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Antioxidants and Wound Healing.

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ANTIOXIDANTS AND WOUND HEALING

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By

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Introduction

Wound healing begins as soon as the injury occurs. The four phases of wound healing are described as hemostasis, inflammation, proliferation, and remodeling. During the inflammation phase of wound healing neutrophils and cytokines produce oxidants, such as reactive oxygen species (ROS) or reactive nitrogen species (RNS). These substances act as free radicals, a highly reactive species that steals electrons from neighboring molecules to satisfy its valence electron needs. This removal of electrons can delay wound healing or produce significant damage within the healthy cells of tissues throughout the body. In response to the oxidative stress occurring at the wound site, antioxidants can be used to quench free radicals and reestablish the necessary environment for wound healing. Antioxidants act by donating electrons to the free radicals, sparing the damaging effects of an oxidation reaction on other molecules and tissues. Therefore, through the promotion of adequate antioxidant intake throughout the wound healing process it is possible to facilitate healthy tissue and to improve the healing outcome. The natural antioxidants frequently cited in literature pertaining to nutrition and wound healing include: Vitamin E, Alpha-Lipoic Acid, Vitamin C, Grape Seed Extract, Coenzyme Q10, Glutathione, and Lutein; all of which promote the development of new tissue in wounds by reducing the concentration of free radicals. In addition to their antioxidative capabilities, each of these compounds contributes to the process of wound healing in unique ways. Thus, these compounds have the potential to provide physiological relief to patients suffering from thermal injuries.

Phases of Wound Healing:

Once a wound is present in the epidermal tissue, it goes through a series of phases during the process of healing and the development of new tissue.

1. Hemostasis:

Immediately upon injury there is a brief period of vasoconstriction, followed by vasodilation and an increase in permeability of the blood vessels (1). This phenomenon is accompanied by an overall increase in blood flow to the surrounding tissues, enabling the infiltration of the wound site with leukocytes such as neutrophils and monocytes. Upon the initiation of hemostasis, the extrinsic cascade resulting in blood-clotting is launched (2). Therefore, this phase refers to the body's natural ability to prevent continuous free bleeding after injury. Fibrinogen in the blood is quickly transformed to fibrin, which promotes the formation of a scab with the aid of blood platelets. This temporary scab provides a protective barrier, shielding the wound from infectious bacteria in the environment (3). The blood clot also acts as the site of large collections of cytokine and growth factor that are released into the wounded tissue upon the activation of platelets to degranulate. This is a critical step in healing because it provides the necessary initiation to draw circulating inflammatory cells to the injured tissue, causes connective tissue to begin contracting, and prepares surrounding blood vessels for revascularization (4).

2. Inflammation:

During inflammation there is an increase in pain, redness, swelling, and warmth at the site of the wound. This phenomenon is due to the increased blood circulation to the area. As the blood vessels become more permeable, some cellular fluid is able to leak out into the extracellular tissue, which creates swelling. The increased permeability of blood vessels also

allows neutrophils and monocytes to enter the wounded tissue (1). Neutrophils clear away wound debris and any harmful microorganisms that may be residing in the tissue, such as bacteria (2). However, neutrophil migration into the wound ceases after the first few days following the initial injury (1). Monocytes are converted to macrophages in the tissue. This transfiguration further amplifies the immune response to foreign debris by producing a respiratory burst that releases reactive oxygen species (ROS), which act to eliminate bacterial infection (2).

3. Proliferation:

Proliferation acts predominantly through formation of new granulation and epithelial tissue. Depending on the size of the wound, this phase will vary in length. Initially, factors released from platelets and macrophages already at the wound site promote fibroblasts to produce glycosaminoglycans and collagen. Both act to form an unstructured connective tissue medium that new cells migrate into during the proliferation phase, making these elements essential to wound healing (1).

Along with the formation of tissue, new capillary growth is necessary to provide oxygen and nutrients to the wound site. The re-growth of vascular tissue can occur in three different ways: angiogenesis, vasculogenesis, arteriogenesis. Angiogenesis refers to the formation of new vascular tissue from previously existing blood vessels. The formation of completely new vascular tissue from endothelial precursor cells is referring to vasculogenesis. When arteries or veins are damaged at the wound site, smaller arterioles and veinoles will develop into mature vessels in the process of arteriogenesis (2).

In addition to critical vascular tissues, the lymph tissue surrounding a wound must be restored for proper functioning of the newly formed tissues. This occurs through a process called

lymphogenesis, which entails the development of new lymph vessels from the pre-existing lymphatic tissue (2).

4. Remodeling

In this final stage, collagen fibers within the new tissue are reorganized to form a more uniform lattice structure. The remodeling process is achieved over longer periods of time, often years, unlike the other immediate phases of wound healing (1). However, at the site of the healed wound there remains a connective tissue scar. This difference in tissue is due to the new collagen matrix of the scar being reconstructed in parallel bundles. This formation differs from areas of unwounded tissue collagen that is constructed in a much more efficient and aesthetically pleasing basket-weave technique (4).

Free Radicals and Antioxidants

Free radicals are missing an electron in their outermost valence shell so they react by stealing electrons from another molecule, a process referred to as oxidation (5). Free radicals that contain oxygen are known as reactive oxygen species (ROS), while free radicals that contain nitrogen are known as reactive nitrogen species (RNS) (6). As a result of oxidation, the molecule under attack must search for an electron to repair its own damage. This is done by stealing an electron from yet another molecule. The oxidative reaction of free radicals is propagated and if the series is allowed to progress, unstable free radicals can destroy healthy cells (5). Free radicals attack and remove electrons from all types of molecules in the cell, including: nucleic acids in DNA, proteins, and polyunsaturated fatty acids in cell membranes or organelle membranes. When free radicals attack proteins they break peptide bonds in the protein backbone, changing the protein structure, and altering its functionality (6). All of these

processes are detrimental to the proliferation of new cells in the healing process of epithelial wounds (5).

There are two types of antioxidants: enzymatic and non-enzymatic. Non-enzymatic antioxidants refer to antioxidants that deter free radicals by preventing oxidative reactions through a process of deactivation, which terminates the oxidative reaction all together (7). This is accomplished by preventing electrons from leaving their orbits and forming free radicals, as well as donating electrons to already existing free radicals. By donating their electrons and satisfying the needs of free radicals, antioxidants spare potential damage to healthy cells and tissues (5).

In contrast, enzymatic antioxidants function by catalyzing the conversion of a free radical into a less harmful product that will not place human tissue in the path of proliferative damage. There are three classes of enzymatic antioxidants: superoxide dismutase, catalase, and glutathione peroxidase. Superoxide dismutase catalyzes a reaction that allows superoxide anions (O_2) to be transformed into a less damaging compound, hydrogen peroxide (H_2O_2). This hydrogen peroxide can then be eliminated by either catalase or glutathione peroxidase. Catalase (CAT) reacts with the hydrogen peroxide to form water (H_2O) and molecular oxygen (O_2):

$$2\ H_2O_2 \xrightarrow{CAT} 2\ H_2O + O_2$$

Additionally, glutathione peroxidase (GPX) catalyzes the reduction of hydroperoxides (ROOH) with the aid of glutathione (GSH) to produce an alcohol, oxidized glutathione (GSSG), and water:

$$ROOH + 2GSH \longrightarrow ROH + GSSG + H_2O$$

The antioxidants of focus in this study are classified as non-enzymatic antioxidants (8).

Effects of Free Radicals in the Body

In the initial stage of healing, hemostasis, increased vasodilation in the surrounding tissue is necessary for healing to occur. However, simultaneous complications of vasodilation have been found. For example, in wounded tissue containing inducible nitric oxide synthase, vasodilation could be over-stimulated which would allow an excess concentration of neutrophils and cytokines to develop (9). These cells are capable of producing O_2^-, the superoxide radical, as a defense mechanism against pathogens. The superoxide radical can be converted to hydrogen peroxide (H_2O_2) which is capable of stimulating the production of other free radicals (10). In addition, nitric oxide synthase stimulates the production of nitric oxide. This substance is capable of producing oxynitrite, a harmful oxygen free radical that increases the oxidative stress and escalates damage to the tissue (9). All of these factors contribute to the wound site becoming dense with free radicals and the cascade effect of tissue damage that may soon follow (10).

While these oxidants (free radicals) may serve as important cellular messengers capable of initiating important cellular and molecular functions, the concentration of these substances is delicate. It has been shown that small concentrations, up to a micromole, of oxidants may activate signals for reactions that are critical for wound healing. For example, they are involved promotion of cytokine and macrophage activity to prevent the invasion of bacteria at the wound site. However, millimolar concentrations of oxidants are capable of depleting the source of antioxidants in the wound and lead to further unintentional tissue damage (10).

The general method for measuring the degree of free radical activity in the body is through the collection of blood samples to assess the lipid peroxidation levels or the depletion of a series of antioxidants often found in the body tissues. In a study at the Burn Center of the

University Hospital of Lyon, 20 patients were evaluated based upon this criterion. Depressed levels up to ten times less, when compared to a Control Group, of antioxidants such as β-carotene, lycopene, lutein, vitamin E, and vitamin C were seen in blood samples taken on day one after the burn injury. The antioxidant levels remained at low concentrations through the fifth day of blood samples. These levels are believed to remain low due to the lack of vascularization in the early stages of healing. Antioxidants obtained through the diet are unable to replenish the wound site due to the inadequate blood supply and damaged blood vessels. Other studies have found that this level is not seen to stabilize until three weeks following the initial injury (11). In order to combat the continual formation of free radicals and effectively prevent extreme levels of oxidative stress, normal levels of antioxidant must be maintained (12). Any malnutrition, including antioxidants, may result in an extended inflammatory phase, decreased production of fibroblasts and collagen, and a less effective angiogenesis stage (13).

According to past studies these free radicals not only have harmful effects at the wound site, but may also result in damage to other body tissues as well. Due to their accumulation at the wound site, free radicals are available to activate complement proteins capable of inducing inflammation in other organs throughout the body (14). Specifically, there is evidence that indicates elevated free radical levels throughout the body in third-degree burns covering 20% or more of the total body surface. This process appears to be the most active during the inflammation stage of healing with the release of oxygen free radicals from neutrophils and cytokines at the wound site. Evidence supports injury to organs such as the heart, lungs, and liver that were not involved with the initial burn wound (15). In animal model studies decreased burn edema and decreased inflammation leading to organ dysfunction has been noted with the use of antioxidant therapies (12).

Vitamin E

Vitamin E is the general term for two groups of compounds distinguished as tocopherols and tocotrienols. Each of these groups is composed of four forms: α, β, ɣ, δ (16). Vitamin E is one of the most common lipid-soluble antioxidants used to combat the harmful effects of free radicals in the skin (17). Vitamin E has increased effectiveness in wound healing due its natural ability to penetrate skin very easily. Oxidative damage will occur when the levels of tocopherols consumed with oxidative stress exceeds the levels of available tocopherols (5). In a study containing 10 patients at the Truman G. Blocker Burn Unit at the University of Texas Medical Branch, significant decreases ($p<0.05$) in the plasma level of Vitamin E were observed for the first 14 days after injury in comparison to a control group. This rapid consumption of available Vitamin E is thought to indicate a role in reducing the amount of oxygen free radicals in the wound (18).

The most well-studied form of Vitamin E, α-tocopherol, has potent antioxidant effects. In the presence of peroxyl radicals, α-tocopherol will donate a hydrogen atom to form a tocopheroxyl radical. When in combination with ascorbic acid (Vitamin C) or glutathione, the initial α-tocopherol antioxidant will be regenerated from the tocopheroxyl radical. This process of regeneration allows small concentrations of α-tocopherol to eliminate larger concentrations of free radicals (18). By eliminating these peroxyl radicals, α-tocopherol ends the self-perpetuating chain reaction of lipid peroxidation (19). Lipid peroxidation is damaging to newly forming tissue because it involves the destruction of polyunsaturated fatty acids in the plasma membranes through oxidation (20). Lipid peroxidation is evaluated by measuring the level of malondialdehyde (MDA) present in the blood, with high levels indicating increased lipid peroxidation. In a randomized study conducted on 36 children admitted to the Burn Unit of the

Joana de Gusmao Children's Hospital, the level of MDA in patients supplemented with Vitamin E, C, and zinc was significantly ($p<0.01$) decreased (21).

Also in recent studies, the incorporation of α-tocopherol into the fibroblasts of tissues helped to prevent the deterioration of newly formed collagen and glycosaminoglycans due to oxidation. This is important because damage to collagen or glycosaminoglycans during the proliferation stage of wound healing can delay or interfere with correct tissue healing (17). Additionally, there is some evidence that by protecting collagen and glycosaminoglycans from oxidative stress, Vitamin E actually promotes a faster rate of wound closure (22). In one particular study, the oral supplementation of a Vitamin E from palm oil resulted in wound size decreasing significantly more in a 10-day span when compared with the wound size of the control group. When analyzing the presence of protein in the same wound, it was found that the control group had significantly less protein than the group treated with Vitamin E from palm oil. This exemplifies that supplementation with Vitamin E from palm oil increased the cellular proliferation within the wound, evidenced by a higher concentration of protein and a faster rate of cell regeneration and wound closure (16).

Alpha-Lipoic Acid

Alpha-lipoic acid and its reduced form, dihydrolipoic acid, are commonly known for their antioxidant activity (23). More specifically, the effectiveness of α-lipoic as an antioxidant is due to dihydrolipoic acid. However, dihydrolipoic acid is unstable alone and must be complexed with lipoic acid, which is easily absorbed by the skin, to produce a therapeutic effect (5). Alpha-lipoic acid acts to satiate the electron needs of several reactive oxidant species (ROS), such as: hydroxyl radicals, superoxide, singlet oxygen, peroxyl radicals, hypochlorous acid, and nitric

oxide (24). As stated previously, each of these substances contributes to oxidative stress and cellular damage. Alpha-lipoic acid produces an affect by either directly eliminating free radicals and preventing harmful oxidation, or by recovering Vitamin E and further increasing the antioxidant power available in the damaged tissue (23). Other antioxidants regenerated by α-lipoic acid are Vitamin C, glutathione, and Coenzyme Q_{10} (25).

In a study based on carrageenan-induced paw edema in rats, α-lipoic acid had a significant anti-inflammatory effect. In fact, the six rats that received 100 mg/kg and 200 mg/kg of enteral supplementation resulted in a 29.6% ($p<0.05$) and 40.7% ($p<0.01$) reduction in carrageenan-induced paw edema by the fourth hour after administration. In the control group, an increased paw volume of 0.54mL was observed after injection of carrageenan. Whereas, in the treatment group receiving 100 mg/kg and 200 mg/kg of enteral supplementation an increased paw volume of only 0.38 mL ($p<0.05$) and 0.32 mL ($p<0.01$) was observed. These values are considered significant data supporting the anti-inflammatory effect of α-lipoic acid (26).

In research thus far, α-lipoic acid has been shown to produce therapeutic significance in diabetic ulcer complications, cataract formation, HIV activation, neurodegenerative disorders, and radiation injury or burns (23). Since α-lipoic acid is both water- and lipid-soluble, it is able to penetrate the lipophilic cellular membranes and occupy aqueous intracellular space equally. This ability to transverse a variety of cellular spaces enables α-lipoic acid to have an effect on free radicals in a wider range of areas within the wounded tissue (25).

Vitamin C (Ascorbic Acid)

Vitamin C, also known as ascorbic acid, is considered an essential component in the synthesis of collagen, glycosaminoglycans, and many other organic factors that make up the

intracellular matrix of bone, skin, and capillary tissues. Vitamin C is needed for the hydroxylation reactions of lysine and proline in procollagen, the precursor to collagen. Without this hydroxylation reaction, procollagen could not be secreted and later converted to active collagen (1). Hydroxyproline, produced in a hydroxylation reaction facilitated by Vitamin C, stabilizes the triple-helix structure of collagen. Hydroxylysine, also produced in a similar hydroxylation reaction, is needed to produce the cross-links in mature collagen that provide the malleable strength in the healing wound tissue (3). Therefore, adequate concentrations of Vitamin C are necessary in the tissue because of this nutrient's impact on the production and physical attributes of collagen. It has been noted that concentrations of Vitamin C below 50% of the normal serum values are typically noted after a burn injury (27).

It has long been evident that Vitamin C is capable of aiding in the improvement of the cellular immune functions in humans, which applies to another commonly encountered delay in wound healing, infection. Due to the exposure to harmful bacterial pathogens or microorganisms and the impaired immune function, wounds are particular susceptible to infection (1). The relationship between burn wounds and immune suppression has been well documented. Patients with burn injuries are considered immunocompromised due to the effect the injury has on the cellular immune system and the decline in humoral immunity. Over the course of the healing process the immune system becomes increasingly overwhelmed, peaking between 7 to 10 days after the initial injury (28). During this critical period infection is a serious risk; sometimes leading to mortality.

During one particular study, volunteers were asked to ingest 2-3g of Vitamin C daily for several weeks. These participants displayed heightened neutrophil activity upon stimulus from an inflammatory response after only a short period of time (1). Further exemplifying the

relationship between Vitamin C and an increased immune response, an animal study was conducted to determine whether Vitamin C protects from suppression of the immune response after a burn injury. A contact hypersensitivity reaction test was performed on the ears of laboratory rats and the percent of ear swelling was measured. It was found that the rat with a burn injury had a swelling response 18.8% of the swelling reaction seen in the control, exemplifying the correlation between burn wounds and immunosuppression ($p<0.0001$). However, the lab rat with a burn injury that received an oral treatment of Vitamin C for seven days immediately following injury had a swelling response 120.0% ($p<0.001$) of the swelling reaction seen in the control (28), thus indicating that the suppressed immune response noted in burn patients may be improved with Vitamin C supplementation. This increase in the effectiveness of neutrophils and the protection from immunosuppression is critical in the inflammation phase of wound healing in order to reduce the risk of infection or sepsis (1).

In addition to its physiological benefits, ascorbic acid functions as a potent antioxidant. Evidence has been provided that large doses of Vitamin C administered after a burn injury result in an improved plasma antioxidant status and a reduction of plasma TBARS (thiobarbituric acid reactive substances), a byproduct of lipid peroxidation (27). The combined ability of Vitamin C to impact collagen formation, free radical formation, and cellular immunity make it an excellent candidate for the promotion of wound healing (1).

Grape Seed Extract

The phenolics found in grape seeds have many benefits in wound healing due to their potent antioxidant power, allowing them to trap and quench free radicals at the wound site (29). In fact, phenolic compounds have the potential to be four to five times as effective in reducing

free radicals when compared to Vitamin C or E (5). These phenolics also enhance the cooperative nature of other antioxidants such as Vitamins C, Vitamin E, the carotenoids, and selenium. This cooperative effect is the result of the phenolics protecting other antioxidant elements against early oxidation, thus improving their availability for reaction with available free radicals (29).

In terms of regeneration of new tissue, grape seed extract is evidenced to promote vascular endothelial growth in skin cells of the dermal layer of tissue (25). An animal model study performed on rabbits, receiving identical wounds, supported this correlation. The animals receiving topical treatments of Grape Seed Extract in varying concentrations with a Eucerin base were shown to have improvements in the production of hydroxyproline, tensile strength, and wound closure time compared to a control. Hydroxyproline, a component of collagen, was seen to increase by approximately 205.7 μg/g of tissue sampled with a 2.0% Grape Seed Extract treatment in comparison to no treatment ($p<0.001$). The tensile strength of healed tissue after the same Grape Seed Extract treatment increased by approximately 190.0 g/cm^2 in comparison to no treatment ($p<0.001$). Finally, the healing time of the wound receiving the 2.0% Grape Seed Extract treatment was reduced by 7 days when compared to the wound given no treatment ($p<0.05$). This study demonstrates that Grape Seed Extract could amplify critical processes occurring during the proliferative phase of wound healing (30).

These phenolics found in Grape Seed Extract also act to inhibit enzymatic processes that influence the production of free radicals and are often linked to inflammatory reactions. Procyanidins, a phenol found in grape seed, prevents the synthesis and release of histamine, serine protease, prostaglandins, and leukotriene. These are substances that promote inflammation and the release of oxidants. Procyanidins also inhibit hyaluronidase, a

proteoglycan-splitting enzyme that attacks surrounding tissue during an inflammatory response after injury (29). By producing an inhibitory control over the inflammation reaction, the phenolics found in grape seed have the potential to decrease the levels of oxidants produced during the inflammatory phase.

Glutathione

As the most abundant intracellular antioxidant, glutathione provides significant protection to the tissue from oxidative stress (32). Glutathione (GSH) specifically targets hydrogen peroxide, acting as an electron acceptor to form an oxidized thiol. This resulting thiol is toxic; glutathione reductase acts to recycle this thiol and reproduce glutathione. Therefore, glutathione maintains the antioxidant concentration at the wound site through regeneration (33). Another potential reaction is via glutathione-S-transferase, where the toxic thiol is degraded and excreted from the cell (32).

Upon injury, the level of glutathione is significantly reduced when compared to normal tissues. In a study reported on diabetic ulcers, the average level of glutathione in wounded tissues was 53.4 pmol/mg wet weight compared to 150.6 pmol/mg wet weight in the control tissues ($p<0.05$). In the same study, it was shown that wounded diabetic mice treated with a topical glutathione monoester healed significantly faster ($p<0.001$) when compared to the wounded diabetic mice treated with 1.0% carboxymethyl cellulose as the control. This experiment suggests that the reduced levels of glutathione found in the wounds significantly altered the overall redox status of the cell, leading to increased complications with healing (32).

In a burn wound, surrounding tissue often becomes damaged as a secondary injury to the initial wound. It is thought that glutathione increases the capacity of a cell to resist this

secondary thermal injury. In a study of this phenomenon, rats were burned using the comb burn model which provides gaps of unharmed tissue in between burn wounds to analyze the secondary injuries that might occur there. Immediately after receiving a comb burn, two different treatments were administered. The first treatment group was administered 5mg/kg of glutathione supplementation in the peritoneum and the second treatment group received the same dose of saline (control). After 10 days, the areas of living and necrotic tissues were quantified by autoradiography. In the glutathione treatment group, the average surviving interspace area was 87.8% of the total 3 cm^2 observed area. In comparison, the control group on average had a surviving interspace area 26.9% of the total 3 cm^2 area. The significant ($p<0.05$) capacity of glutathione supplementation to save surrounding tissue from secondary injuries induced by a primary burn wound is demonstrated by increased area of surviving interspace tissue (33).

Lutein

Lutein and its structural isomer, zeaxanthin, are among a large class of antioxidants knows as carotenoids. The antioxidant power of lutein comes from its ability to physically quench singlet oxygen radicals (34). There is evidence supporting the decline in a broad range of antioxidant concentrations in skin tissues after a burn injury occurs. Normally, antioxidants levels will begin to stabilize after approximately 3 weeks post trauma when vascularization returns and new tissue is in the stages of growth. There is an extended deficiency of carotenoids after burn injury, observed even after the levels of other antioxidants begin to normalize (35). This phenomenon is understood to occur because select carotenoids serve as precursors for Vitamin A. Evidence supports an increased demand for Vitamin A during the inflammatory process; as a result, an increased conversion of carotenoids to Vitamin A may be noted. This would explain the transitory concentration of carotenoids identified after a burn injury (36).

Therefore, unless patients are provided supplementary carotenoids these plasma values may remain depleted for extended periods of time (35).

A study was conducted to determine the effect of oral and topical applications of carotenoids (lutein and zeaxanthin) on multiple aspects of skin health. Notable results were obtained regarding the concentration of superficial skin lipids, lipid peroxidation, skin elasticity, and skin hydration. Members of the trial received an oral treatment of 5mg lutein and 0.3mg zeaxanthin twice a day, and a topical treatment of 50ppm lutein and 3ppm zeaxanthin twice a day. After only 2 weeks, an approximately 20% increase in surface lipids was determined; however, after 12 weeks this increase peaked at an approximately 60% rise in surface lipids ($p<0.05$). Acting as a potent antioxidant in the skin, the same treatment decreased lipid peroxidation, as evidenced by a decrease in the amount of malondialdehyde (MDA) generated. At 12 weeks into treatment the level of MDA had been reduced to approximately 0.4mg per 100mg lipid compared to the approximately 1.1mg measured for the placebo treatment ($p<0.05$). A continual rise in skin elasticity and hydration was documented over the course of the 12 week study, ultimately peaking at an almost 70% increase in skin elasticity and an over 80% increase in skin hydration ($p<0.05$) at week 12 (37).

In support of the data above, another study was conducted by administering a treatment of multiple carotenoids (including lutein) and observing changes in skin thickness and density through ultrasound measurements. A 6.57% increase in skin density over 12 weeks was measured for the carotenoid treatment ($p<0.007$); in comparison, no significant change was noted during the trial period in the placebo. A 15.99% increase in skin thickness over 12 weeks was noted for the same carotenoid treatment ($p<0.0001$). Again, no significant changes were documented for the placebo treatment (38). Both of these studies provide evidence that

carotenoids, such as lutein, may be found beneficial in the stabilization of oxidative stress right after an injury and the progression cell proliferation and new tissue growth.

Coenzyme Q_{10} (Ubiquinol)

The coenzyme Q_{10} molecule is unique because it is the only lipid-soluble antioxidant that is naturally synthesized by the human body (39). More specifically, the antioxidant capacity of this molecule is found in the reduced form, $CoQ_{10}H_2$ (40). Due to its hydrophobic nature, coenzyme Q_{10} is able to maneuver within the membrane phospholipid bilayer (39). A sizable concentration of $CoQ_{10}H_2$ is located in cell membranes along with natural enzymes capable of reducing coenzyme Q_{10} to $CoQ_{10}H_2$ (40). Due to its presence within cell membranes, this molecule is found in close correlation with unsaturated fatty acids often subject to lipid peroxidation. Coenzyme Q_{10} acts by being locally available to protect vulnerable membranes from oxidation by free radicals (41). In fact, when cell membranes become oxidized, $CoQ_{10}H_2$ is the first antioxidant consumed to prevent membrane damage. This is important because the cell membrane is at increased risk of oxidation by the reactive oxygen species (ROS) produced in response to a cutaneous injury, such as a burn wound (40).

The presence of coenzyme Q_{10} also provides a regenerative effect, sustaining effective levels of antioxidants in damaged tissues. In the presence of $CoQ_{10}H_2$ the antioxidant α-tocopherol (Vitamin E) will be spared (40). Upon the oxidation of Vitamin E to a tocopheroxyl radical, coenzyme Q_{10} is capable of regenerating the initial active form of Vitamin E (39). Without coenzyme Q_{10} present in tissues, the regeneration of Vitamin E can be very slow (41). Additionally, coenzyme Q_{10} possesses the ability to regenerate itself. After encountering free radicals, coenzyme Q_{10} is transformed to an ubisemiquinone radical. Through the action of the

electron transport chain or quinone-specific reductases, the original antioxidant will be reproduced. This ability to recycle antioxidants for continued use critically reduces the levels of oxidative stress that may be produced after an injury occurs (39).

A particular study focused on the effects an oral administration of coenzyme Q_{10} has on collagen regrowth, myeloperoxidase (MPO) activity, malondialdehyde (MDA) and superoxide dismutase (SOD) concentrations in skin-incised mice. The natural collagen in undamaged skin is in the form of three helical polypeptide chains. However, in wounded epithelial tissue the presence of collagen-like polymer (CLP) will be evident. CLP, characterized by a single polypeptide chain, was used to detect increased collagen production in the injured tissue of the test subjects. Coenzyme Q_{10} raised the CLP levels 16.26 ± 2.99 mg/g tissue higher than the control ($p<0.05$). The presence of increased collagen levels in the subjects treated with coenzyme Q_{10} indicates the potential for enhanced tissue repair in the initial stages of wound healing (40).

Next, the level of MPO activity was used to assess the neutrophil concentration at the wound site. Neutrophils gather at the wound site as a part of the inflammatory response to injury. At an oral dose of 100 mg/kg, coenzyme Q_{10} significantly inhibited the MPO activity to only 13.11 ± 3.64 μU/g tissue compared to the control value of 17.20 ± 3.06 μU/g tissue ($p<0.05$). The decreased MPO activity is related to a reduced concentration of neutrophils at the wound site. Therefore, coenzyme Q_{10} is shown to produce an anti-inflammatory role in wound healing (40).

Measurements of MDA and SOD levels provide a good indication of the degree of lipid peroxidation present at the wound site. The control animals exhibited MDA level of 8.78 ± 2.65 μg/g tissue; however, the animals treated with coenzyme Q_{10} exhibited a MDA level of only 2.55

± 1.53 μg/g tissue. The levels of SOD, an enzymatic antioxidant, were increased in subjects treated with coenzyme Q_{10}. The SOD levels in the subjects treated with coenzyme Q_{10} were approximately 3.64 ± 0.32 U/mL compared to the control values of 2.43 ± 0.32 U/mL. The SOD levels are understood to decrease at the wound site as a normal response to the presence of reactive oxygen species (ROS). Since coenzyme Q_{10} is the first antioxidant used in the tissue, it may provide a sparing effect of SOD present. The inhibitory role on MDA levels and sparing effect of SOD provide evidence of the antioxidant potential of coenzyme Q_{10} in vivo (40).

Vitamin E-TPGS

This research involved the use of Vitamin E-TPGS as a solubilizer for the antioxidants studied and a major gel constituent. This compound, formally known as D-α-tocopheryl polyethylene glycol succinate, has been found to have applications in drug delivery. Due to its amphiphilic nature, Vitamin E-TPGS acts by enhancing the cellular uptake of the drug it is combined with (42). Specifically the compound is able to solubilize water-insoluble medications, or antioxidants for this research (43). Other modes of use include: an absorption enhancer, emulsifier, additive, permeation enhancer, and stabilizer (42).

Current research indicates the potential for topical applications of Vitamin E-TPGS based upon its high compatibility with human skin (43). This compatibility is the result of the natural ability of vitamin E to transverse the dermis easily, enhancing skin permeation and the delivery of topical medications into the cell membrane. Cream formulations involving vitamin E and its succinate esters are very few, but have been reported (5, 43).

Conclusion

The current research surrounding the use of antioxidants to promote wound healing is relatively new. However, significant evidence has been compiled regarding the positive effects that Vitamin E, Alpha-Lipoic Acid, Vitamin C, and Grape Seed Extract, Glutathione, Lutein, and Coenzyme Q_{10} have on multiple aspects of the process of wound healing and overall skin health. The time needed for complete healing, collagen formation and stability, infection prevention, and collective antioxidant power can be manipulated using antioxidants to develop the ideal therapeutic environment for the wounded tissue. Currently, antioxidants are being administered to the wound site via standard enteral nutrition practices. Implications arise with this mode of distribution due to the vascular damage apparent in the initial stages of healing. Since there is relatively no vascularization to the damaged tissue during this period, it is difficult to obtain appropriate concentrations of antioxidants to combat the oxidative reactions produced by free radicals. This has made it difficult to determine the true capacity that antioxidants have on the advancement of healing. It is our hypothesis that the development of new research pertaining to the topical application of antioxidants in a gel medium will allow for the necessary concentrations to be obtained in order to promote fast and effective healing with minimal scarring.

Abstract

Neutrophils and cytokines present during an inflammatory response produce oxidants, such as reactive oxygen species (ROS) or reactive nitrogen species (RNS). These oxidants act as free radicals, a highly reactive species that steal electrons from nearby molecules to satisfy their valence electron needs. The removal of electrons by free radicals produces damage within the healthy cells of tissue. Antioxidants can be used to reduce this oxidative stress and reestablish the necessary environment for wound healing by donating electrons to the free radicals, sparing the damaging effects oxidation causes to other molecules. The standard procedure for administering supplemental antioxidants is through enteral delivery. However, the inflammation and vascular damage experienced with a burn wound produces a notable decrease in the blood profusion to the damaged tissues. In contrast, this research focuses on a topical antioxidant treatment applied directly to the surface of the wound. By applying the gel topically, a higher concentration of antioxidants will be able to permeate the damaged tissue and quench enough free radicals to provide a therapeutic effect. Additionally, the gel developed by this research is comprised of a large percentage of Vitamin E-TPGS. This is a product capable of stabilizing moisture at the wound site; drawing fluid from the moist center and redistributing it to the drier perimeters of the wound. It is hypothesized the need for surgical debridement may decrease as a result of use of this topical application. Antioxidants often referred to in literature discussing nutrition and wound care include the following: Vitamin E, α-Lipoic Acid, Vitamin C, Grape Seed Extract, Coenzyme Q_{10}, Glutathione, and Lutein. These antioxidants were incorporated into a gel formula, using a factorial method, based on their antioxidant potential as evidenced by the existing literature. In order to identify the most effective combination of these antioxidants, one-, two-, three-, four-, and five-component antioxidant gels representing every combination of

the test antioxidants were produced. This resulted in a compilation of 35 gels for comparison. Each gel was tested on the basis of viscosity, pH, and antioxidant capacity. Antioxidant capacity was determined using the Ferric Reducing Antioxidant Plasma (FRAP) Assay, a spectrophotometric evaluation. A three-antioxidant gel composed of α-Lipoic Acid, Coenzyme Q_{10}, and Mixed Tocopherols (with and without micronized silver) was chosen as the final formulation. The storage stability of the final formulation was then evaluated once per week (over a total of 7 weeks) by measuring changes in pH, viscosity, and FRAP assay. In collaboration with Dr. Joseph Molnar at Wake Forest School of Medicine, an animal model trial will be conducted in the Spring Semester 2013 to determine the viability of the gel in comparison to a negative control and silver sulfadiazine, the current standard treatment protocol.

Material and Methods

Raw Materials

Seven antioxidants were selected for inclusion in a trial to develop a topical antioxidant gel. The antioxidants evaluated in a factorial design included: mixed tocopherols, α-lipoic acid, ascorbyl palmitate, grape seed extract, glutathione, lutein, and coenzyme Q_{10}. All antioxidants were sourced from Pure Bulk, with the exception of mixed tocopherols (Archer Daniels Midland). Additional components of the following gels may include: grape seed oil, squalene and Vitamin E-TPGS. Grape seed oil (Bella Famiglia, Flavor Delight, Richburg, SC), squalene (Sigma Aldrich), and Vitamin E-TPGS (Antares) were evaluated for effectiveness in producing a topical gel with specific physical characteristics.

Establishing a Procedure

The objective was to develop an amorphous Vitamin E-TPGS-based gel formulation that is stable at room temperature and with a consistency similar to that of commercial honey. Initially, this required determining the percentage of water needed to produce a gel with that desired consistency. Five gels were developed at varying percentages of water (Table 1). Each gel was prepared by adding the heated (60 °C) Deionized Distilled Water (DDW) slowly to the laboratory beaker containing Vitamin E-TPGS and stirring using a Cole-Parmer, Model 50004-00, Digital Reversing Mixer until a homogenous solution was formed.

Table 1	
Gel	**Formulation (% by Wt.)**
1	2.5% H_2O 97.5% Vitamin E-TPGS
2	5.0% H_2O 95.0% Vitamin E-TPGS
3	10.0% H_2O 90.0% Vitamin E-TPGS
4	12.0% H_2O 88.0% Vitamin E-TPGS
5	15.0% H_2O 85.0% Vitamin E-TPGS

After seven days, all five of the prepared gels had crystallized. The crystallization indicated an inability to maintain an amorphous physical state at room temperature.

The next gels developed were modified to include one antioxidant, at varying concentrations, to the gel formulation. It was hypothesized that the use of additional lipophilic compounds would prevent crystallization and allow for amorphous gel formation. The first set of gels were produced at 0.5% antioxidant, 10.0% DDW, and 89.5% Vitamin E-TPGS by weight (Table 2). The second set of gels were produced at 1.5% antioxidant, 10.0% DDW, and 88.5% Vitamin E-TPGS by weight (Table 2). Each gel was developed by mixing the antioxidant into a 250 mL laboratory beaker of Vitamin E-TPGS using a Cole-Parmer, Model 50004-00, Digital Reversing Mixer at 250 RPM. The heated DDW (60 °C) was added slowly to the beaker while being continually mixed at 250 RPM. The gel was stirred until a homogenous solution was formed.

Table 2	
Gel	**Formulation (% by Wt.)**
1	0.5% α-Lipoic Acid 10% H_2O 89.5% Vitamin E-TPGS
2	1.5% α-Lipoic Acid 10% H_2O 88.5% Vitamin E-TPGS
3	0.5% Lutein 10% H_2O 89.5% Vitamin E-TPGS
4	1.5% Lutein 10% H_2O 88.5% Vitamin E-TPGS

After five days, all four of the prepared gels had crystallized.

The next gels developed were modified to include grape seed oil. This was done in an effort to prevent the crystallization of the Vitamin E-TPGS and water solutions by further increasing the lipophile concentration. Grape seed oil was chosen because its density is similar to that of Vitamin E-TPGS and the other gel components. Additionally, the percentage of water in each gel was decreased. In order to determine the concentration of grape seed oil needed, a set of trial gels was developed at varying percentages of grape seed oil (Table 3). Each gel was produced by mixing the grape seed oil into a laboratory beaker containing Vitamin E-TPGS using a Cole-Parmer, Model 50004-00, Digital Reversing Mixer at 250 RPM. The heated DDW (60 °C) was slowly added to the beaker still being stirred at 250 RPM. The gel was allowed to mix until a homogenous solution was formed.

Table 3	
Gel	**Formulation (% by Wt.)**
1	0.5% Grape Seed Oil 5.0% H_2O 94.5% Vitamin E-TPGS
2	1.0% Grape Seed Oil 5.0% H_2O 94.0% Vitamin E-TPGS
3	2.5% Grape Seed Oil 5.0% H_2O 92.5% Vitamin E-TPGS
4	5.0% Grape Seed Oil 5.0% H_2O 90% Vitamin E-TPGS
5	7.5% Grape Seed Oil 5.0% H_2O 87.5% Vitamin E-TPGS
6	10.0% Grape Seed Oil 5.0% H_2O 85.0% Vitamin E-TPGS

After 2 days, all six of the prepared gels had crystallized. This result indicated that the mixture still did not contain enough, or the right form of, lipophile to keep the gel constituents in an amorphous state.

The next gels developed were modified to include squalene instead of grape seed oil. Again, the objective was to prevent the crystallization of the Vitamin E-TPGS and water solutions. Similar to grape seed oil, squalene was chosen because it has a density comparable to the other compounds of the gel but with a different chemical structure. It was hypothesized that the more rigid structure of squalene, in addition to its lipophilic nature, might prevent crystallization in the prepared gels. With this set of gels, the Vitamin E-TPGS solution was prepared on a Fisher-Scientific Isotemp Heated Stir Plate at 105 °C and the Cole-Parmer, Model 50004-00, Digital Reversing Mixer speed was increased to 800 RPM for one minute for a final mix.

Two gels (Table 4) were produced by mixing the squalene and antioxidant into a laboratory beaker containing the Vitamin E-TPGS using a Cole-Parmer, Model 50004-00, Digital Reversing Mixer at 500 RPM. The heated DDW (60 °C) was slowly added to the beaker still being mixed at 500 RPM. The solution was kept on a hot plate at 105 °C during the entire preparation. The gel was allowed to mix until a homogenous solution was formed. The rotational speed of the Cole-Parmer, Model 50004-00, Digital Reversing Mixer was then increased to 800 RPM for one minute.

Table 4

Gel	Formulation (% by Wt.)
1	0.55g α-Lipoic Acid 5.38g H_2O 10.13g Squalene 89.97g Vitamin E-TPGS **Total Lipophile Concentration: 100.65g**
2	1.66g α-Lipoic Acid 5.24g H_2O 10.67g Squalene 90.27g Vitamin E-TPGS **Total Lipophile Concentration: 102.60g**

After four days, both of the prepared gel had started to crystallize. Approximately 30% of the mixture had crystallized in Gel 1. The crystallization was not as prevalent in Gel 2; approximately 5% of the gel exhibited crystallization. Therefore, it was determined that the total amount of crystallization had been decreased with the use of squalene.

The next gels developed were modified to include a higher concentration of squalene. The objective was to prevent the remaining crystallization of the Vitamin E-TPGS and water solutions. Additionally, the Cole-Parmer, Model 50004-00, Digital Reversing Mixer rotational speed was increased to 800 RPM during the addition of gel constituents, further increased to 1200 RPM for two minutes, reduced to 800 RPM during addition of heated DDW, and increased

to 1000 RPM for a final mix. The alterations in speeds were an attempt to produce a more homogenous mixture through an even distribution of the lipophiles throughout the gel solution.

Four gels (Table 5) were produced by mixing the squalene and antioxidant into a laboratory beaker containing Vitamin E-TPGS using a Cole-Parmer, Model 50004-00, Digital Reversing Mixer at 800 RPM. The rotational speed was increased to 1200 RPM for two minutes and then reduced back to 800 RPM. The heated DDW (60 °C) was slowly added to the beaker still being mixed at 800 RPM. The solution was kept on a hot plate at 105 °C during the entire preparation. The gel was allowed to mix until a homogenous solution was formed. The rotational speed of the Cole-Parmer, Model 50004-00, Digital Reversing Mixer was then increased to 1000 RPM for one minute.

Table 5

Gel	Formulation (% by Wt.)
1	1.5% α-Lipoic Acid 8.5% Squalene 10.0% H_2O 80.0% Vitamin E-TPGS
2	0.5% α-Lipoic Acid 9.5% Squalene 10.0% H_2O 80.0% Vitamin E-TPGS
3	1.5% Glutathione 8.5% Squalene 10.0% H_2O 80.0% Vitamin E-TPGS
4	0.5% Glutathione 9.5% Squalene 10.0% H_2O 80.0% Vitamin E-TPGS

An additional gel (Table 6) was produced by mixing the squalene into a 250 mL laboratory beaker containing Vitamin E-TPGS using a Cole-Parmer, Model 50004-00, Digital Reversing Mixer at 800 RPM. The rotational speed was increased to 1200 RPM for two minutes and then reduced back to 800 RPM. Glutathione was dissolved in DDW at 105 °C. The heated

DDW and glutathione mixture was slowly added to the beaker still being mixed at 800 RPM. The solution was kept on a hot plate at 105 °C during the entire preparation. The gel was allowed to mix until a homogenous solution was formed. The rotational speed of the Cole-Parmer, Model 50004-00, Digital Reversing Mixer was then increased to 1000 RPM for one minute.

Table 6

Gel	Formulation (% by Wt.)
5	0.5% Glutathione 9.5% Squalene 10.0% H_2O 80.0% Vitamin E-TPGS

After two days, Gel 1 and 2 stayed in solution; no crystallization was observed. Gel 2 exhibited some cloudiness, while Gel 1 remained completely clear. Gel 3, 4, 5 all developed phase separation. The procedure for Gel 1 and 2 was confirmed as the most effective technique and was used to produce subsequent gels.

Finally, the thioyl ester in glutathione produced a strong odor making it unable to be used effectively as a potential product. Thus, glutathione was eliminated from any future testing. Lutein was eliminated because it lacked the heat stability necessary for the heated mixing procedure developed for preparation of the gels.

Gel Production

In order to determine the gel with the most potent antioxidant capacity and consistent stability, every possible combination of the five remaining antioxidants was produced. These gels were then analyzed on the basis of pH, viscosity, and antioxidant capacity. These values were then compared to determine the most favorable antioxidant combination.

All of the gels were prepared in an identical procedure. The squalene and antioxidant(s) were weighed out on Denver Instrument, Top Loading Balance for each gel formulation. The lipophile(s) and squalene were mixed into the Vitamin E-TPGS using a Cole-Parmer, Model 50004-00, Digital Reversing Mixer at 800 RPM on a hot plate heated to 105 °C. The rotational speed was then increased to 1200 RPM for 2 minutes. The rotational speed was decreased to 800 RPM and the heated DDW (160 °C) was added slowly. Finally, the speed was increased again to 1000 RPM for 1 minute.

First, five gels were produced using only one antioxidant per gel. The antioxidant concentration in these gels was maintained at 0.5% by weight. These gels were composed of 80.0% Vitamin E-TPGS, 10.0% DDW, 9.5% squalene, and 0.5% antioxidant by weight. The same five gels were then reproduced with an antioxidant concentration of 1.5% by weight. Each gel consisted of 80.0% Vitamin E-TPGS, 10.0% DDW, 8.5% squalene, and 1.5% antioxidant by weight (Table 7).

Table 7: 1 Antioxidant Gels

31412 23 2	**31612 25 1**	**31612 25 2**	**31612 25 4**	**32112 26 1**
0.5% α-Lipoic Acid	0.5% Grape Seed Extract	0.5% Coenzyme Q10	0.5% Ascorbyl Palmitate	0.5% Mixed Tocopherols
31412 23 1	**---**	**31612 25 3**	**31612 25 5**	**32112 26 2**
1.5% α-Lipoic Acid	1.5% Grape Seed Extract	1.5% Coenzyme Q10	1.5% Ascorbyl Palmitate	1.5% Mixed Tocopherols

The next set of gels produced included a combination of two antioxidants (Table 8). Each gel was 80.0% Vitamin E-TPGS, 10.0% DDW, 9.0% squalene, and 1.0% equal distribution of antioxidants by weight. Therefore, each antioxidant was included at 0.5% by weight. Throughout the following procedures, the percentage of squalene is altered to accommodate for a fluctuating percentage of antioxidant by weight. As the percentage of antioxidant in the gel increases, the percentage of squalene in the gel decreases. The objective was to maintain the concentration of lipophile at 10.0% by weight for each gel.

Table 8: 2 Antioxidant Gels				
041112 34 1	**041112 34 2**	**041112 34 3**	**041112 34 4**	**041112 34 5**
1. α-Lipoic Acid 2. Ascorbyl Palmitate	1. α-Lipoic Acid 2. Grape Seed Extract	1. α-Lipoic Acid 2. Mixed Tocopherols	1. Ascorbyl Palmitate 2. Mixed Tocopherols	1. Grape Seed Extract 2. Mixed Tocopherols
041812 36 1	**041812 36 2**	**041812 36 3**	**041812 36 4**	**041812 36 5**
1. α-Lipoic Acid 2. Coenzyme Q10	1. Ascorbyl Palmitate 2. Grape Seed Extract	1. Ascorbyl Palmitate 2. Coenzyme Q10	1. Grape Seed Extract 2. Coenzyme Q10	1. Coenzyme Q10 2. Mixed Tocopherols

Following the same progression, the next set of gels produced included a combination of three antioxidants (Table 9). Each gel was 80.0% Vitamin E-TPGS, 10.0% DDW, 8.5% squalene, and 1.5% antioxidants by weight.

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Table 9: 3 Antioxidant Gels				
041812 36 6	**041812 36 7**	**041812 36 8**	**041812 37 1**	**041812 37 2**
1. α-Lipoic Acid 2. Ascorbyl Palmitate 3. Grape Seed Extract	1. Ascorbyl Palmitate 2. Grape Seed Extract 3. Mixed Tocopherols	1. Grape Seed Extract 2. Mixed Tocopherols 3. Coenzyme Q10	1. α-Lipoic Acid 2. Grape Seed Extract 3. Coenzyme Q10	1. α-Lipoic Acid 2. Mixed Tocopherols 3. Coenzyme Q10
041812 37 3	**041812 37 4**	**041812 37 5**	**041812 37 6**	**041812 37 7**
1. α-Lipoic Acid 2. Ascorbyl Palmitate 3. Mixed Tocopherols	1. Ascorbyl Palmitate 2. Mixed Tocopherols 3. Coenzyme Q10	1. α-Lipoic Acid 2. Grape Seed Extract 3. Mixed Tocopherols	1. α-Lipoic Acid 2. Ascorbyl Palmitate 3. Coenzyme Q10	1. Ascorbyl Palmitate 2. Grape Seed Extract 3. Coenzyme Q10

The next set of gels produced included a combination of four antioxidants (Table 10). Each gel was 80.0% Vitamin E-TPGS, 10.0% DDW, 8.0% squalene, and 2.0% equal distribution of antioxidants by weight.

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Table 10: 4 Antioxidant Gels				
042512 49 1	**042512 49 2**	**042512 49 3**	**042512 49 4**	**042512 49 5**
1. Coenzyme Q10 2. α-Lipoic Acid 3. Ascorbyl Palmitate 4. Grape Seed Extract	1. α-Lipoic Acid 2. Ascorbyl Palmitate 3. Grape Seed Extract 4. Mixed Tocopherols	1. Ascorbyl Palmitate 2. Grape Seed Extract 3. Mixed Tocopherols 4. Coenzyme Q10	1. α-Lipoic Acid 2. Grape Seed Extract 3. Mixed Tocopherols 4. Coenzyme Q10	1. Ascorbyl Palmitate 2. α-Lipoic Acid 3. Mixed Tocopherols 4. Coenzyme Q10

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The final gel produced was a combination of all five antioxidants (Table 11). This gel consisted of 80.0% Vitamin E-TPGS, 10.0% DDW, 7.5% squalene, and 2.5% equal distribution of antioxidants by weight.

Table 11: 5 Antioxidant Gels
042512 49 6
1. α-Lipoic Acid 2. Ascorbyl Palmitate 3. Grape Seed Extract 4. Mixed Tocopherols 5. Coenzyme Q10

Viscosity

Each gel was placed in a heated water bath at 25°C and allowed to equilibrate in temperature before testing. Once at the appropriate temperature, each gel was removed from its original container and placed in the designated container provided with viscometer. The viscosity was analyzed for each gel sample using a viscometer (A and D Vibro. Viscometer, Model SV-100, Japan) under the standard operating procedure. The viscosity (Pa-s) and temperature (°C) of each gel was recorded for comparison.

pH

Each gel was diluted to a 97.0% water and 3.0% gel solution. The dilution was prepared by measuring 3.0g of each gel into a laboratory beaker then adding 97.0g of heated (60 °C) DDW. The components were mixed with a Cole-Parmer, Model 50004-00, Digital Reversing Mixer until a homogenous solution was formed. The pH of each gel solution was measured using a pH meter (Mettler-Totedo Seven Multi, pH Meter) under the standard operating procedure. These measurements were recorded for comparison.

Antioxidant Capacity

Each gel was diluted to a 97.0% water and 3.0% gel solution. The dilution was prepared by measuring 3.0g of each gel into a laboratory beaker then adding 97.0g of heated (60 °C) DDW. The components were mixed with a Cole-Parmer, Model 50004-00, Digital Reversing

Mixer until a homogenous solution was formed. A FRAP (Ferric Reducing Antioxidant Plasma) Assay was used to determine the antioxidant capacity of each gel solution. The FRAP reagent was produced using the method of Benzie and Strain (1996, Analytical Biochemistry 239: 70-76). For each gel solution, 5 cuvettes were prepared with 30.0μL gel solution and 1.0mL FRAP reagent. A blank cuvette was prepared using 30.0μL DDW and 1.0mL FRAP reagent. The cuvettes were allowed to react for 4 minutes before all 6 cuvettes were loaded into a spectrophotometer (Thermo Scientific, Biomate 3S UV-Visible Spectrophotometer) for colorimetric analysis at 593nm.

The measurements for both absorbance and concentration of each gel solution, as compared to a standard curve, were recorded for further comparison.

Results

The results of each test were evaluated on the basis of: antioxidant capacity using the FRAP assay, desired viscosity (10-15 Pa/s), and the properties of each of the antioxidant components. Ultimately, five gels were chosen as the most appropriate for a topical antioxidant gel application. This selection was based on desired physical characteristics and their antioxidant capacity for counteracting free radical damage in burns

The first gel selected was 042512 49 1 (Table 15), composed of: 0.5% coenzyme Q_{10}, 0.5% α-lipoic Acid, 0.5% ascorbyl palmitate, and 0.5% grapeseed extract by weight. This gel was selected for having the third highest antioxidant capacity of all the multi-antioxidant samples and a viscosity (15.2 Pa/s) reasonably close to the desired limits. Alpha-lipoic acid is both a water- and lipid-soluble antioxidant; thereby, extending its therapeutic potential in both membranes and intracellular spaces. This antioxidant is also capable of regenerating both the coenzyme Q_{10} and ascorbyl palmitate constituents of the gel, further propagating its effectiveness. These attributes make α-lipoic acid a desirable component in the treatment of damaged tissues. In conjunction, grapeseed extract has been identified to promote vascular endothelial growth in dermal tissue. This is of significant importance when treating a burn wound in the proliferation phase of healing.

The second gel selected was 041812 37 3 (Table 14), composed of: 0.5% α-lipoic acid, 0.5% ascorbyl palmitate, and 0.5% mixed tocopherols by weight. This gel was selected for having the fifth highest antioxidant capacity of all the multi-antioxidant samples and a viscosity (12.8 Pa/s) that falls well within the desired limits. As identified above, the α-lipoic acid included in the gel assists in a number of important factors contributing to wound healing.

However when considering regeneration of antioxidants in this particular gel, the α-lipoic acid is only able to regenerate the ascorbyl palmitate component. Additionally, in previous studies mixed tocopherols (Vitamin E) have been noted to penetrate the skin easily. In a topical antioxidant gel, this attribute is critical to the effectiveness of the treatment by increasing the concentration of antioxidants available in the wounded tissue. This gel was also chosen to exemplify the properties of a gel produced without grape seed extract.

The third gel selected was 041812 37 2 (Table 14), composed of: 0.5% α-lipoic acid, 0.5% mixed tocopherols, and 0.5% coenzyme Q_{10} by weight. This gel was selected for having the sixth highest antioxidant capacity of all the multi-antioxidant samples and a viscosity (12.2 Pa/s) that falls well within the desired limits. In addition to the qualities that both α-lipoic acid and the mixed tocopherols contribute, coenzyme Q_{10} provides its own degree of potentially beneficial elements as well. Coenzyme Q_{10} is the first antioxidant used to protect lipid membranes from oxidation due to its naturally occurring presence within the cell membrane. This antioxidant is also able to regenerate α-tocopherol, a component of mixed tocopherols. In summary, there are many opportunities for regeneration in the composite gel. The α-lipoic acid is able to regenerate the coenzyme Q_{10}, which is able to regenerate the α-tocopherol in the mixed tocopherols. This chain of regeneration may provide further protection from oxidation by free radicals at the wound site. Again, this was another gel chosen to exemplify the properties of a gel produced without grape seed extract.

The fourth gel selected was 041812 36 7 (Table 15), composed of: 0.5% ascorbyl palmitate, 0.5% grapeseed extract, and 0.5% mixed tocopherols by weight. This gel was selected for having the fourth highest antioxidant capacity of all the multi-antioxidant samples and a viscosity (14.5 Pa/s) that falls within the desired limits. Additionally, the gel has the capacity to

exhibit all of the positive attributes identified above for ascorbyl palmitate, grape seed extract, and mixed tocopherols.

The fifth gel selected was 042512 49 3 (Table 15), composed of: 0.5% ascorbyl palmitate, 0.5% grapeseed extract, 0.5% mixed tocopherols, and 0.5% coenzyme Q_{10} by weight. This gel was selected for having the third highest antioxidant capacity of all the multi-antioxidant samples and a viscosity (15.2) reasonably close to the desired limits. This gel has the capacity to exhibit all of the positive attributes identified above for ascorbyl palmitate, grape seed extract, mixed tocopherols, and coenzyme Q_{10}.

While some of the single antioxidant gels (Table 12) exhibited a higher antioxidant capacity, none were chosen for final comparison. This was due to the increased potential for healing in a gel that was composed of several multi-functional antioxidants rather than the benefits only one antioxidant may provide.

Table 12

Gel	AOX*	pH	Absorbance	Concentration	Viscosity (Pa-s)	Temperature (°C)
1-Antioxidant						
31412 23 1	LA	4.115	0.40236	0.69492	7.33	24.7
31412 23 2	LA	4.3	0.35688	0.6195	14.2	24.7
31612 25 1	GS	4.624	1.59516	2.68092	15.0	24.7
31612 25 2	CQ	4.585	0.4302	0.74136	10.2	25.4
31612 25 3	CQ	6.624	0.1464	0.2688	11.6	25.0
31612 25 4	AP	4.107	0.30444	0.53196	11.5	24.9
31612 25 5	AP	4.017	1.11756	1.8858	14.7	24.9
31612 26 1	MT	4.588	0.6348	1.0988	12.2	25.0
32112 26 2	MT	4.639	0.4584	0.7884	10.1	25.1

*LA: α-lipoic acid; GS: grape seed extract; CQ: coenzyme Q_{10}; AP: ascorbyl palmitate; MT: mixed tocopherols

Table 13						
Gel	**AOX***	**pH**	**Absorbance**	**Concentration**	**Viscosity (Pa-s)**	**Temperature (°C)**
2-Antioxidant						
041112 34 1	LA, AP	4.231	0.38736	0.67008	12.2	25.0
041112 34 2	LA, GS	4.383	0.43656	0.75192	14.1	25.0
041112 34 3	LA, MT	4.336	0.26472	0.46584	12.6	25.0
041112 34 4	AP, MT	4.374	0.57864	0.98856	12.7	25.0
041112 34 5	GS, MT	4.704	0.67356	1.1466	12.5	24.9
041812 36 1	LA, CQ	4.355	0.2222	0.412	14.3	24.8
041812 36 2	AP, GS	4.384	0.3900	0.6912	14.3	24.9
041812 36 3	AP, CQ	4.263	0.3162	0.5682	12.2	24.9
041812 36 4	GS, CQ	4.660	0.2698	0.4910	14.2	25.0
041812 36 5	CQ, MT	4.631	0.2160	0.4014	14.3	24.9

*LA: α-lipoic acid; GS: grape seed extract; CQ: coenzyme Q_{10}; AP: ascorbyl palmitate; MT: mixed tocopherols

Table 14						
Gel	**AOX***	**pH**	**Absorbance**	**Concentration**	**Viscosity (Pa-s)**	**Temperature (°C)**
3-Antioxidant						
041812 36 6	LA, AP, GS	4.286	0.3974	0.7036	13.5	24.9
041812 36 7	AP, GS, MT	4.413	0.4880	0.8542	14.5	24.7
041812 36 8	GS, MT, CQ	4.692	0.3548	0.6328	13.8	24.7
041812 37 1	LA, GS, CQ	4.409	0.2624	0.4788	16.5	24.8
041812 37 2	LA, MT, CQ	4.366	0.4628	0.81254	12.2	24.7
041812 37 3	LA, AP, MT	4.262	0.4656	0.817	12.8	24.8
041812 37 4	AP, MT, CQ	4.257	0.3944	0.6984	14.6	24.9
041812 37 5	LA, GS, MT	4.397	0.3818	0.6776	14.0	24.6
041812 37 6	LA, AP, CQ	4.199	0.3326	0.5958	14.5	24.6
041812 37 7	AP, GS, CQ	4.363	0.4364	0.7682	17.0	24.8

*LA: α-lipoic acid; GS: grape seed extract; CQ: coenzyme Q_{10}; AP: ascorbyl palmitate; MT: mixed tocopherols

Table 15						
Gel	**AOX***	**pH**	**Absorbance**	**Concentration**	**Viscosity (Pa-s)**	**Temperature (°C)**
4-Antioxidant						
042512 49 1	CQ, LA, AP, GS	4.386	0.5334	0.9302	15.2	24.7
042512 49 2	LA, AP, GS, MT	4.496	0.3830	0.6798	16.3	24.8
042512 49 3	AP, GS, MT, CQ	4.759	0.4418	0.7772	17.2	24.8
042512 49 4	LA, GS, MT, CQ	4.529	0.2934	0.5304	13.7	24.9
042512 49 5	AP, LA, MT, CQ	4.454	0.2606	0.4758	11.6	24.8

*LA: α-lipoic acid; GS: grape seed extract; CQ: coenzyme Q_{10}; AP: ascorbyl palmitate; MT: mixed tocopherols

Table 16						
Gel	**AOX***	**pH**	**Absorbance**	**Concentration**	**Viscosity (Pa-s)**	**Temperature (°C)**
5-Antioxidant						
042512 49 6	LA, AP, GS, MT, CQ	4.505	0.4016	0.7106	13.3	24.8

*LA: α-lipoic acid; GS: grape seed extract; CQ: coenzyme Q_{10}; AP: ascorbyl palmitate; MT: mixed tocopherols

Final Gel Selection

After consultation with Dr. Joseph Molnar of Wake Forest School of Medicine, a specialist in Wound Care and author of *Nutrition and Wound Healing*, one gel was chosen for final evaluation. This gel (041812 37 2; Table 3) and was composed of: α-lipoic acid, mixed tocopherols, and coenzyme Q_{10}. In addition to the high antioxidant capacity and desired viscosity discussed above, the combination of antioxidants in this gel was determined to be highly effective in treating burn wounds as determined by previous literature published.

Alpha-lipoic acid is easily absorbed by the skin and is specific in quenching reactive oxidant species (ROS), such as: hydroxyl radicals, superoxide, singlet oxygen, peroxyl radical, hypochlorous acid, and nitric oxide. Evidence supports that these ROS are produced abundantly in the event of a cutaneous injury, such as a burn. Fortunately due to the unique property of being both water- and lipid-soluble, the benefits of this antioxidant extend into both cellular membranes and intracellular spaces of tissue. Alpha-lipoic acid is also capable of regenerating vitamin E and coenzyme Q_{10}, both of which are also included in the gel.

The mixed tocopherols (Vitamin E) also possess the capacity to penetrate the skin easily. Mixed tocopherols, specifically α-tocopherols, are effective in eliminating the peroxyl radicals that damage lipid membranes. In newly formed tissue, such as a healing burn wound, the destruction of the polyunsaturated fatty acids of plasma membranes produced by lipid peroxidation is detrimental. Thus, indicating the importance that protection from lipid peroxidation may provide. Furthermore, α-tocopherol inhibits the oxidation of newly formed collagen and glycosaminoglycans present in tissue regrowth, preventing complications in the proliferation stage of wound healing and supporting healthy new tissue.

Finally, coenzyme Q_{10} is a hydrophobic antioxidant that protects the lipid membrane of cells from peroxidation. In fact, coenzyme Q_{10} is the first antioxidant consumed in the presence of oxidative stresss at the cell membrane due to its naturally occurring presence there. Therefore, it is increasingly important to maintain appropriate concentrations to prevent oxidative damage. In terms of regeneration, coenzyme Q_{10} is not only able to regenerate itself but also the active form of Vitamin E. Without this critical antioxidant the regeneration of Vitamin E proceeds slowly, limiting the concentration of antioxidants available to quench free radicals.

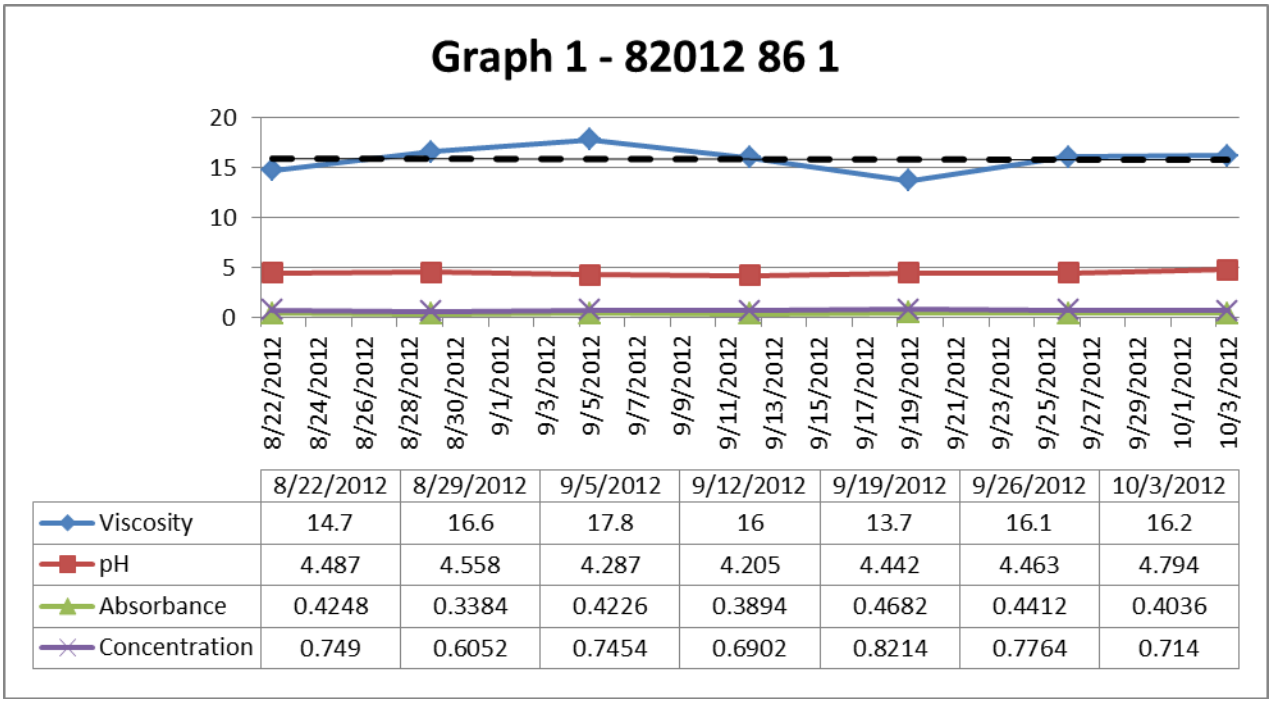
The gels 042512 49 1, 041812 36 7, and 042512 49 3 were eliminated due to the presence of grape seed extract. Since the gel was being produced for a potential animal model trial, all antioxidants must be Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration. Grape seed extract has not been approved because of its multicomponent antioxidant composition, and the identification and reproducibility of all its components has not been established. The gel 041812 37 3 was eliminated despite its close resemblance to the final gel chosen. Both ascorbyl palmitate and coenzyme Q_{10} have been identified as possessing antioxidant functions significant to healing in tissue wounds. The formulation including Coenzyme Q_{10} was ultimately selected because it is the first antioxidant depleted when the body is exposed to oxidative stress, particularly in situations of burn injury. Additionally, the compound is easily identifiable and already present in body tissue without supplementation.

Stability Progression

The selected gel (041812 37 2) was then reproduced in pairs and tested on the basis of stability over time. The first gel in each pair was produced as: 80.5% Vitamin E-TPGS, 9.5% DDW, 8.5% Squalene, 1.5% lipophile (3 antioxidant blend). The second gel in each pair was produced as: 79.5% Vitamin E-TPGS, 9.5% DDW, 8.5% Squalene, 1.5% lipophile (3 antioxidant blend), and 1.0% silver (Alfa Aesar silver powder, spherical, APS 0.5-1.0 u 99.9% Ag). Silver was incorporated into the second gel due to its well-recognized antimicrobial affects in burn wounds; in fact, silver sulfadiozine is the current standard treatment therapy.

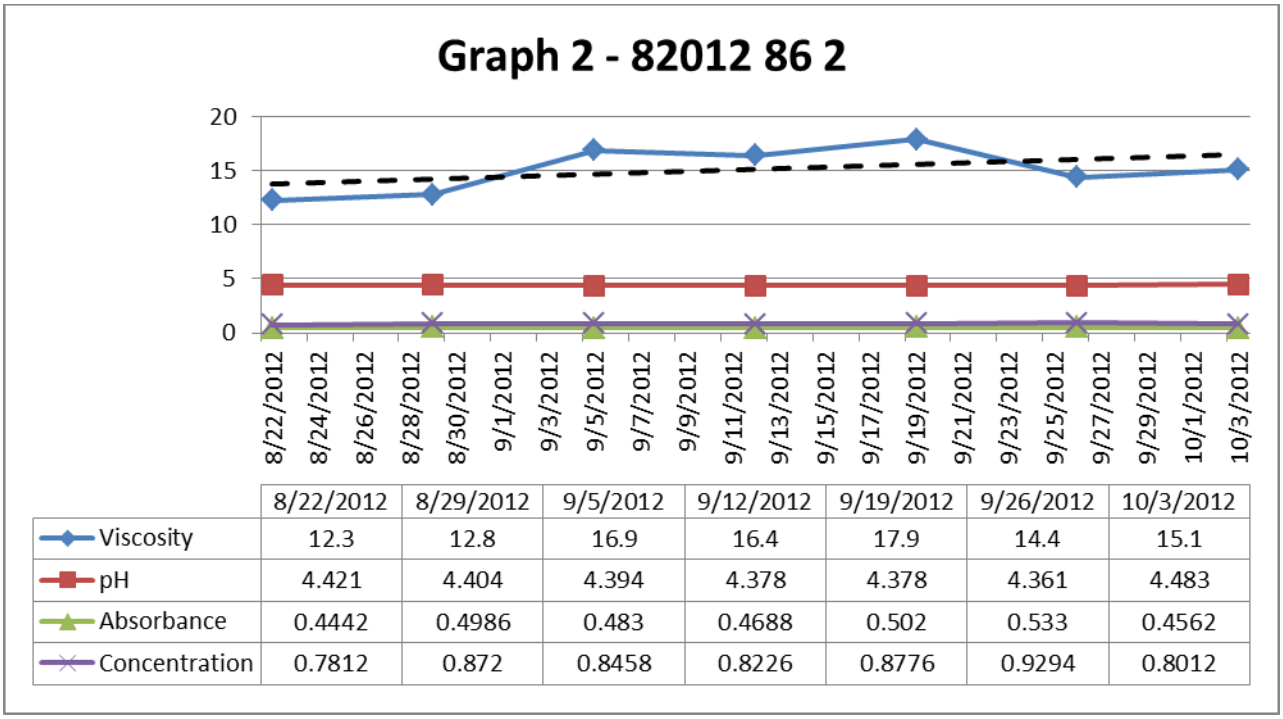
Pair 1	**82012 86 1**	w/o silver
	82012 86 2	silver
Pair 2	**82712 99 1**	w/o silver
	82712 99 2	silver
Pair 3	**9312 103 1**	w/o silver
	9312 103 2	silver
Pair 4	**91012 107 1**	w/o silver
	91012 107 2	silver

A new gel was prepared each week, tested 2 days later, and then re-tested every 7 days. The first pair of gels, 82012 86 1 and 82012 86 2, were tested over the course of 7 weeks (Graph 1,2). The second pair of gels, 82712 99 1 and 82712 99 2, were tested over the course of 6 weeks (Graph 3,4). The third pair of gels, 9312 103 1 and 9312 103 2, were tested over the course of 5 weeks (Graph 5,6). The final pair of gels, 91012 107 1 and 91012 107 2, were tested over the course of 4 weeks (Graph 7,8).



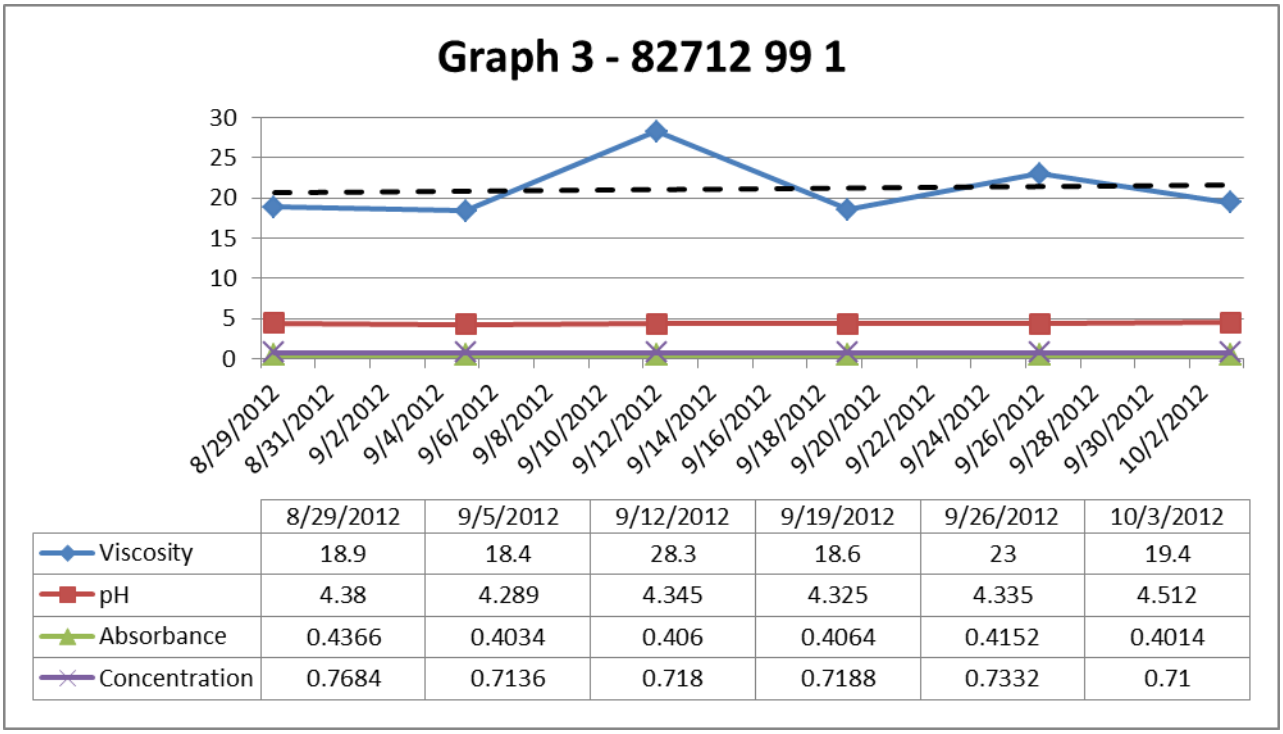
	8/22/2012	8/29/2012	9/5/2012	9/12/2012	9/19/2012	9/26/2012	10/3/2012
Viscosity	14.7	16.6	17.8	16	13.7	16.1	16.2
pH	4.487	4.558	4.287	4.205	4.442	4.463	4.794
Absorbance	0.4248	0.3384	0.4226	0.3894	0.4682	0.4412	0.4036
Concentration	0.749	0.6052	0.7454	0.6902	0.8214	0.7764	0.714

*Dashed line (---) indicates trend line for Viscosity



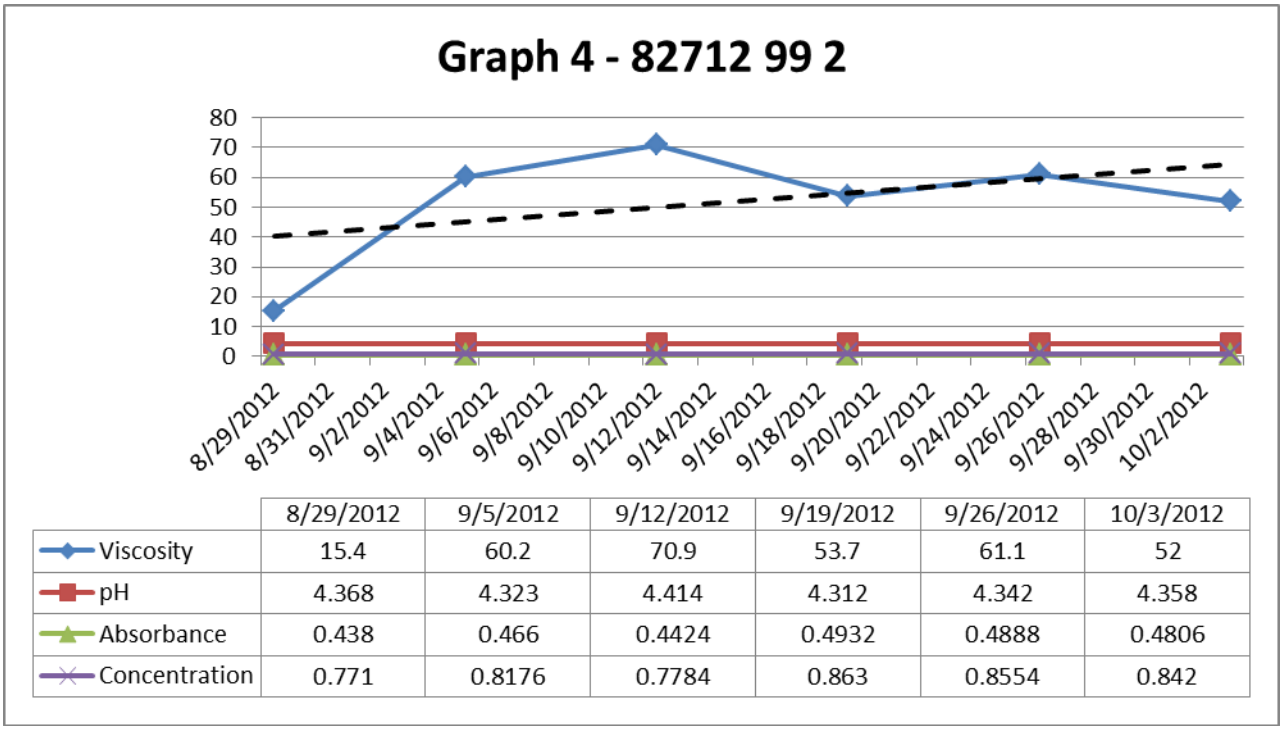
	8/22/2012	8/29/2012	9/5/2012	9/12/2012	9/19/2012	9/26/2012	10/3/2012
Viscosity	12.3	12.8	16.9	16.4	17.9	14.4	15.1
pH	4.421	4.404	4.394	4.378	4.378	4.361	4.483
Absorbance	0.4442	0.4986	0.483	0.4688	0.502	0.533	0.4562
Concentration	0.7812	0.872	0.8458	0.8226	0.8776	0.9294	0.8012

*Dashed line (---) indicates trend line for Viscosity



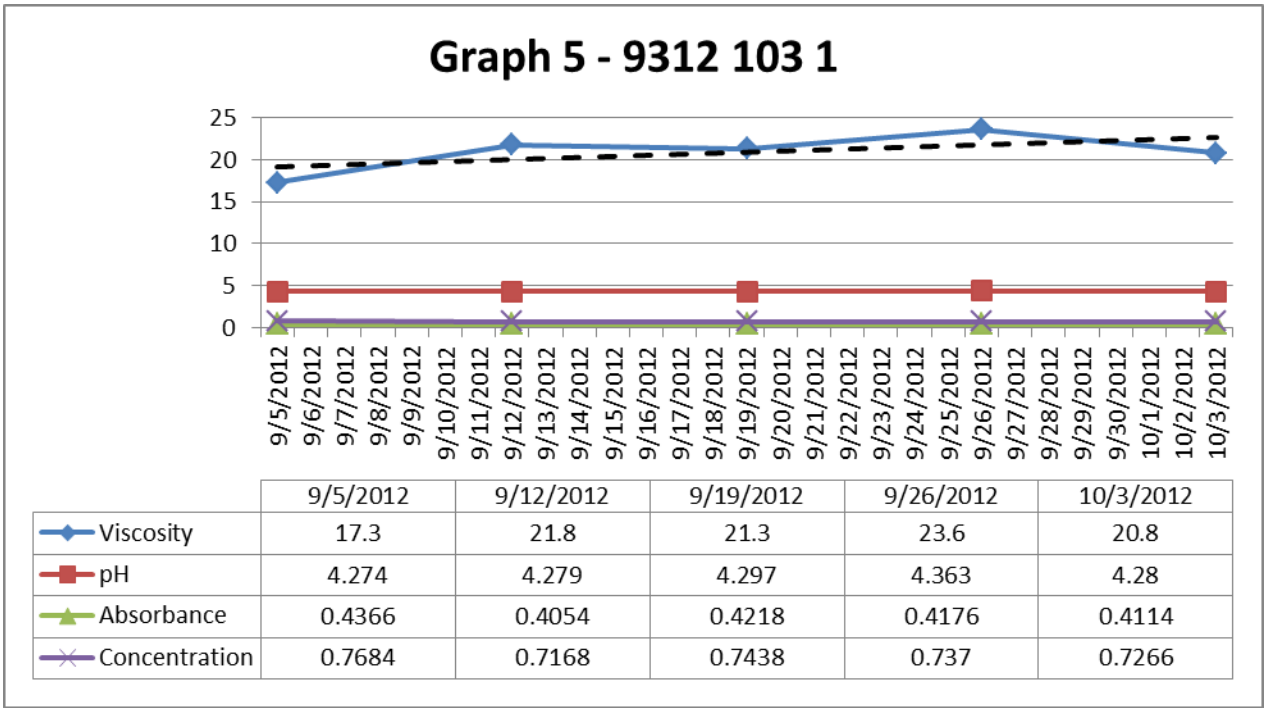
	8/29/2012	9/5/2012	9/12/2012	9/19/2012	9/26/2012	10/3/2012
Viscosity	18.9	18.4	28.3	18.6	23	19.4
pH	4.38	4.289	4.345	4.325	4.335	4.512
Absorbance	0.4366	0.4034	0.406	0.4064	0.4152	0.4014
Concentration	0.7684	0.7136	0.718	0.7188	0.7332	0.71

*Dashed line (---) indicates trend line for Viscosity



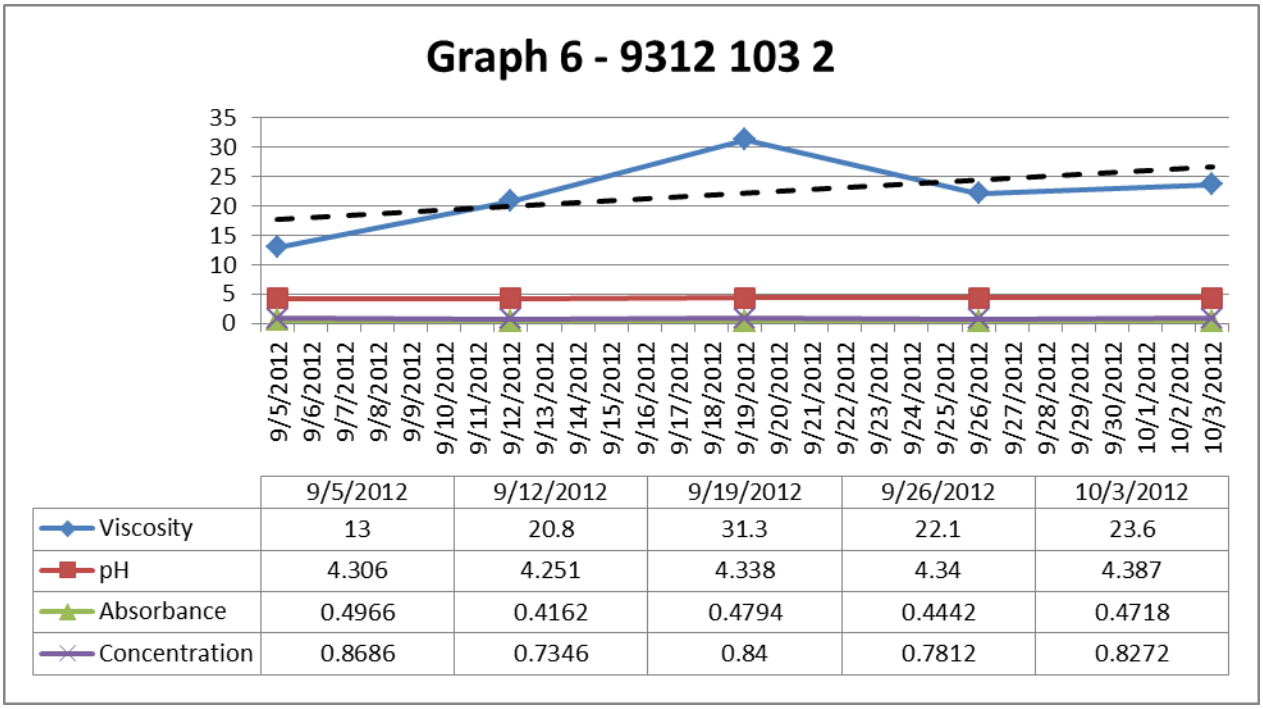
	8/29/2012	9/5/2012	9/12/2012	9/19/2012	9/26/2012	10/3/2012
Viscosity	15.4	60.2	70.9	53.7	61.1	52
pH	4.368	4.323	4.414	4.312	4.342	4.358
Absorbance	0.438	0.466	0.4424	0.4932	0.4888	0.4806
Concentration	0.771	0.8176	0.7784	0.863	0.8554	0.842

*Dashed line (---) indicates trend line for Viscosity



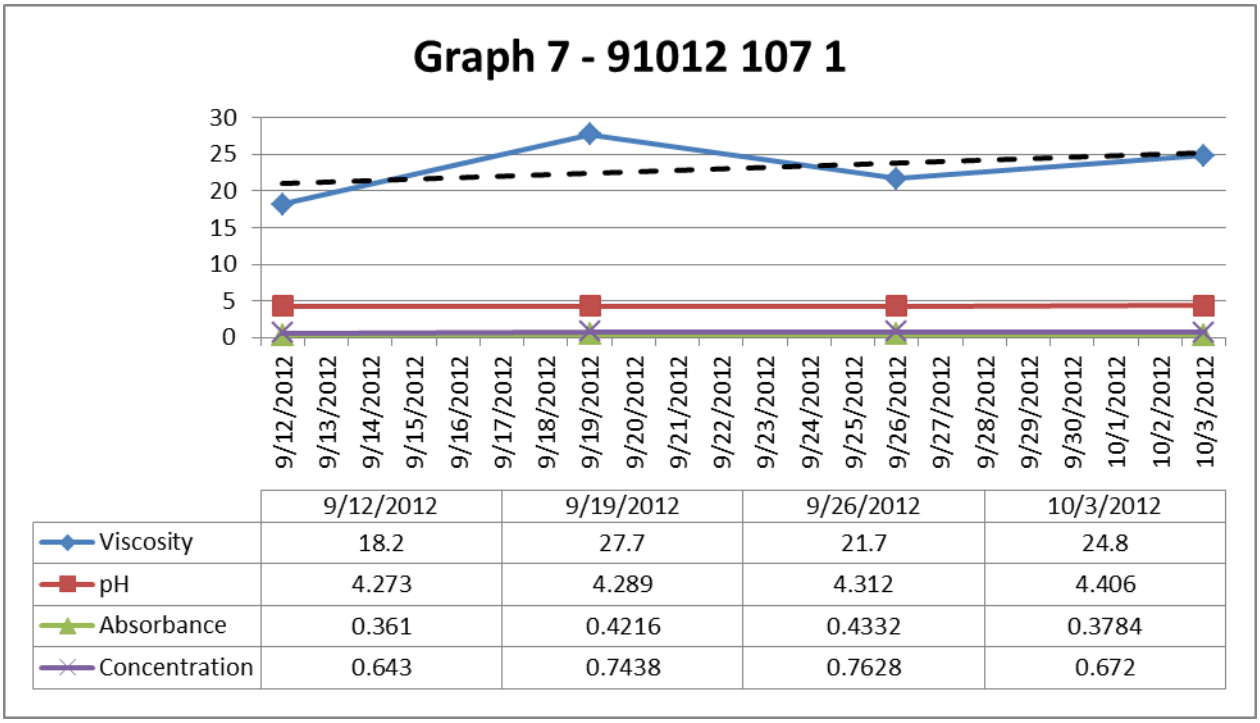
	9/5/2012	9/12/2012	9/19/2012	9/26/2012	10/3/2012
Viscosity	17.3	21.8	21.3	23.6	20.8
pH	4.274	4.279	4.297	4.363	4.28
Absorbance	0.4366	0.4054	0.4218	0.4176	0.4114
Concentration	0.7684	0.7168	0.7438	0.737	0.7266

*Dashed line (---) indicates trend line for Viscosity



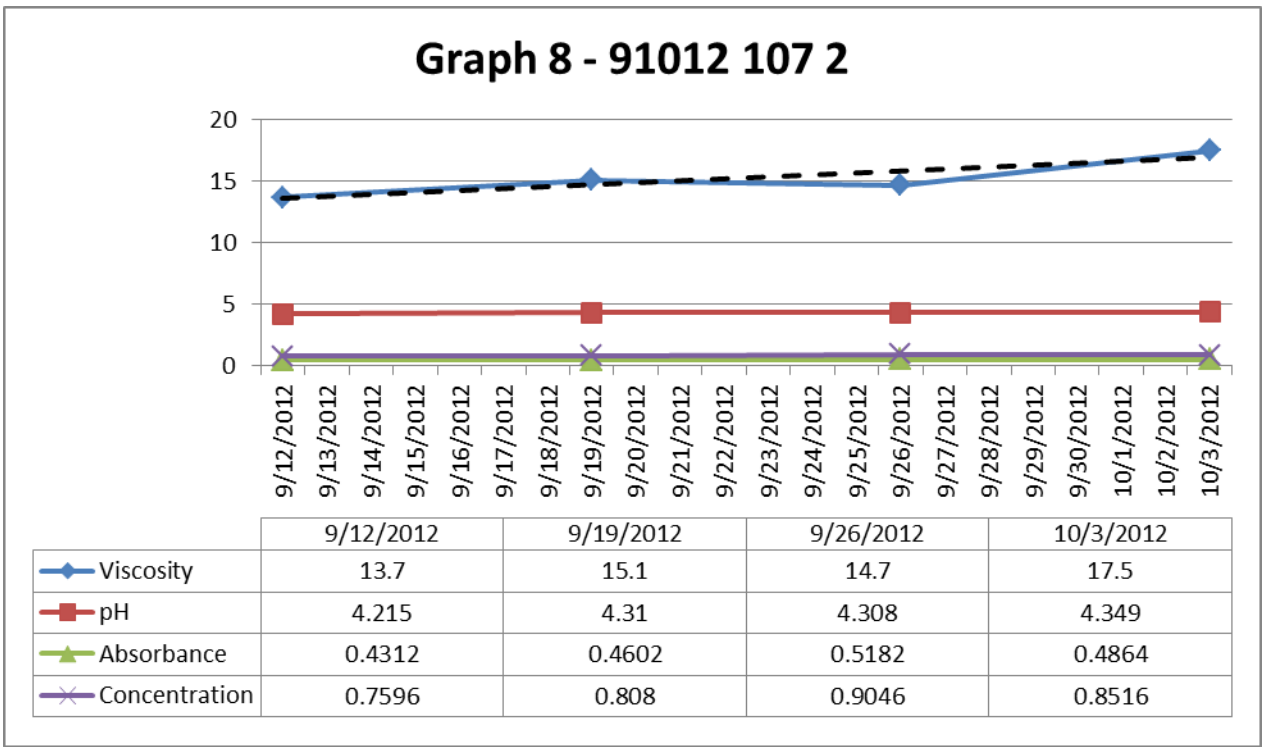
	9/5/2012	9/12/2012	9/19/2012	9/26/2012	10/3/2012
Viscosity	13	20.8	31.3	22.1	23.6
pH	4.306	4.251	4.338	4.34	4.387
Absorbance	0.4966	0.4162	0.4794	0.4442	0.4718
Concentration	0.8686	0.7346	0.84	0.7812	0.8272

*Dashed line (---) indicates trend line for Viscosity



	9/12/2012	9/19/2012	9/26/2012	10/3/2012
Viscosity	18.2	27.7	21.7	24.8
pH	4.273	4.289	4.312	4.406
Absorbance	0.361	0.4216	0.4332	0.3784
Concentration	0.643	0.7438	0.7628	0.672

*Dashed line (---) indicates trend line for Viscosity



	9/12/2012	9/19/2012	9/26/2012	10/3/2012
Viscosity	13.7	15.1	14.7	17.5
pH	4.215	4.31	4.308	4.349
Absorbance	0.4312	0.4602	0.5182	0.4864
Concentration	0.7596	0.808	0.9046	0.8516

*Dashed line (---) indicates trend line for Viscosi

Discussion

Beneficial outcomes have been noted in previous studies of wound healing with the reduction of free radical damage using both enteral and topical antioxidant therapy.

The antioxidants used in the final gel formulation were: mixed tocopherols, α-lipoic acid, coenzyme Q_{10}, and additional α-tocopherol in the form of Vitamin-E TPGS. Alpha-tocopherol, a component of mixed tocopherols, eliminates peroxyl radicals and protects the cell membrane from oxidation (19). This antioxidant can also protect collagen and glycosaminoglycans from oxidation, which may speed the rate of wound closure (22). Alpha-lipoic acid is another antioxidant that functions to protect the cell from lipid peroxidation by eliminating reactive oxidant species (ROS), such as: hydroxyl radicals, superoxide, singlet oxygen, peroxyl radicals, hypochlorous acid, and nitric oxide (24). In addition to its direct effect on free radicals, α-lipoic acid also acts to regenerate vitamin E, vitamin C, glutathione, and coenzyme Q_{10} (25). Finally, coenzyme Q_{10} is the only lipid-soluble antioxidant that is naturally synthesized in the body (39). It is also the first antioxidant consumed upon the infiltration of free radicals in order to protect unsaturated fatty acids in the cell membranes from oxidation (40). This function is critical due to the integral role of coenzyme Q_{10} in the electron transport chain occurring in the mitochondria. Coenzyme Q_{10} transfers electrons from primary substrates to the oxidase system while simultaneously transferring protons to the exterior of the mitochondrial membrane. This action creates a proton gradient across the membrane (41). This antioxidant is also capable of regenerating both itself and vitamin E, extending the availability of antioxidants to quench free radicals (41, 39).

The development of a topical antioxidant gel containing Vitamin E-TPGS as a lipophile solubilizer is unique in two elements: topical application and moisture stabilization. The physiology of burn wounds inhibits an adequate concentration of antioxidants from reaching the wound site. Due to significant vascular damage and inflammation, enteral administration of antioxidant supplementation may have limited effectiveness. Therefore, by applying the antioxidants directly to the surface of the wound with a topical gel this complication might be avoided.

Next, Vitamin E-TPGS possesses the capability to stabilize wound moisture. The typical burn wound is characterized as fluid at the center with a dryer outer edge that often requires surgical debridement. Vitamin E-TPGS acts to absorb the excess moisture and redistribute it to drier portions of the wound. It is hypothesized that this will decrease the need for surgical debridement and the development of scar tissue.

After establishing a procedure and selecting the final gel formulation, a stability trial was conducted to determine the constancy of the gel's characteristics over time. Stability of the gel was considered to ensure that the gel would remain at its maximal therapeutic value over time. The final gel formulation was reproduced in pairs; in which, one gel contained 1.0% silver in addition to the other gel components. Silver was included due to its anti-microbial affects in burn wounds. A new set of gels was produced each week (day 1), tested two days later (day 3), and then re-tested every seven days (day 10, 17, 24, etc.). The first pair of gels was tested over the course of 7 weeks (Graph 1,2). The second pair of gels was tested over the course of 6 weeks (Graph 3,4). The third pair of gels was tested over the course of 5 weeks (Graph 5,6). The fourth, and final, pair of gels was tested over the course of 4 weeks (Graph 7,8). Eight gels were

prepared in total. Measurements for viscosity, pH, and antioxidant capacity were recorded over the extended period of time (Graph 1-8).

Overall, the values for pH and antioxidant capacity over time remained markedly similar to the original values for all eight of the test gels. The most variance was seen in the measurement of viscosity. The average viscosity of the eight gels at the first testing period was approximately 15 Pa-s. The average viscosity rose to approximately 25 Pa-s over 3 weeks before stabilizing. The addition of silver to the final gel formulation did not appear to affect the viscosity measured; both the gels with and without silver exhibited a similar trend of increasing viscosity during the stability trial. Even though this rise was noted, the gels still remained spreadable and useful in the application as a topical therapy since the gel will become less viscous as it warms on the skin. According to Warren K. Hopkins (Yasoo Health, March 2013), in the laboratories of Eastman Chemical Company Vitamin E-TPGS was shown to take up to three weeks before stabilizing into its final structural form when mixed with water. This may provide explanation for the inconsistency in viscosity measurements during the first few weeks of the stability trial. However in future productions, it may be advantageous to begin with a less viscous gel to accommodate for the physical changes over time. Ultimately, results of the stability trial indicate that the gel will remain at its most potent antioxidant potential and at a steady amorphous consistency throughout storage.

In the spring of 2013, an animal model trial to evaluate the effectiveness of the final formulation of the antioxidant gel will be conducted at Wake Forest School of Medicine under the supervision of Dr. Joseph Molnar. This project has been designed to test the hypothesis that this new topical therapy will provide beneficial effects on the progression of a burn wound through the phases of healing. Pigs have been chosen as the animal model due to the similarities

between their skin and that of humans. The animals will receive standardized burn wounds, administered under anesthesia and according to an established Wake Forest School of Medicine protocol. Each animal will then receive the following treatments for comparison: no treatment (negative control), silver sulfadiazine (positive control), and the antioxidant gel produced with and without silver (experimental). Results of testing will be determined using standardized digital photography, scanning laser Doppler, thermal dilution probe, florescence dye evaluation, and histology. The rate of epithelialization, or rate of healing, will be determined by evaluation of standard digital photography on days 0, 3, 7, 10, 14, 17, and 21. The rate of revascularization and blood flow present at the wound site will be determined through scanning laser Doppler, thermal dilution probe, and florescence dye evaluation on days 0, 3, and 7. Histology obtained on days 3 and 7 will aid in the determination of revascularization and total vascular damage. The results of this trial will indicate whether the proposed antioxidant gel is a viable therapy in the treatment of thermal wounds.

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