for Mac OSX version 6.0a. Data in graph is represented as an N=6 for each group.
A.4.5 Immunohistochemistry Analysis of Periwound Tissue Vascularization

After 3 days and 7 seven days post-wounding, young and aged Fischer 344 rats were humanely euthanized and peri-wound tissue, livers, and spleen were harvested and preserved in 10% neutral, phosphate buffered formalin. The peri-wound tissue was paraffin-embedded, cut into 5-micron sections, and mounted onto microscope slides at the USF Morsani College of Medicine Histology Core. One set of slides was stained with hematoxylin and eosin and mounted with cover slips for morphological analysis. Another set of slides was probed for the endothelial cell marker CD31 to visualize blood vessels. These slides were rehydrated through a series of xylene and ethanol washes, and antigen was retrieved via standard heat-mediated citrate buffer method. Slides were incubated in 0.3% H$_2$O$_2$ in dH$_2$O to inhibit endogenous peroxide activity. Immunohistochemical analysis of the slides was performed using the VECTASTAIN Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA). Slides were blocked for 60 minutes in normal blocking serum, and then incubated in anti-CD31 antibody (1:25, ab28364, Abcam) over night at 4 degrees Celsius in a humidified container. Slides were then incubated in biotinylated anti-rabbit secondary antibody in normal blocking serum for 30 mints at room temperature, followed by VECTASTAIN ABC reagent containing avidin and biotinylated horseradish peroxidase solution for 30 minutes at room temperature. Slides were developed using the VECTOR NovaRED Peroxidase (HRP) Substrate Kit (Vector Laboratories Inc., Burlingame, CA). Slides were counterstained with hematoxylin, washed, mounted with coverslips, sealed with clear nail polish, and visualized and scanned with light microscope via Moffitt Core Facilities. The entire periwound tissue was analyzed for blood vessels. The number of CD31+ blood vessels in the peri wound tissue were counted between control and treated groups as a measure of wound
vascularization. Statistical analysis was performed via one-way ANOVA with Tukey’s post-hoc analysis. Results were considered significant when p<0.05.

**A.4.6 Migration Analysis of young and aged HDFs**

Cells were grown on 6-well poly-d lysine coated plates in DMEM +Glutamax 4.5g/L glucose media with 10% FBS and 1% penicillin and streptomycin until 75-80% confluent. Cells were changed to DMEM phenol red free 5mM glucose + L-Glutamine media with 10% FBS and 1% penicillin and streptomycin and appropriate cells were treated with 5mM βHB for 72 hours. Media was replaced every 24 hours. After 72 hours, cells were then serum starved for 18 hrs with DMEM phenol red free 4.5 g/L glucose + L-Glutamine media with 0.2% FBS and 1% penicillin and streptomycin. There was not a need to use mytomycin to stop proliferation since used the serum free media. After 18 hours, two scratches were created per well using a 200 µL pipette tip. Wells were rinsed with 1mL RT Hank’s BBS to remove floating and loose cells. Wells were visualized using phase contrast microscopy and a picture was taken for baseline comparison. After 18 hours, cells were fixed with 4% paraformaldehyde for 15 minutes, rinsed with RT 1x PBS, and a cover slip was placed in the well using VECTASHIELD mounting media with DAPI (Vector Laboratories Inc., Burlingame, CA) and sealed with clear nail polish. Wells were visualized using phase contrast and fluorescent microscopy, and number of cells that migrated into scratch area was counted. Statistical analysis was performed via one-way ANOVA with Tukey’s post-hoc analysis. Results were considered significant when p<0.05.
A.4.7. Proliferation Analysis of young and aged HDFs

75,000 young and aged HDF cells were seeded on 6-well poly-d lysine coated plates in DMEM phenol red free 5mM glucose + L-Glutamine media with 10% FBS and 1% penicillin and streptomycin with or without the presence of 5 mM βHB. Media was replaced every 24 hours. Total cell number was counted at 24, 48, 72, 96, and 120 hours post seeding. At given time point, media was removed and cells were washed with 1 mL sterile RT 1x PBS, cells were suspended using 250 µL (0.25% Trypsin in Hank’s Phenol Red BBS) and 750 µL of appropriate media (with or with out βHB). Total cell density was measured using hemocytometry. Two-way ANOVA with Tukey’s post-hoc analysis, results were considered significant when p<0.05. Error Bars Represent mean (SD).

A.4.8. Lactate Quantification of young and aged HDFs

Young and aged primary HDFs were seeded with 250,000 cells on 6-well poly-d lysine coated plates in DMEM phenol red free 5mM glucose + L-Glutamine media with 10% FBS and 1% penicillin and streptomycin with or without the presence of 5 mM βHB. Extracellular lactate was measured in 10 µL of cellular media at 24, 48 and 72 hours post-seeding using Nova Biomedical© Lactate Plus (Nova Biomedical, Waltham, MA) blood lactate measuring meter. Two-way ANOVA with Tukey’s post-hoc analysis, results were considered significant when p<0.05. Error Bars Represent mean (SD).
A.5. References for Appendix A


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This appendix contains the original publications for the following references:


Demonstration of the Rat Ischemic Skin Wound Model

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URL: http://www.jove.com/video/52637
DOI: doi:10.3791/52637

Keywords: Medicine, Issue 98, Wound Healing, Ischemia, rat, animal model; chronic wounds, laser Doppler, bipedicled flap

Date Published: 4/1/2015


Abstract

The propensity for chronic wounds in humans increases with aging, disease conditions such as diabetes and impaired cardiovascular function, and unrelied pressure due to immobility. Animal models have been developed that attempt to mimic these conditions for the purpose of furthering our understanding of the complexity of chronic wounds. The model described herein is a rat ischemic skin flap model that permits a prolonged reduction of blood flow resulting in wounds that become ischemic and resemble a chronic wound phenotype (reduced vascularization, increased inflammation and delayed wound closure). It consists of a bipedicled dorsal flap with 2 ischemic wounds placed centrally and 2 non-ischemic wounds lateral to the flap as controls. A novel addition to this ischemic skin flap model is the placement of a silicone sheet beneath the flap that functions as a barrier and a scaffold to prevent revascularization and reduce contraction as the wounds heal. Despite the debate of using rates for wound healing studies due to their quite distinct anatomic and physiologic differences compared to humans (i.e., the presence of a paravascular cambium muscle, short life-span, increased number of hair follicles, and their ability to heal infected wounds) the modifications employed in this model make it a valuable alternative to previously developed ischemic skin flap models.

Video Link

The video component of this article can be found at http://www.jove.com/video/52637/

Introduction

Effective drug development and other wound healing therapeutics require appropriate in vivo models, despite known problems in translating findings in animal models to human therapies.1 What follows is a description of a detailed protocol for the use of a rat model of ischemic skin wound healing to investigate mechanisms that further the understanding of pathological wound healing. The rat species, often employed due to its wide availability, size and simple anatomy, is used for wound healing studies as it is large enough to provide a suitable skin area for incisional and excisional wounding, imaging and tissue collection.2 However it should be taken into careful consideration that the skin of a rat and a human are different anatomically, with rats being referred to as loose-skinned animals. This distinct characteristic allows for wound contraction, rather than epithelialization to contribute significantly to the closing of the rat skin wounds.3 Additionally, the presence of a subcutaneous paravascular cambium carnosus muscle in rats, contributes to healing by both contraction and collagen formation.4,5 These very important anatomical distinctions were considered in the development of the rat ischemic skin wound model and specific modifications were implemented to decrease wound contraction and reduce the influence of the paravascular cambium carnosus muscle.6

In diabetic foot ulcers, venous leg ulcers, and pressure ulcers, healing is delayed and these wounds are considered chronic. The wounds are characterized by excessive inflammation, which prevents the wound from progressing to the next phase of wound repair.6 One of the main features in the development of a chronic wound is localized tissue ischemia (reduced blood flow) contributing to the inability to clear inflammation. At the time of which this model was being developed and validated (2003-4), there were no standardized animal models that could provide enough tissue to test induction of angiogenesis in the wound bed, a key stage during normal wound healing and the motivation for developing this model.7 That said, the model presented here is a modification of the ischemic wound model originally described by Schwartz et al.8 and subsequently used in modified form by Chan et al.9

In the modified ischemic wound model, changes were made to circumvent the above mentioned anatomical characteristics of the rat that lead to healing by contraction rather than epithelialization: (1) Two full-thickness excisional wounds are created within a bipedicled dorsal skin flap and the paravascular cambium muscle is removed from the wound bed by dissecting just above the muscle fascia. (2) The flap itself has more narrow dimensions, ensuring that blood supply is random and the wounds located at the midpoint of the flap are ischemic. (3) A silicone sheet is inserted beneath the flap, preventing revascularization and reperfusion of the flap from underlying tissue. Wound contraction is limited (not eliminated) by anchoring or suturing the flap to the silicone sheet.10
The model has recently been used in studies ranging from hyperbaric oxygen effects on ischemic wound healing to ischemic wound healing in young versus aged rats and has proven to be a reliable model of prolonged tissue ischemia. The dimensions of the bipedicled flap have also been adapted to different rat strains, including Sprague Dawley (11 cm long by 2 cm wide) and F344 rats (10.5 cm long by 3.0-3.5 cm wide) and other species, including swine and mice. This video utilizes the F344 Sprague Dawley rat strain in the demonstration of the ischemic skin wound model.

NOTE: Approval for all animal procedures presented below was obtained from the University of South Florida's Animal Care Committee (IACUC) and abide by all requirements of the Animal Welfare Act and the Guide for Care and Use of Laboratory Animals.

### Protocol

1. **Preparation of Silicone Sheets and Surgical Instruments**
   1. **Prep** fresh strip (10.5 cm x 3.0 cm) of non-reinforced 0.01 thickness, medical grade silicone sheeting and sterilize using an autoclave.
   2. Clean and sterilize suitable surgical instruments (scissors, forceps and drapes or towels to create a sterile field during surgery).

2. **Experimental Animals**
   1. Use adult male or female rats weighing in range from 250-350 g obtained from a commercial breeder. If aged rats are utilized, they should be >350 g in order to ensure better survival post-surgery. Prior to the start of any experiments acclimate all animals for at least 7 days under standard conditions of a 12 hr light-dark cycle with food and water ad libitum.

3. **Anesthesia, Pre-operative Analgesia and Operative Preparation**
   1. Induce general anesthesia by using isoflurane at 3%-4% via an induction chamber and maintain (via use of a nose cone) at 1%-2% with O₂ during skin preparation and surgery. Monitor the depth of anesthesia by observation of the rate and depth of respiration, interdigital pinch or palpebral blink reflex.
   2. In a location remote from the sterile surgical area, place the rat in the prone position and shave the dorsum with clippers from the base of the neck down approximately 11 cm. Stencil with permanent marker, outline for the 3.0 cm x 10.5 cm flap (see Figure 1A).
   3. Move the rat to a clean, designated surgical area equipped with an approved heating pad and sterile surgical drapes or towels. Inject 5 mg/kg Ketoprofen subcutaneously prior to the first surgical incisions for pain management. Additional fluids (saline) can be given (up to 3 cc) subcutaneously as needed.
   4. Prepare the skin further by scrubbing first with 70% isopropyl alcohol and second with 0.2% chlorhexidine; then apply sterile drapes to create a sterile field. 10% povidone-iodine (Betadine) can also be used.
   NOTE: An antibiotic (ampicillin at 15 mg/kg) can be administered subcutaneously, but if good aseptic technique is used it is not required.

4. **Creating Excisional Wounds and Bipedicled Flap**
   1. Using a sterile, disposable 5 mm biopsy punch tool, create two circular "ischemic" wounds in the center of the designated flap area (Figure 1B). The depth of the wound should be deep to (not through) the underlying fascia of the parascapular muscle (Figure 1B inset).
   2. Using forceps lift the skin in the middle of the wound outline created by the punch biopsy and then use iris scissors (with curved tips) to excise the circular piece of tissue (including the parascapular muscle). The result will be a full-thickness wound with the fascia as the base of the wound.
   NOTE: The excised tissue (wound plug) can be snap frozen in liquid nitrogen or fixed in 10% buffered formalin for later processing as control, normal skin.
   3. Create a bipedicled flap by making incisions with a sterile scalpel on each side of the ischemic wounds along the pre-drawn lines (Figure 1C) that are 10.5 cm in length and 3.0 cm apart. The depth of the incisions should be down to the parascapular muscles. Using iris scissors, separate the parascapular fascia from the parascapular muscles, being careful to keep the fascia intact as the "base" of the 6 mm punch (Figure 1D).
   4. Take 1 sterile pre-cut silicone sheet and place it in between the parascapular fascia and the parascapular muscles (Figure 1E) ensuring that the sheet does not buckle or fold. Using black, non-absorbable sutures (size 4.0) close both incisions by anchoring the silicone sheet to the skin with at least 8 interrupted stitches on each side, along the length of the flap (Figure 1F and 1G).
   5. Using a sterile, disposable biopsy punch tool, create two internal control "non-ischemic" wounds (down to the anterior fascia of the parascapular muscle) 1 cm lateral to the ischemic wounds on either side of the flap (Figure 1G).
   6. Place a ruler below the wounds and take digital photos for wound measurement purposes (see Figure 3A). At this time, blood flow (perfusion) can be monitored by use of laser Doppler or other manipulations ( topical drug placement) performed.
   7. Apply an approved liquid adhesive both cranial and caudal to the wounds and a transparent film dressing to keep the wound environment moist and clean (sterile). An additional dressing may be placed at the caudal end of the flap to prevent the animal from removing the most caudal sutures.
5. Post-operative Procedures

1. Place animals in cages (singly housed) equipped with shallow feeders so as to prevent the surgical site from rubbing against the feeder. Animals should not be left unattended or returned to the company of other animals until they regain sufficient consciousness to maintain normal recumbency and exhibit purposeful movement. Heating mats should be placed under half of the cage for up to 2 days during recovery.
2. To manage pain post-operatively, administer Ketoprofen (0 mg/kg) subcutaneously to animals the following morning and 1x per day for up to 48 hr post-surgery. Animals should also be monitored daily for prolonged signs of pain, weight loss or surgical site infections.

6. Subsequent Wound Measurements and Dressing Changes

1. Measure ischemic and non-ischemic wounds frequently under general anesthesia using isoleucine at 3%-4% via an induction chamber and maintained (via a nose cone) at 1%-2% with O₂ as in step 3.1.
2. Remove the dressing gently as to not pull the adhesive from the skin. At this time additional digital photos are taken for wound measurements, topical treatments re-applied, laser Doppler imaging (LDI) or other manipulations performed to suit the investigator's needs.
3. Apply adhesive and a clean dressing to allow the animal to recover as in step 3.1.

7. Wound Collection and Euthanasia

1. Harvest ischemic and non-ischemic wounds (on days the investigator deems appropriate) while the animal is under general anesthesia using isoleucine at 3%-4% via an induction chamber and maintained (via a nose cone) at 1%-2% with O₂ as in step 3.1.
2. Using a scalpel, make a square shaped excision around the wound to include some healthy tissue around the wound. Place the excision into a 1.5 ml snap cap tube and snap freeze in liquid nitrogen (store at -80°C) for future molecular analysis or incubate in 10% buffered formalin at RT for histological processing.
   *NOTE:* The wound excisions can also be cut in half to provide more samples for analysis.
3. After wound tissue removal, euthanize the animal using the approved method of CO₂ inhalation.

Representative Results

The rat ischemic wound healing model protocol should take approximately 20 min per animal if performed efficiently. Prior to application of a dressing the model should appear as represented in Figure 1A. It will be important to verify that the bipedicled flap and wounds therein are ischemic. Subcutaneous oxygen tension (PscO₂) at the level of the wound has been measured during validation of this model by placing a polarographic electrode in the subcutaneous tissue between the two ischemic wounds. PscO₂ values were in the critically ischemic range (23-40 mmHg). Since the development of this model the use of LDI has increasingly been used to measure blood perfusion and this technique will give adequate information about the ischemic state of the bipedicled flap.

Briefly, the LDI technique is based on the emission of a beam of laser light carried by a fiber-optic probe. The measuring depth depends on the tissue properties and wavelength of the laser light. In normal skin, an instrument with a probe with standard fiber separation (0.25 mm) and a 780 nm wavelength laser, measuring depth will be on the order of 0.5-1 mm. A representative Doppler image (left) for a dorsal bipedicled flap showing both a perfused area (left of the suture line) and the non-perfused area (right of the suture line) is shown in Figure 2.

In addition to measuring subcutaneous oxygen tension, one can also use probes or common biochemical markers to establish that the wounds in the flap are ischemic. PECAM-1 or CD31, normally found on vascular endothelial cells, is a marker for new vessel formation in the wounds. Various markers for reactive oxygen species, found to be elevated in ischemic wounds, are available commercially often as fluorescent-tagged antibodies or supernova indicators such as dithydroethidium (DHE).

Wound area measurements to track wound closure may be represented in a variety of ways. Commonly, wound area is quantified from digital images of the wounds over the time course of healing using a formula such as (6 mm punch biopsy area = π r² = 3.14 x 9 = 26.26 mm²) at day 0 with data presented as percentage of initial wound area or wound surface area on a particular day can be quantified as in Guidos et al. For the purposes of this demonstration, wound closure progression is represented as percent of initial wound area over a time course of 28 days. Using the free software ImageJ, a digital picture is opened and the scale is set using 10 mm on the ruler in the image (Figure 3A). A line drawn 10 mm in length equates to a pixel count (inset in Figure 3A) which can be converted into a unit of choice. In this case (mm). Next, the circumference of the wound is traced on the image (Figure 3B) and once the measure command is given, the area is presented in mm (inset in Figure 3B). Data can then be presented as percentage of initial wound area on the y-axis and days on the x-axis (Figure 3C).
Figure 1. Photographs depicting steps during surgery to create ischemic wounds, the bipedicled flap, and non-ischemic wounds. (A) Pre-surgical hair removal and skin preparation of an anesthetized rat receiving a pre-surgical dose of analgesics (Ketoprofen) subcutaneously for pain management. (B and inset) Ischemic wounds are created inside the 2 incision markers by utilizing a sterile punch biopsy tool. (C) Incisions are made along the marked lines down to the paraspinal muscle and (D) the bipedicled flap is raised to show the separation of the flap (with the panniculus carnosus fascia intact) from the muscle layer below. (E) A sheet of sterile silicone (black arrow) is placed between the fascia of the panniculus carnosus and the paraspinal muscle. (F) Black, non-absorbable sutures (size 4.0) close both incisions by anchoring the silicone sheet to the skin with multiple interrupted stitches along the length of the flap (G). Two non-ischemic wounds (black arrows) are created using a sterile punch biopsy tool lateral to the bipedicled flap on both sides. Please click here to view a larger version of this figure.
Figure 2. Representative laser Doppler image of blood perfusion post-surgery. The right panel shows a black and white image of 2 ischemic wounds (black arrows) in the middle of the bipedicted flap and 1 non-ischemic wound (single black arrow) lateral to the flap. The suture line has been highlighted in white. The left panel shows the Doppler image of the same area depicted in the right panel. Areas with brighter colors are more perfused than areas with dark blue. This difference in perfusion between the non-ischemic area (left) and the ischemic area (right) is clear and can be followed along the entire length of the suture line. Note that the wounds on both sides appear bright, as blood cells are still present to some degree. Please click here to view a larger version of this figure.
Figure 3. Wound measurement and data representation. (A) Digital photographs depicting wound area measurement using software (ImageJ), conversion of pixels to (mm) (black arrows) and the method to capture an accurate circumference (single black arrow) of the wound. (B) Statistical data (mean ± SEM of wound area on a given day) can then be presented as percentage of initial wound size (day 0) on the y-axis and days on the x-axis (C). The line graph presents data analyzed using a 2-way ANOVA with Sidak's multiple comparisons test. *** represents significantly higher (P = 0.0004) than non-ischemic at the same time point (day 10), N = 8 wounds per group. Please click here to view a larger version of this figure.

**Discussion**

Wound healing in rats has often been the subject of debate due to their ability to heal infected wounds and high rate of interanimal variability. One of the original goals of the model during its development was to decrease this variation. Modifications to the width of the flap, reducing the number of wounds with specific placement (centered on the flap with consistent cranio-caudal location) and introduction of a silicone sheet has accomplished this goal. Wound healing by contraction has also been reduced and healing by epithelialization, as in humans, is the measured outcome. Adaptation of the model to a different strain of rat, i.e., the F344, has also proven successful and reproduces the degree of ischemia observed using Sprague Dawley rats. Overall the surgical techniques (biopsy, suturing and wound excision) required for this model are easily acquired by most students and technicians with limited surgical experience.

To achieve consistency with this model while performing multiple surgeries, it was found that it is important to create the ischemic wounds prior to elevation of the flap for silicone sheet placement. Additionally, not puncturing through the parietal muscosus fascia is critical to provide a viable wound bed to remain over the silicone. This silicone acts not only to prevent vascular regrowth but also as a "splint" that encourages wound re-epithelialization. The application of the adhesive and dressings to prevent infection and maintain a moist environment for wound healing is also important. Product choice can be what is preferred or used in the researcher's animal facility. However, it is not uncommon for some of the animals to be able to remove their dressings, no matter what type of adhesive/dressing combination is used.

The bipedicled flap should remain visible throughout a time course of healing which is approximately 28 days, depending on rat strain and other co-morbidities present. Rarely, abscesses can form in the flap (particularly near sutures) and seromas may form under the flap. Fluid can be drained and antibiotics administered if necessary. However, if the flap loses viability and becomes necrotic it is recommended that that animal no longer be used. Wound excision for biochemical analysis does introduce variability due to (1) some normal tissue must be retained for support.
(2) the choice of tissue homogenization and preparation for isolation of RNA, DNA or protein and (3) inherent interanimal variability. One could consider this last point a limitation to the model and it was found that reducing the size of the flap (<2.0 cm) or flap trauma can cause necrosis, indicating that minor variations in factors such as temperature or stress levels, may also lead to biochemically detectable variation between wound samples from one rat to another.

In summary, this model, with a longitudinal bipolarized flap ranging from 2.0-3.0 cm in width and a strategically placed silicone sheet, is a reliable model of prolonged tissue ischemia. Once the user is adept at using the techniques to create a consistent ischemic wound, they should be able to adapt it to additional species and species of rodents (mice included). The excisional wounds can be treated topically, or systemic treatments utilized to further explore the mechanism(s) involved in chronic wound formation, exaggerated inflammatory responses, aberrant angiogenesis and delayed wound closure.

Disclosures
The authors have nothing to disclose.

Acknowledgements
The authors would like to acknowledge funding support from the University of South Florida, Department of Surgery (Mark Wu, MD, PhD).

References
Effects of exogenous ketone supplementation on blood ketone, glucose, triglyceride, and lipoprotein levels in Sprague–Dawley rats

Shannon L. Kesl¹, Angela M. Poff¹, Nathan P. Ward¹, Tina N. Fiorelli¹, Csilla Ari¹, Ashley J. Van Putten¹, Jacob W. Sherwood¹, Patrick Arnold² and Dominic P. D’Agostino¹

Abstract

Background: Nutritional ketosis induced by the ketogenic diet (KD) has therapeutic applications for many disease states. We hypothesized that oral administration of exogenous ketone supplements could produce sustained nutritional ketosis (>0.5 mM) without carbohydrate restriction.

Methods: We tested the effects of 28-day administration of five ketone supplements on blood glucose, ketones, and lipids in male Sprague–Dawley rats. The supplements included: 1,3-butanediol (BD), a sodium/potassium β-hydroxybutyrate (βHB) mineral salt (BMS), medium chain triglyceride oil (MCT), BMS + MCT 1:1 mixture, and 1,3-butanediol acetoacetate diester (KE). Rats received a daily 5–10 g/kg dose of their respective ketone supplement via intragastric gavage during treatment. Weekly whole blood samples were taken for analysis of glucose and βHB at baseline and, 0.5, 1, 4, 8, and 12 h post-gavage, or until βHB returned to baseline. At 28 days, triglycerides, total cholesterol and high-density lipoprotein (HDL) were measured.

Results: Exogenous ketone supplementation caused a rapid and sustained elevation of βHB, reduction of glucose, and little change to lipid biomarkers compared to control animals.

Conclusions: This study demonstrates the efficacy and tolerability of oral exogenous ketone supplementation in inducing nutritional ketosis independent of dietary restriction.

Keywords: Ketogenic diet, Ketone ester, Ketone supplement, Appetite, β-hydroxybutyrate, Hyperketonemia, Triglycerides

Background

Emerging evidence supports the therapeutic potential of the ketogenic diet (KD) for a variety of disease states, leading investigators to research methods of harnessing the benefits of nutritional ketosis without the dietary restrictions. The KD has been used as an effective non-pharmacological therapy for pediatric intractable seizures since the 1920s [1–3]. In addition to epilepsy, the ketogenic diet has elicited significant therapeutic effects for weight loss and type-2 diabetes (T2D) [4]. Several studies have shown significant weight loss on a high fat, low carbohydrate diet without significant elevations of serum cholesterol [5–12]. Another study demonstrated the safety and benefits of long-term application of the KD in T2D patients. Patients exhibited significant weight loss, reduction of blood glucose, and improvement of lipid markers after eating a well-formulated KD for 56 weeks [13]. Recently, researchers have begun to investigate the use of the KD as a treatment for acne, polycystic ovary syndrome (PCOS), cancer, amyotrophic lateral sclerosis (ALS), traumatic brain injury (TBI) and Alzheimer’s disease (AD) with promising preliminary results [14–26].

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The classical KD consists of a 4:1 ratio of fat to protein and carbohydrate, with 80–90 % of total calories derived from fat [27]. The macronutrient ratio of the KD induces a metabolic shift towards fatty acid oxidation and hepatic ketogenesis, elevating the ketone bodies acetoacetate (AcAc) and β-hydroxybutyrate (β-HB) in the blood. Acetone, generated by decarboxylation of AcAc, has been shown to have antiinflammatory properties [28–32]. Ketone bodies are naturally elevated to serve as alternative metabolic substrates for extra-hepatic tissues during the prolonged reduction of glucose availability, suppression of insulin, and depletion of liver glycogen, as occurs during starvation, fasting, vigorous exercise, calorie restriction, or the KD. Although the KD has clear therapeutic potential, several factors limit the efficacy and utility of this metabolic therapy for widespread clinical use. Patient compliance to the KD can be low due to the severe dietary restriction - the diet being generally perceived as unpalatable - and intolerance to high-fat ingestion. Maintaining ketosis can be difficult as consumption of even a small quantity of carbohydrates or excess protein can rapidly inhibit ketogenesis [33, 34]. Furthermore, enhanced ketone body production and tissue utilization by the tissues can take several weeks (keto-adaptation), and patients may experience mild hypoglycemic symptoms during this transitional period [35].

Recent studies suggest that many of the benefits of the KD are due to the effects of ketone body metabolism. Interestingly, in studies on T2D patients, improved glycemic control, improved lipid markers, and retraction of insulin and other medications occurred before weight loss became significant. Both β-HB and AcAc have been shown to decrease mitochondrial reactive oxygen species (ROS) production [36–39]. Veech et al. have summarized the potential therapeutic uses for ketone bodies [28, 40]. They have demonstrated that exogenous ketones favorably alter mitochondrial bioenergetics to reduce the mitochondrial NAD couple, oxidize the co-enzyme Q, and increase the ΔG (free enthalpy) of ATP hydrolysis [41]. Ketone bodies have been shown to increase the hydraulic efficiency of the heart by 28 %, simultaneously decreasing oxygen consumption while increasing ATP production [42]. Thus, elevated ketone bodies increase metabolic efficiency and as a consequence, reduce superoxide production and increase reduced glutathione [28]. Sullivan et al. demonstrated that mice fed a KD for 10–12 days showed increased hippocampal uncoupling proteins, indicative of decreased mitochondrial-produced ROS [43]. Bough et al. showed an increase of mitochondrial biogenesis in rats maintained on a KD for 4–6 weeks [44, 45]. Recently, Shimazu et al. reported that β-HB is an exogenous and specific inhibitor of class I histone deacetylases (HDACs), which confers protection against oxidative stress [38]. Ketone bodies have also been shown to suppress inflammation by decreasing the inflammatory markers TNF-α, IL-6, IL-8, MCP-1, E-selectin, I-CAM, and PAI-1 [8, 46, 47]. Therefore, it is thought that ketone bodies themselves confer many of the benefits associated with the KD.

Considering both the broad therapeutic potential and limitations of the KD, an oral exogenous ketone supplement capable of inducing sustained therapeutic ketosis without the need for dietary restriction would serve as a practical alternative. Several natural and synthetic ketone supplements capable of inducing nutritional ketosis have been identified. Desrochers et al. elevated ketone bodies in the blood of pigs (>0.5 mM) using exogenous ketone supplements: (R, S)-1,3 butanediol-acetooacetate monoesters and diester [48]. In 2012, Clarke et al. demonstrated the safety and efficacy of chronic oral administration of a ketone monoester of R-βHB in rats and humans [49, 50]. Subjects maintained elevated blood ketones without dietary restriction and experienced little to no adverse side effects, demonstrating the potential to circumvent the restrictive diet typically needed to achieve therapeutic ketosis. We hypothesized that exogenous ketone supplements could produce sustained hyperketonemia (>0.5 mM) without dietary restriction and without negatively influencing metabolic biomarkers, such as blood glucose, total cholesterol, HDL, LDL, and triglycerides. Thus, we measured these biomarkers during a 28-day administration of the following ketone supplements in rats: naturally-derived ketogenic supplements included medium chain triglyceride oil (MCT), sodium/potassium-βHB mineral salt and sodium/potassium-βHB mineral salt + medium chain triglyceride oil 1:1 mixture (BMS + MCT) and synthetically produced ketogenic supplements included 1,3-butanediol (BD), 1,3-butanediol acetooacetate diester/ ketone ester (KE).

**Methods**

**Synthesis and formulation of ketone supplements**

KE was synthesized as previously described [29]. BMS is a novel agent (sodium/potassium-βHB mineral salt) supplied as a 50 % solution containing approximately 375 mg/g of pure β-HB and 125 mg/g of sodium/potassium-βHB mineral salt. Both KE and BMS were developed and synthesized in collaboration with Savind Inc. Pharmaceutical grade MCT oil (~65 % caprylic triglyceride; 45 % capric triglyceride) was purchased from Now Foods (Bloomington, IL). BMS was formulated in a 1:1 ratio with MCT at the University of South Florida (USF), yielding a final mixture of 25 % water, 25 % pure β-HB and 50 % MCT. BD was purchased from Sigma-Aldrich (Prod # B84785, Milwaukee, WI).

**Daily gavage to induce dietary ketosis**

Animal procedures were performed in accordance with the University of South Florida Institutional Animal
Care and Use Committee (IACUC) guidelines (Protocol #0006R). Juvenile male Sprague–Dawley rats (275–325 g, Harlan Laboratories) were randomly assigned to one of six study groups: control (water, n = 11), BD (n = 11), KE (n = 11), MCT (n = 10), BMS (n = 11), or BMS + MCT (n = 12). Caloric density of standard rodent chow and dose of ketone supplements are listed in Table 1. On days 1–14, rats received a 5 g/kg body weight dose of their respective treatments via intragastric gavage. Dosage was increased to 10 g/kg body weight for the second half of the study (days 15–28) for all groups except BD and KE to prevent excessive hyperketonemia (ketoacidosis). Each daily dose of BMS would equal ~1000–1500 mg of βHB, depending on the weight of the animal. Intragastric gavage was performed at the same time daily, and animals had ad libitum blood for analysis of total cholesterol and lipoprotein profile data were analyzed using One-Way ANOVA. Basal blood ketone and blood glucose levels were controlled to the applicable time points using a Two-Way ANOVA. Correlation between blood βHB and glucose levels in ketone supplemented rats was compared to controls using ANCOVA analysis. Organ and body weights were analyzed using One-Way ANOVA. Basal blood ketone and blood glucose levels were analyzed using Two-Way ANOVA. All mean comparisons were carried out using Tukey’s multiple comparisons post-hoc test.

Results
Effect of ketone supplementation on triglycerides and lipoproteins
Baseline measurements showed no significant changes in triglycerides or the lipoproteins (data not shown). Data represent triglyceride and lipoprotein concentrations measured after 4 weeks of daily exogenous ketone supplementation. No significant change in total cholesterol was observed at 4 weeks for any of the ketone treatment groups compared to control. (Fig. 1a). No

<table>
<thead>
<tr>
<th>Macronutrient Information</th>
<th>Standard Diet</th>
<th>Water</th>
<th>BMS + MCT</th>
<th>BMS</th>
<th>MCT</th>
<th>KE</th>
<th>BD</th>
</tr>
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<tr>
<td>% Cal from Fat</td>
<td>18.0</td>
<td>0.0</td>
<td>50.0</td>
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<td>% Cal from Carbohydrates</td>
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<tr>
<td>Dose 0–14 Days (g/kg)</td>
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<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
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</tr>
<tr>
<td>Dose 15–28 Days (g/kg)</td>
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</table>
significant difference was detected in triglycerides for any ketone supplement compared to control (Fig. 1b). MCT supplemented animals had a significant reduction in HDL blood levels compared to control ($p < 0.001$) (Fig. 1c). LDL levels in ketone-supplemented animals did not significantly differ from controls (Fig. 1d).

Ketone supplementation causes rapid and sustained elevation of $\beta$HB
Over the 28-day experiment, ketone supplements administered daily significantly elevated blood ketone levels without dietary restriction (Fig. 2a, b). Naturally derived ketogenic supplements including MCT (5 g/kg) elicited a significant elevation in blood $\beta$HB at 4 h, which was no longer significant at 8 h. BMS + MCT (5 g/kg) did not elicit a significant elevation in blood $\beta$HB at any time point. For days 14–28, BMS + MCT (10 g/kg) and MCT (10 g/kg) elevated blood $\beta$HB levels within 30 min and remained significantly elevated for up to 12 h. We observed a delay in the peak elevation of blood $\beta$HB: BMS + MCT peaked at 8 h instead of at 4 h and MCT at 4 h instead of at 1 h. Blood $\beta$HB levels in the BMS group did not show significant elevation at any time point, even after dose escalation (Fig. 2a). Synthetically derived ketogenic supplements including KE and BD supplementation rapidly elevated blood $\beta$HB within 30 min and was sustained for 8 h. For the rats receiving ketone supplementation in the form of BD or the KE, dosage was kept at 5 g/kg to prevent adverse effects associated with hyperketonemia. The Precision Xtra™ ketone monitoring system measures $\beta$HB only; therefore, total blood ketone levels ($\beta$HB + AcAc) would be higher than measured. For each of these groups, the blood $\beta$HB profile remained consistent following daily ketone supplementation administration over the 4-week duration. (Fig. 2b).

Ketone supplementation causes a significant decrease of blood glucose
Administration of ketone supplementation significantly reduced blood glucose over the course of the study (Fig. 3a, b). MCT (5 g/kg) decreased blood glucose compared to control within 30 min which was sustained for
Fig. 2 Effects of ketone supplementation on blood [βHB]. a, b Blood [βHB] levels at times 0, 0.5, 1, 4, 8, and 12 h post intragastric gavage for ketone supplements tested. a BMS + MCT and MCT supplementation rapidly elevated and sustained significant [βHB] elevation compared to controls for the duration of the 4-week dose escalation study. BMS did not significantly elevate [βHB] at any time point tested compared to controls. b BD and KE supplements, maintained at 5 g/kg, significantly elevated [βHB] levels for the duration of the 4-week study. Two-Way ANOVA with Tukey’s post hoc test, results considered significant if \( p < 0.05 \). Error bars represent mean (SD).
Fig. 3. Effects of ketone supplementation on blood glucose. 

**a** Blood glucose levels at times 0, 0.5, 1, 4, 8, and 12 h (for 10 dose) post intragastric gavage for ketone supplements tested. 

- Ketone supplements BMS + MCT and MCT significantly reduced blood glucose levels compared to controls for the duration of the 4-week study. BMS significantly lowered blood glucose only at 8 h/week 1 and 12 h/week 3.

- KE, maintained at 5 g/kg, significantly reduced blood glucose compared to controls from week 1–4. BD did not significantly affect blood glucose levels at any time point during the 4-week study. Two-Way ANOVA with Tukey's post hoc test, results considered significant if p < 0.05. Error bars represent mean (SD).
8 h at baseline and at week 1. MCT (10 g/kg) likewise decreased blood glucose within 30 min and lasted through the 12 h time point during weeks 2, 3, and 4. BMS + MCT (5 g/kg) lowered blood glucose compared to control from hours 1–8 only at week 1. BMS + MCT (10 g/kg) lowered blood glucose compared to control within 30 min and remained low through the 12 h time point at weeks 2, 3, and 4. Rats supplemented with BMS had lower blood glucose compared to control at 12 h in week 4 (10) (Fig. 3a). Administration of BD did not significantly change blood glucose levels at any time point during the 4-week study. KE (5 g/kg) significantly lowered blood glucose levels at 30 min for week 1, 2, 3, and 4 and was sustained through 1 h at weeks 2–4 and sustained to 4 h at week 3. (Fig. 3b).

Hyperketonemia suppresses blood glucose levels
At baseline, 4 h after intragastric gavage, the elevation of blood ketones was inversely related to the reduction of blood glucose compared to controls following the administration of MCT (5 g/kg) (p = 0.008) and BMS + MCT (5 g/kg) (p = 0.039). There was no significant correlation between blood ketone levels and blood glucose levels compared to controls for any other ketone supplemented group at baseline (Fig. 3a). At week 4, 4 h after intragastric gavage, there was a significant correlation between blood ketone levels and blood glucose levels (r² = 0.8619, p < 0.0001; r² = 0.6365, p = 0.0057). Linear regression analysis, results considered significant if p < 0.05.

Fig. 4 Relationship between blood ketone and glucose levels: a BMS + MCT (5 g/kg) supplemented rats demonstrated a significant inverse relationship between elevated blood ketone levels and decreased blood ketone levels (r² = 0.4314, p = 0.0203). b At week 4, BMS + MCT (10 g/kg) and MCT (10 g/kg) showed a significant correlation between blood ketone levels and blood glucose levels (r² = 0.8619, p < 0.0001; r² = 0.6365, p = 0.0057). Linear regression analysis, results considered significant if p < 0.05.
blood glucose levels compared to controls in MCT (10 g/kg) and BMS + MCT (10 g/kg) \((p < 0.0001, p < 0.0001)\) (Fig. 4b).

**Ketone supplementation changes organ weight and decreases body weight**

At day 29 of the study, animals were euthanized and brain, lungs, liver, kidneys, spleen and heart were harvested and weighed. Organ weights were normalized to body weight. Ketone supplementation did not significantly change brain, lung, kidney, or heart weights compared to controls (Fig. 5a, b, d, f). MCT supplemented animals had significantly larger livers compared to their body weight \((p < 0.05)\) (Fig. 5c). Ketone supplements BMS + MCT, MCT and BD caused a significant reduction in spleen size \((BMS + MCT p < 0.05, MCT p < 0.001, BD p < 0.05)\) (Fig. 5e). Rats administered KE gained significantly less weight over the entire study compared to controls. BMS + MCT, BMS, and BD supplemented rats gained significantly less weight than controls during weeks 2 – 4, and MCT animals gained less

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**Fig. 5** Effects of ketone supplementation on organ weight. Data is represented as a percentage of organ weight to body weight. a, b, d, f Ketone supplements did not significantly affect the weight of the brain, lungs, kidneys or heart. c Liver weight was significantly increased as compared to body weight in response to administered MCT ketone supplement compared to control at the end of the study \((p < 0.001)\). e Rats supplemented with BMS + MCT, MCT, and BD had significantly smaller spleen percentage as compared to controls \((p < 0.05, p < 0.001, p < 0.05)\). Two-Way ANOVA with Tukey’s post hoc test; results considered significant if \(p < 0.05\). Error bars represent mean (SD).
weight than controls at weeks 3 – 4 (Fig. 6). Increased gastric motility (increased bowel evacuation and changes to fecal consistency) was visually observed in rats supplemented with 10 g/kg MCT, most notably at the 8 and 12-h time points. All animals remained in healthy weight range for their age even though the rate of weight gain changed with ketone supplementation [53–54]. Food intake was not measured in this study. However, there was not a significant change in basal blood glucose or basal blood ketone levels over the 4 week study in any of the rats supplemented with ketones (Fig. 7).

Discussion
Nutritional ketosis induced with the KD has proven effective for the metabolic management of seizures and potentially other disorders [1–26]. Here we present evidence that chronic administration of ketone supplements can induce a state of nutritional ketosis without the need for dietary carbohydrate restriction and with little or no effect on lipid biomarkers. The notion that we can produce the therapeutic effects of the KD with exogenous ketone supplementation is supported by our previous study which demonstrated that acutely administered KE supplementation delays central nervous system (CNS) oxygen toxicity seizures without the need for dietary restriction [29]. We propose that exogenous ketone supplementation could provide an alternative method of attaining the therapeutic benefits of nutritional ketosis, and as a means to further augment the therapeutic potential of the KD.

Ketone supplementation causes little to no change in triglycerides and lipoproteins
One common concern regarding the KD is its purported potential to increase the risk of atherosclerosis by elevating blood cholesterol and triglyceride levels [55, 56]. This topic remains controversial as some, but not all, studies have demonstrated that the KD elevates blood levels of cholesterol and triglycerides [57–62]. Kwiterovich and colleagues demonstrated an increase in low-density lipoprotein (LDL) and a decrease in high-density lipoprotein (HDL) in epileptic children fed the classical KD for two years [27]. In this study, total cholesterol increased by ~130 %, and stabilized at the elevated level over the 2-year period. A similar study demonstrated that the lipid profile returned to baseline in children who remained on the KD for six years [63]. Children typically remain on the diet for approximately two years then return to a diet of common fat and carbohydrate ingestion [64]. The implications of these findings are unclear, since the influence of cholesterol on cardiovascular health is controversial and macronutrient sources of the diet vary per study. In contrast to these studies, the majority of recent studies have suggested that the KD can actually lead to significant benefits in biomarkers of metabolic health, including blood lipid profiles [65–72]. In these studies, the
KD positively altered blood lipids, decreasing total triglycerides and cholesterol while increasing the ratio of HDL to LDL [68–77]. Although, the KD is well-established in children, it has only recently been utilized as a strategy to control seizures in adults. In 2014, Schoeler and colleagues reported on the feasibility of the KD for adults, concluding that 39% of individuals achieved > 50% reduction in seizure frequency, similar to the results reported in pediatric studies. Patients experienced similar gastrointestinal adverse events that have been previously described in pediatric patients, but they did not lead to discontinuation of the diet in any patient [78].

With oral ketone supplementation, we observed a significant elevation in blood βHB without dietary restriction and with little change in lipid biomarkers (Fig. 1). Over the 4 week study, MCT-supplemented rats demonstrated decreased HDL compared to controls. No significant changes were observed in any of the triglycerides or lipoproteins (HDL, LDL) with any of the remaining exogenously applied ketone supplements. It should be noted that the rats used for this study had not yet reached full adult body size [79]. Their normal growth rate and maturation was likely responsible for the changes in triglyceride and lipoprotein levels observed in the control animals over the 4 week study (baseline data not shown, no significant differences) [80, 81]. Future studies are needed to investigate the effect of ketone supplementation on fully mature and aged animals. Overall, our study suggests that oral ketone supplementation has little effect on the triglyceride or lipoprotein profile after 4 weeks. However, it is currently unknown if ketone supplementation would affect lipid biomarkers after a longer duration of consumption. Further studies are needed to determine the effects of ketone supplements on blood triglyceride and lipoproteins after chronic administration and as a means to further enhance the hyperketonemia and improve the lipid profile of the clinically implemented (4:1) KD.

LDL is the lipoprotein particle that is most often associated with atherosclerosis. LDL particles exist in different sizes: large molecules (Pattern A) or small molecules (Pattern B). Recent studies have investigated the importance of LDL-particle type and size rather than total concentration as being the source for cardiovascular risk [56]. Patients whose LDL particles are predominantly small and dense (Pattern B) have a greater risk of
cardiovascular disease (CVD). It is thought that small, dense LDL particles are more able to penetrate the endothelium and cause in damage and inflammation [82–85]. Volek et al. reported that the KD increased the pattern and volume of LDL particles, which is considered to reduce cardiovascular risk [73]. Though we did not show a significant effect on LDL levels for ketone supplements, future chronic feeding studies will investigate the effects of ketone supplementation on lipidomic profile and LDL particle type and size.

**Therapeutic levels of hyperketonemia suppress blood glucose levels**

We demonstrated that therapeutic ketosis could be induced without dietary (calorie or carbohydrate) restriction and that this acute elevation in blood ketones was significantly correlated with a reduction in blood glucose (Figs. 2, 3 and 4). The BMS ketone supplement did not significantly induce blood hyperketonemia or reduced glucose in the rats. The KE supplemented rats trended towards reduced glucose levels; however, the lower dose of this agent did not lower glucose significantly, as reported previously in acute response of mice [59]. MCTs have previously been shown to elicit a slight hypoglycemic effect by enhancing glucose utilization in both diabetic and non-diabetic patients [86–88]. Kashiwaya et al. demonstrated that both blood glucose and blood insulin decreased by approximately 50 % in rats fed a diet where 30 % of calories from starch were replaced with ketone esters for 14 days, suggesting that ketone supplementation increases insulin sensitivity or reduced hepatic glucose output [89]. This ketone-induced hypoglycemic effect has been previously reported in humans with IV infusions of ketone bodies [90, 91]. Recently, Mikkelson et al. showed that a small increase in βHB concentration decreases glucose production by 14 % in post-absorptive health males [92]. However, this has not been previously reported with any of the oral exogenous ketone supplements we studied. Ketones are an efficient and sufficient energy substrate for the brain, and will therefore prevent side effects of hypoglycemia when blood levels are elevated and the patient is keto-adapted. This was most famously demonstrated by Owen et al. in 1967 wherein keto-adapted patients (starvation induced therapeutic ketosis) were given 20 IU of insulin. The blood glucose of fasted patients dropped to 1–2 mM, but they exhibited no hypoglycemic symptoms due to brain utilization of ketones for energy [93]. Therefore, ketones maintain brain metabolism and are neuroprotective during severe hypoglycemia. The rats in the MCT group had a correlation of blood ketone and glucose levels at week 4, whereas the combination of BMS + MCT produced a significant hypoglycemic correlation both at baseline and at week 4. No hypoglycemic symptoms were observed in the rats during this study. Insulin levels were not measured in this study; however, future ketone supplementation studies should measure the effects of exogenous ketones on insulin sensitivity with a glucose tolerance test. An increase in insulin sensitivity in combination with our observed hypoglycemic effect has potential therapy implications for glycemic control in T2D [40]. Furthermore, it should be noted that the KE metabolizes to both AcAc and βHB in 1:1 ratio [29]. The ketone monitor used in this study only measures βHB as levels of AcAc are more difficult to measure due to spontaneous decarboxylation to acetone; therefore, the total ketone levels (βHB + AcAc) measured were likely higher, specifically for the KE [14]. Interestingly, the 10 g/kg dose produced a delayed blood βHB peak for ketone supplements MCT and BMS + MCT. The higher dose of the ketogenic supplements elevated blood levels more substantially, and thus reached their maximum blood concentration later due to prolonged metabolic clearance. It must be noted that the dosage used in this study does not translate to human patients, since the metabolic physiology of rats is considerably higher. Future studies will be needed to determine optimal dosing for human patients.

**Effects of ketone supplementation on organ weight and body weight percentage**

Ketone supplementation did not affect the size of the brain, lungs, kidneys or heart of rats. As previously mentioned, the rats were still growing during the experimental time frame; therefore, organ weights were normalized to body weight to determine if organ weight changed independently to growth. There could be several reasons why ketones influenced liver and spleen weight. The ratio of liver to body weight was significantly higher in the MCT supplemented animals (Fig. 5). MCTs are readily absorbed in the intestinal lumen and transported directly to the liver via hepatic portal circulation. When given a large bolus, such as in this study, the amount of MCTs in the liver will likely exceed the β-oxidation rate, causing the MCTs to be deposited in the liver as fat droplets [94]. The accumulated MCT droplets in the liver could explain the higher liver weight to body weight percentage observed with MCT supplemented rats. Future toxicology and histological studies will be needed to determine the cause of the observed hepatomegaly. It should be emphasized that the dose in this study is not optimized in humans. We speculate that an optimized human dose would be lower and may not cause hepatomegaly or potential fat accumulation. Nutritional ketosis achieved with the KD has been shown to decrease inflammatory markers such as TNF-α, IL-6, IL-8, MCP-1, E-selectin, I-CAM, and PAI-1 [8, 46], which may account for the observed decrease in spleen weight. As previously mentioned, Veech and colleagues demonstrated that exogenous
supplementation of 5 mM βHB resulted in a 28% increase in hydraulic work in the working perfused rat heart and a significant decrease in oxygen consumption [28, 41, 42]. Ketone bodies have been shown to increase cerebral blood flow and perfusion [95]. Also, ketone bodies have been shown to increase ATP synthesis and enhance the efficiency of ATP production [14, 28, 40]. It is possible that sustained ketosis results in enhanced cardiac efficiency and O₂ consumption. Even though the size of the heart did not change for any of the ketone supplements, further analysis of tissues harvested from the ketone-supplemented rats will be needed to determine any morphological changes and to understand changes in organ size. It should be noted that the Harlan standard rodent chow 2018 is nutritionally complete and formulated with high-quality ingredients to optimize gestation, lactation, growth, and overall health of the animals. The same cannot be said for the standard American diet (SAD). Therefore, we plan to investigate the effects of ketone supplements administered with the SAD to determine if similar effects will be seen when the micronutrient deficiencies and macronutrient profile mimics what most Americans consume.

MCT oil has recently been used to induce nutritional ketosis although it produces dose-dependent gastrointestinal (GI) side effects in humans that limit the potential for its use to significantly elevate ketones (>0.5 mM). Despite these limitations, Azzam and colleagues published a case report in which a 43-year-old man had a significant decrease in seizure frequency after supplementing his diet with 4 tablespoons of MCT oil twice daily [96]. An attempt to increase his dosage to 5 tablespoons twice daily was halted by severe GI intolerance. Henderson et al. observed that 20% of patients reported GI side effects with a 20 g dose of ketogenic agent AC-1202 in a double-blind trial in mild to moderate Alzheimer’s patients [24]. We visually observed similar gastrointestinal side effects (loose stools) in the rats treated with MCT oil in our study. Rats were closely monitored to avoid dehydration, and gastric motility returned to normal between 12–24 h. Interestingly, the BMS + MCT supplement elevated [βHB similarly to MCT oil alone, without causing the adverse gastrointestinal effects seen in MCT-supplemented rats. However, this could be due to the fact in a 10 g/kg dose of BMS + MCT, only 5 g/kg is MCT alone, which is less than the 10 g/kg dose that elicits the GI side effects. This suggests that this novel combination may provide a more useful therapeutic option than MCT oil alone, which is limited in its ability to elevate ketones in humans.

Exogenously delivered ketone supplements significantly altered rat weight gain for the duration of the study (Fig. 6). However, rats did not lose weight and maintained a healthy range for their age. Rats have been shown to effectively balance their caloric intake to prevent weight loss/gain [97–99]. Due to the caloric density of the exogenous ketone supplements (Table 1) it is possible for the rats to eat less of the standard rodent chow and therefore less carbohydrates while maintaining their caloric intake. Food intake was not measured for this study. However, if there was a significant carbohydrate restriction there would be a significant change in basal blood ketone and blood glucose levels. As the hallmark to the KD, carbohydrate restriction increases blood ketone levels and reduces blood glucose levels. Neither an increase in basal blood ketone levels nor a decrease in basal blood glucose levels was observed in this study (Fig. 7). Additionally, if there were an overall blood glucose decrease due to a change in food intake, this would not explain the rapid reduction (within 30 min) in blood glucose correlated with an elevation of blood ketone levels after an intragastric bolus of ketone supplement (Figs. 2, 3 and 4).

Conclusions
Several studies have investigated the safety and efficacy of ketone supplements for disease states such as AD and Parkinson’s disease, and well as for parenteral nutrition [40, 48–50, 100–103]. Our research demonstrates that several forms of dietary ketone supplementation can effectively elevate blood ketone levels and achieve deleted: therapeutic nutritional ketosis without the need for dietary carbohydrate restriction. We also demonstrated that ketosis achieved with exogenous ketone supplementation can reduce blood glucose, and this is inversely associated with the blood ketone levels. Although preliminary results are encouraging, further studies are needed to determine if oral ketone supplementation can produce the same therapeutic benefits as the classic KD in the broad-spectrum of KD-responsive disease states. Additionally, further experiments need to be conducted to see if the exogenous ketone supplementation affects the same physiological features as the KD (i.e., ROS, inflammation, ATP production). Ketone supplementation could be used as an alternative method for inducing ketosis in patients uninterested in attempting the KD or those who have previously had difficulty implementing the KD because of palatability issues, gall bladder removal, liver abnormalities, or intolerance to fat. Additional experiments should be conducted to see if ketone supplementation could be used in conjunction with the KD to assist and ease the transition to nutrition ketosis and enhance the speed of keto-adaptation. In this study we have demonstrated the ability of several ketone supplements to elevate blood ketone levels, providing multiple options to induce therapeutic ketosis based on patient need. Though additional studies are needed to determine the therapeutic potential of ketone supplementation, many patients that previously
able were unable to benefit from the KD may now have an alternate method of achieving therapeutic ketosis. Ketone supplementation may also represent a means to further augment ketonemia in those responsive to therapeutic ketosis, especially in those individuals where maintaining low glucose is important.

Abbreviations

AcAc: 2-oxoacetate; AL: amyotrophic lateral sclerosis; AD: Alzheimer’s; bHB: beta-hydroxybutyrate; BMS: sodium/potassium bHB mineral salt; BMS + MCT: BMS + MCT 1:1 mixture; BD: 1,3-butanediol; CNS: central nervous system; HDL: high density lipoprotein; HDACs: histone deacetylases; LDL: low density lipoprotein; KD: ketogenic diet; KE: 1,3-butanediol ketone ester; MCT: medium chain triglyceride oil; PCOS: polycystic ovary syndrome; ROS: reactive oxygen species; SAD: standard American diet; TBI: traumatic brain injury; T2D: type-2 diabetes.

Competing interests

International Patent # PCT/US2014/031237, University of South Florida, D.P. D’Agostino, S. Kerd, P. Arnold, “Compositions and Methods for Producing Elevated and Sustained Ketosis,” P. Arnold (Savind) has received financial support (ONR N000140610105 and N000140910244) from D.P. D’Agostino (USF) to synthesize ketone esters. The remaining authors have no conflicts of interest.

Authors’ contributions

Conceived and designed the experiments: SK, AP, NW, TF, CA, DP. Performed the experiments: SK, AP, NW, TF, CA, JS, AVP. Analyzed the data: SK, AP, DP. Contributed reagents/materials/analysis tools: PA. Helped draft the manuscript: SK, AP, NW, CA, DP. All authors read and approved the final manuscript.

Acknowledgements

The authors would like to thank Savind Inc for manufacturing some of the ketone supplements, Jay Dean for the use of his Hyperbaric Research Laboratory, and the ONR and Scivation Inc for funding the project.

Grants

This study was supported by the Office of Naval Research (ONR) Grant N000140610105 (D.P.), and a Morsani College of Medicine Department of Pharmacology and pharmaceutical departmental grant.

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Received: 10 September 2015 Accepted: 28 January 2016

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