Investigator Initiated Research Protocol Format

If the protocol is not for GCRC review you only need to complete and submit the following information to the IRB.

I. Protocol Information

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b. **IRB Protocol Number**: 12-189

c. **Study Phase**: 2 and 3

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III. Investigators

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V. Study Information: Biomedical, Drug, Biologic, Tissue/Blood/Other Specimen

Abstract

We previously demonstrated that thermal injury depletes plasma alpha-tocopherol in burn patients [1]. However, plasma changes may reflect only short-term alterations in vitamin E nutriture. Alpha-tocopherol concentrations in adipose tissue are generally accepted as reflecting long-term vitamin E status [2]. Our recent findings demonstrate that burn injury significantly depleted stores of vitamin E in adipose tissue of children by nearly 50% within one month of injury [3]. The consequences of this severe and rapid depletion are unknown because adipose tissue alpha-tocopherol normally takes years to deplete. Vitamin E deficiency in humans is manifested by a progressive, peripheral neuropathy [4-5], a symptom frequently observed in burn patients [6]. The progression to tocopherol deficiency is hypothesized to be first manifested as an increase in markers of oxidative stress and the depletion of adipose tissue alpha-tocopherol stores. The daily supplementation of vitamin E was proven safe in children ages 8-17 in a large randomized, placebo-controlled clinical trial as a preventative treatment for non-alcoholic fatty liver disease (NAFLD), as discussed below [7]. **What is not known** is the degree to which vitamin E supplements can reverse alpha-tocopherol depletion, reverse oxidative stress, and improve post-burn outcomes, including lung function and impaired wound healing in burn patients.

Our **long-term goal** is to improve the quality of life of the burn patient by preventing pathophysiology that may result from oxidative stress. The **objectives of this application** are to **a)** attenuate alpha-tocopherol depletion in burn patients by vitamin E supplementation, **b)** to prevent or reverse oxidative stress, **c)** to collect pilot data on the effect of vitamin E supplementation on lung function and impaired wound healing. Oxidative stress greatly contributes to multi-organ dysfunction syndrome (MODS) and causes decreases in lung function [8-9]. Roughly 90% of MODS patients have pulmonary problems. Scavengers of reactive oxygen and nitrogen species such as alpha-tocopherol are significantly depleted in patients after burn injury [10]. Our **central hypothesis** is that the administration of high doses of alpha-tocopherol will prevent or restore levels of vitamin E in adipose tissue and reverse the oxidative state present in burn patients. We will assess the degree to which vitamin E supplements can reverse alpha-tocopherol depletion and improve the post-burn oxidative state.

We will test this hypothesis by pursuing the following three specific aims in burn patients. We will administer vitamin E supplements to burn subjects for either days 1-15 or days 16-30 of the study (n= 21 per group, 18-85 years, ≥40% total body surface area burns). The two groups are necessary for our reverse crossover design. We will administer vitamin E to consenting patients at the Shriners Hospitals for Children (SHC) and Blocker Burn Unit (BBU) at the University of Texas Medical Branch (UTMB) in Galveston, the Burn Intensive Care Unit at Memorial Hermann Hospital (BICU-MHH) in Houston, and the Parkland Health and Hospital System Burn Unit (PHHS-BU) in Dallas.

Aim 1: To determine the degree that supplemental Vitamin E will attenuate alpha-tocopherol depletion. Our working hypothesis is that oral vitamin E supplements given on either days 1-15 or days 16-30 will adequately prevent or reverse alpha-tocopherol depletion by measuring alpha-tocopherol in plasma and adipose tissue over 30 days. Supplements will be given according to subject age: 800 IU/day to ages 16-17, and 1200 IU/day to ages 18-85.

Aim 2: To determine if supplemental Vitamin E reduces markers of oxidative stress in burn patients. Our working hypothesis is that supplemental vitamin E will significantly decrease oxidative stress markers in adipose tissue, plasma, and urine. We will measure, plasma malondialdehyde, and urinary biomarkers of oxidative stress, such as isoprostanes, on a weekly basis and/or at every surgery (at least 4 times in 30 days).

Aim 3: To collect preliminary data to establish the relationship between oxidative stress and pulmonary pathophysiology and impaired wound healing after burn injury. Our working hypothesis is that pulmonary changes and impaired wound healing associated with burn injury are caused by increased oxidative stress. The oral administration of vitamin E will significantly reduce lung markers of oxidative stress and improve lung function and wound healing. These data will be used to determine the number of subjects needed for a clinical study on the effect of vitamin E supplementation on lung function and wound healing.

We anticipate that this research will yield the following **outcomes**: A) improve our understanding of the metabolism of alpha-tocopherol in thermally injured patients, and B) demonstrate the mechanism by which vitamin E administration **reverses** the oxidative stress induced by burn injury. These outcomes have an important **positive impact** because they will lay the foundation for the development of effective, safe, and economic therapeutic interventions to treat burn injury-associated metabolic abnormalities. We anticipate that vitamin E supplements will prevent vitamin E depletion, reduce oxidative stress, attenuate the development of lung dysfunction and impaired wound healing, reduce hospital stay, and improve the quality of life of burn patients.

Significance

Our published observations in pediatric patients [11] emphasize that severe burn injury induces a hyper-

catabolic state [12] that causes a severe depletion of alpha-tocopherol stores and further suggest that inadequate vitamin E is being supplied to these children despite the enteral feedings provided to the subjects [3]. The plasma depletion of alpha-tocopherol was associated with elevation in markers of oxidation, and if the levels of antioxidants were not restored the patients did not survive [10]. The severity of the alpha-tocopherol depletion from adipose tissue that occurred within a few weeks of burn injury in pediatric patients is stunning because adipose tissue reflects long-term vitamin E status and changes in adipose tissue alpha-tocopherol generally take years to occur [3, 13].

The recommended daily allowance (RDA) [14] for vitamin E is 7 mg alpha-tocopherol daily for children with a reference weight of 20 kg and BMI of 15 kg/m^2, who are aged 4 to 8 y (the average age of the children in our study was 7 y). The current practice of the Shriners Hospital for Children and the Blocker Burn Unit is to determine the exact caloric need via determination of resting energy expenditure and multiply by 1.4. The enteral feed used provides alpha-tocopherol 15 IU (10 mg) per 1000 kcal. It is expected that based on the calculation of caloric requirements, the vitamin E intakes during the first weeks of hospitalization of all the children at the Shriners Hospital met or exceeded the RDA for normal healthy children. By contrast, the estimated daily rate of loss equates to an approximate loss of 5 mg alpha-tocopherol daily. Given that absorption of vitamin E is less than 50% [15], it is clear that the **vitamin E intakes by these pediatric burn patients are less than their requirements**. Our clinical trial will supplement the subjects between 800-1200 IU/day, depending on their ages, an amount that will exceed their normal requirements, but may be necessary in this pathological state.

Vitamin E deficiency in humans causes a peripheral neuropathy due to a dying back of large caliber fibers in sensory neurons [4-5]. Remarkably, peripheral neuropathy in the burn patient is also common [6]. Moreover, it is well known that the burn patient is in a hyper-catabolic state and has an elevated metabolic rate [16]; and that depletion of fat stores, massive lipolysis, and the development of fatty liver are all components of the disorder [16]. Thus, the trauma of the burn patient and its sequelae suggest that there is a labile pool of alpha-tocopherol that can be drawn upon during stress. These findings also suggest that **the typical adverse metabolic consequences observed in these patients, fatty liver and peripheral neuropathy, may result from inadequate vitamin E status.**

The 2011 Treatment of NAFLD in Children (TONIC) clinical trial in children tested whether treatment with metformin, an insulin sensitizer, or vitamin E, led to improvements in biochemical and histological features of non-diabetic children with biopsy-proven NAFLD [7, 17]. Lavine *et.al.* used vitamin E (400 IU b.i.d.), or placebo for 96 weeks. The primary outcome measure chosen for the trial was improvement in serum alanine aminotransferase (ALT) levels with treatment as compared to placebo. An improvement in ALT is defined as reduction in serum ALT levels to below 50% of the baseline values or into the normal range (40 U/L or less) during the last 48 weeks of treatment. Histological improvement is defined by changes in liver histology between a baseline and end-of-treatment liver biopsy in regards to (1) steatohepatitis, (2) NAFLD Activity Score, consisting of scores for steatosis, lobular inflammation, and hepatocellular injury (ballooning), and (3) fibrosis score. Between September 2005 and September 2007, 173 children were enrolled into TONIC at 10 clinical centers in the United States. The final results showed significant changes in ALT levels from baseline with vitamin E supplementation compared to placebo groups at weeks 24 (p<0.005) and 48 (p<0.04), as well as significant improvements in ballooning degeneration (p<0.02), changes in NAFLD activity scores (p<0.02), and resolution of nonalcoholic steatohepatitis (p<0.006) with daily vitamin E supplementation. Thus, there is clinical evidence suggesting that vitamin E will be beneficial in pediatric burn patients and the TONIC clinical trial showed that vitamin E supplementation in pediatric NAFLD patients was safe and prevented fatty liver.

Innovation

The proposed research is **innovative** because a dietary antioxidant, alpha-tocopherol, will be used to attenuate Vitamin E short- and long-term losses in plasma and adipose, reverse the oxidative stress of burn injury and, in the process, decrease the secondary consequences of burn injury, including lung injury and impaired wound healing. Also:

a) It is the first study whose focus is to **alleviate the severe vitamin E depletion in burn patients** by administering supplemental vitamin E. We have previously reported that adult patients with severe burns had very high levels of markers of oxidation and very low levels of antioxidants. If the redox status of these patients was not restored, the patients died [10]. We have recently shown that the long-term vitamin E status of burn patients decreases by half as early as 3 weeks after injury [3]. The patients are entering the operating room with consistently low vitamin E status. The decrease in vitamin E results in less scavenging of reactive oxygen (ROS) and reactive nitrogen (RNS) species, which we have shown decreases lung compliance and increases lung fibrosis in the ovine model of burn and smoke inhalation [18]. The proposed study will create a more complete picture of vitamin E metabolism in response to supplementation and improve our understanding of the therapeutic interventions with vitamin E in humans.

b) Preliminary data will be collected that shows attenuation of the lung pathophysiology and impaired

wound healing after burn injury by administering a **relatively inexpensive and simple vitamin** instead of more complex antioxidants and pharmaceutical interventions. It is a dietary supplement that is **abundant** and **approved for clinical use** that we propose will normalize the oxidation status of burn patients. Vitamin E's actions and side effects are known, and it is easy to administer. This is important because significant oxidation occurs in burn injury and may directly cause its sequelae of lung dysfunction and impaired wound healing, and vitamin E provides an easy method of reversing oxidation. We will utilize this study to determine whether the normalization of oxidation will correct the pathophysiology associated with burn injury.

Approach

Because of the nature of our proposed research, we will present the Background separately for each Specific Aim. The Preliminary Studies, Research Design, and Potential Problems with Alternative Strategies are common for both Aims and will be presented following Specific Aim 3.

Specific Aim 1: To determine the degree that supplemental Vitamin E will attenuate alpha-tocopherol depletion. **Specific Aim 2:** To determine the degree that supplemental Vitamin E reduces markers of oxidative stress in burn patients.

Background

Vitamin E is a peroxyl radical scavenger that halts the radical-mediated chain reaction of lipid peroxidation of polyunsaturated fatty acids (PUFA) [19-21]. PUFA are particularly susceptible to oxidation because of their easily-abstracted bis-allylic hydrogens [22]. Due to the kinetic properties of alpha-tocopherol, only small amounts are required to be present to reduce oxidants, and only 1 tocopherol molecule is needed to protect ~1000 lipid molecules [22-23].

We plan to evaluate plasma and adipose tissue alpha-tocopherol in Specific Aim 1. Barbosa *et al.* showed that a combination of antioxidants [vitamin C, vitamin E, and zinc (average of 900 mg/d, 400 mg/d, and 7 mg/d, respectively)] produced a significant increase in circulating vitamin E and decrease in malondialdehyde, which is a marker of oxidative stress, in burn children [24]. Our previous mechanistic studies using deuterium labeled alpha-tocopherol in an ovine model of burn and smoke injury have shown that the injury to the lung and skin is reflected in a higher depletion of liver alpha-tocopherol, suggesting mobilization of vitamin E from the liver [25]. However, these studies also suggested that in addition to depletion of alpha-tocopherol from the lung and liver, other tissues were also depleted [25]. The present investigation suggests that in face of severe thermal injury in humans that the adipose tissue can release alpha-tocopherol to protect other tissues. Given, the well-appreciated hyper-metabolic and profoundly catabolic state and disordered lipid metabolism, there is concern that depletion of vitamin E may be a very significant problem in patients with burn injury.

The depletion of alpha-tocopherol observed in the pediatric patients with burn injury perhaps results from the oxidative stress the patients are experiencing. It is not clear from the available data if the alpha-tocopherol is being redistributed to other sites, or if it is being depleted (oxidized) as a result of lipid peroxidation. Thus, we plan to measure plasma and adipose tissue alpha- and gamma-tocopherol concentrations, in addition to biomarkers of oxidative stress, such as malondialdehyde and F_2-isoprostanes. Plasma asymmetric dimethylarginine (ADMA), which is an endogenous inhibitor of nitric oxide synthase, will also be measured.

Specific Aim 3: To collect preliminary data to establish the relationship between oxidative stress and pulmonary pathophysiology, as well as impaired wound healing, after burn injury

Background

In Specific Aim 3, we will collect preliminary data to examine both lung pathophysiology and impaired wound healing and burn injury:

Lung Pathophysiology

Histologically, fibrosis correlates with increased mortality in patients with acute respiratory distress syndrome (ARDS) [26]. Most lung function studies in patients following burn injury are directed at the immediate post burn period [27-29]. Mlcak, *et.al.* investigated late residual pulmonary impairment in 17 patients with a 67 ± 32% total body surface area (TBSA) burn injury eight years post-injury. The children were not diagnosed with inhalation injury at the time of admission for their burns. However, after eight years, 13 of the 17 children were diagnosed by bronchoscopy with inhalation injury and nine patients had obstructive and restrictive diseases processes. The decrease in lung function may have been caused by altered lung mechanics, impaired gas exchange, and chest wall scarring [28]. A characteristic of restrictive lung disease is the excessive deposition of collagen, contributing to fibrosis [30]. The histological features of acute lung injury (ALI) correlate with duration of injury, and an alteration in lung structure is an important histological feature that explains changes in pulmonary function. However, there is no effective and efficient method of attenuating the pulmonary

dysfunction after burn injury. We believe that by administration of vitamin E immediately upon injury, we can prevent the long-term lung damage. Although our proposed study will not be analyzing patient pulmonary status years after the burn injury, we provide evidence from our ovine model that suggests that we will observe significant changes in pulmonary status at a much earlier time period [18].

ALI from burn and smoke inhalation triggers an exaggerated inflammatory cascade. Lung tissue is injured by proteases, cytokine expression, and high concentrations of ROS and RNS. Oxidative stress contributes to the overall pathophysiology of the injury because of the synthesis and secretion of inflammatory molecules such peroxynitrite [31-32], and ARDS patients are associated with increased oxidative stress [33]. There are many defenses of protection from free radical processes, including preventative antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase that eliminate species involved in the initiation of free radical chain reactions [34-35]. Small molecule antioxidants such as Vitamin E are capable of preventing cellular damage and scavenging and repairing oxidized radicals [36]. Although a number of the patients do not sustain smoke inhalation, they may still have impaired pulmonary function from oxidative stress that may be normalized by vitamin E treatment.

Wound Healing

In Specific Aim 3, we will also evaluate the relationship between oxidative stress and impaired wound healing. Wound healing is comprised of three overlapping events: inflammation, proliferation and remodeling. Inflammatory cells and fibroblasts contribute to the excess disorganized extracellular matrix within this highly vascularized tissue[37-38]. Normal wound healing relies on the inflammatory response to prevent infection and to drive the fibrotic responses that close the wound. However, the inflammatory response from excess oxidative and nitrosative stress in burn injury further impairs the healing process. We will examine wound healing time in burn patients with and without vitamin E supplementation.

Barbosa, *et al*. evaluated wound healing in burn children (n=17) after oral supplementation with vitamin E, C, and zinc [24]. They analyzed the number of days to complete tissue repair and clinically observed the presence of a new stable layer of epithelium that completely covered the burn without bleeding or ulceration. Patients given supplemental vitamin E, C, and zinc had a significantly lower number of days to heal ($p < 0.001$) [24]. Vitamin E has also been shown to be beneficial in the treatment of wounds in a variety of models, including scar formation from acute surgical wounds[39], and rats with skin lesions from diabetes[40].

Preliminary Studies

We have performed several studies that support the rationale for our specific hypotheses and illustrate our ability to carry out the proposed studies.

i) Pediatric burn patients have depleted long-term vitamin E by 50% as early as 3 weeks after injury. To test the hypothesis that thermal injury depletes body stores of vitamin E, we have measured alpha-tocopherol concentrations in adipose tissue samples from pediatric subjects (n=8), who were followed up to one year following burn injury [11]. During the first week following injury, adipose tissue alpha-tocopherol concentrations were within the expected normal range of 199 ± 40 nmol/g adipose tissue, but were substantially lower at weeks two and three (133 ± 13 and 109 ± 8 nmol/g adipose, respectively). Individual rates of decrease, estimated by linear regression, showed that adipose tissue alpha-tocopherol decreased on average at 6.1 ± 0.6 nmol/g daily. Importantly, we observed that the depletion of tocopherol and triglyceride concentrations in the adipose tissue were not parallel. Thus, the adipose tissue tocopherol depletion cannot be explained solely by tissue catabolism.

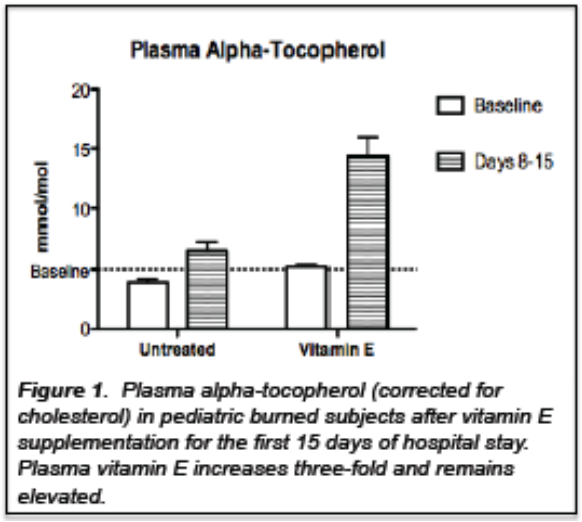


Figure 1. *Plasma alpha-tocopherol (corrected for cholesterol) in pediatric burned subjects after vitamin E supplementation for the first 15 days of hospital stay. Plasma vitamin E increases three-fold and remains elevated.*

ii) Plasma alpha-tocopherol triples after treatment with vitamin E for fifteen days. We measured plasma alpha-tocopherol concentrations in burn subjects (n=10; 'Untreated', age: 14.4 ± 11.7 y; TBSA: 38 ± 8.3%) and burn subjects treated with alpha-tocopherol for fifteen days (n=3, 'Vitamin E', age: 11 ± 1.5; TBSA: 45.5 ± 20.5%). After admittance into the hospital, plasma alpha-tocopherol concentrations were within the expected range of 4.6 ± 0.65 mmol/mol cholesterol, but substantially increased by two- and three-fold after two weeks (10.5 ± 3.87 mmol/mol **Figure 1**).

iii) Adipose alpha-tocopherol increases after treatment with vitamin E for fifteen days. We measured alpha-tocopherol

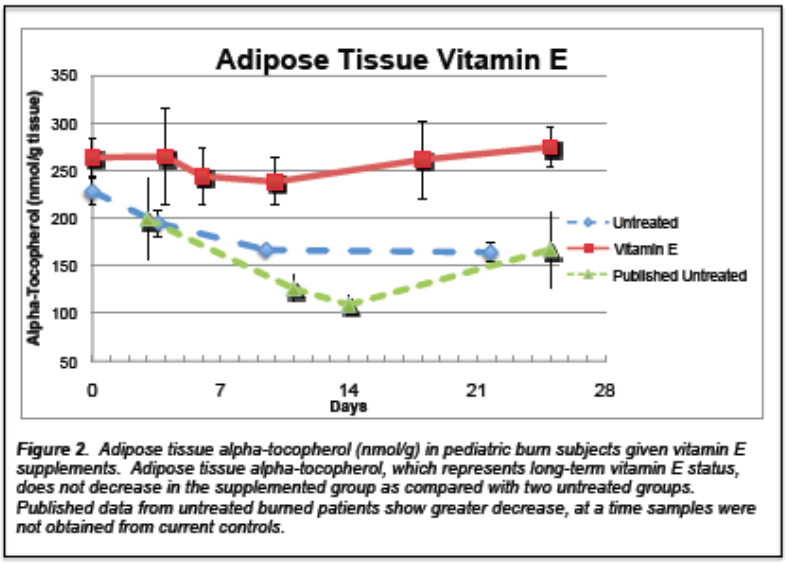


Figure 2. *Adipose tissue alpha-tocopherol (nmol/g) in pediatric burn subjects given vitamin E supplements. Adipose tissue alpha-tocopherol, which represents long-term vitamin E status, does not decrease in the supplemented group as compared with two untreated groups. Published data from untreated burned patients show greater decrease, at a time samples were not obtained from current controls.*

concentrations in adipose tissue, which represents long-term vitamin E status. Measurements were taken from burn subjects treated with vitamin E (n=3, 'Vitamin E', red line, age: 11 ± 1.5; TBSA: 45.5 ± 20.5) and compared to two different groups of burn subjects without vitamin E treatment (n=10, 'Untreated', blue line, age: 14.4 ± 11.7; TBSA: 38 ± 8.3; n=7, 'Published Untreated'[3], green line, age: 7.1 ± 4.7; TBSA: 68 ± 14.1). Vitamin E supplementation prevented the decrease in adipose tissue alpha-tocopherol concentrations relative to the observations in the untreated groups (**Figure 2**). This study is ongoing.

iv) Supplemental vitamin E attenuates lung injury in the ovine model. Our well-established ovine model of acute lung injury is used to study burn and smoke inhalation because of the close similarity between sheep and human lungs. Three weeks after combined burn (20%, third degree) and smoke inhalation (36 breaths of cotton smoke), **(A)** mutagenic ROS such as MDA, **(B)** hydroxyproline, and **(D)** lung water content significantly increased, while **(C)** lung diffusion capacity significantly decreased **(Figure 3)**. When similarly-injured sheep were nebulized with tocopherol (700 mg) during the first 48 hours after burn and smoke inhalation, these variables were returned to normal three weeks after injury **(Figure 3)**.

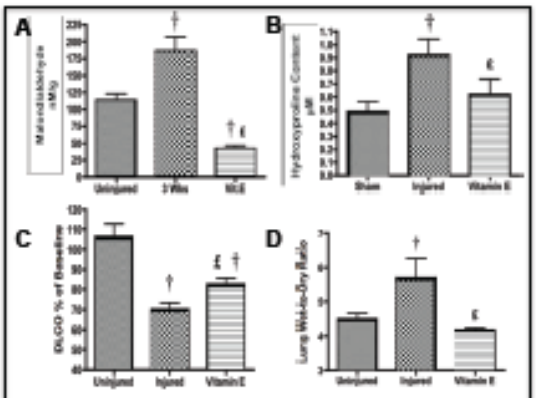


Figure 3. *Effect of burn and smoke inhalation in the ovine model with and without vitamin E. Increases in the mutagenic ROS, lung hydroxyproline, lung DLCO and edema were significantly attenuated with vitamin E.*

v) Supplemental vitamin E increases wound healing in the ovine model. Our well-established ovine model of acute lung injury was used to study wound healing. We present evidence **(Figure 4)** that the scavenging of oxidative stress by vitamin E in the first 48 hours after burn and smoke inhalation in the ovine model significantly increased wound healing. **Figure 4A** and **4B** quantify and illustrate the beneficial effect of vitamin E in wound healing after 3 weeks.

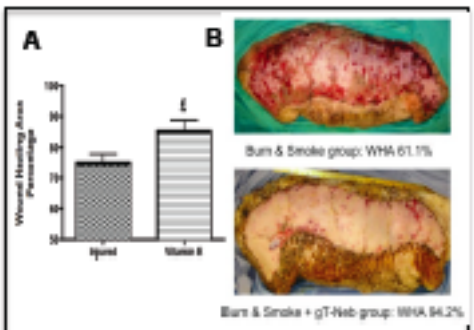


Figure 4. *Increases in Wound Healing after Vitamin E*

vi) Supplemental vitamin E attenuates PaO_2/FiO_2, plasma conjugated dienes, and nitrotyrosine in the ovine model. Vitamin E treatment largely prevented the increase in pulmonary permeability index and moderated the increase in lung lymph flow (52.6 ± 6.2 ml/min, compared with 27.3 ± 6.0 ml/min, respectively), increased the PaO_2/FiO_2 ratio, ameliorated both peak and pause airway pressure increases, and decreased plasma conjugated dienes and 3-nitrotyrosine after 48 hours [41].

Research Design

Subjects

A total of 42 burn subjects from the Shriners Hospital for Children and Blocker Burn Unit (BBU), the BICU-MHH, or the PHHS-BU will participate in this study. Written informed consent will be obtained from each subject, or their guardian or power of attorney, before participation. Inclusion and exclusion criteria are listed in **Tables 1 and 2**, respectively. Subjects will be randomly assigned to the early or delayed vitamin E treated group and will be matched for age, race, TBSA, and sex to avoid confounding from possible differences in vitamin E status. The presence of inhalation injury and sepsis will also be considered and matched. The two groups are necessary for our reverse crossover design.

Table 1. *Subject Inclusion Criteria*
• Age: 18 - 85 years
• ≥40% TBSA burn

Table 2. *Subject Exclusion Criteria*
• Bleeding disorders
• Positive hepatitis or HIV screens
• Pregnancy (women)

Screening Tests and Preliminary Testing

We will use a two-step screening approach **(Figure 5)**. Eligibility of subjects will be determined on the basis of age (18-85 years) and TBSA burn (≥40%). A physical and history examination will screen subjects for hepatitis, HIV, and pregnancy (women). Eligible participants will be enrolled in the study and randomized into the 2 groups within the first week of admission.

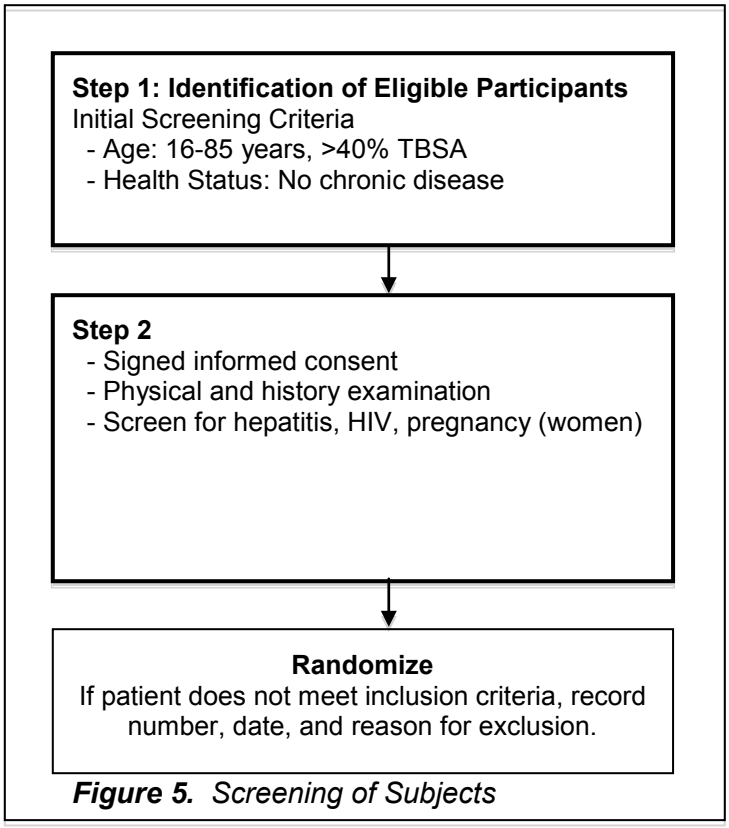


Figure 5. *Screening of Subjects*

Experimental Design

i) Overview of Study Design

We will administer vitamin E (800-1200 IU RRR alpha-tocopherol) to burn subjects (n= 42, 18-85 years) after screening and voluntary consent who have ≥40% TBSA assessed by either the Lund and Browder chart or the 'rule of nines' method during excisional surgery [42] **(Figure 6)**. Vitamin E will be supplemented orally in either a liquid form, or if/when the subject can eat independently, a pill form. It will be administered daily on either days 1-15 or days 16-30 of the study, and plasma, urine, and adipose will be collected on a weekly basis, and/or at every surgery. All subjects will receive similar standard medical care and treatment from the time of emergency admission until their discharge. Plasma, urine, and adipose tissue will be collected from both groups.

ii) Detailed Presentation of Study Design – Experimental Procedures

Pre-Study. Subjects will be admitted into the Shriners Hospital for Children and the BBU, the BICU-MHH, or the PHHS-BU. The subjects will then be screened for our study. Once stable, the subjects or their legal guardians will be approached by research nurses to obtain unbiased consent (in the preferred language for the families and subjects). In accordance with the standards of care at Shriners and the BBU, the BICU-MHH, or the PHHS-BU, within 48 hours the subjects will be resuscitated with fluid and will undergo total burn wound excision and grafting in the operating room.

Day 1. Samples of adipose tissue, plasma, and urine will be collected on the first day of the study. We will also document information such as date of burn, date of birth, total TBSA, third-degree TBSA, and type of burn. The samples will serve as baseline data before vitamin E supplementation. **Blood samples** will be collected in chilled tubes containing EDTA to measure substrate concentrations. The samples will be placed on ice, and plasma will be separated by centrifugation within 30 min of collection. The plasma samples will be stored at -80ºC until analyses are performed. **Adipose tissue** will be obtained from the subject during their weekly surgery for removal of eschar and skin grafting until the wounds are healed. Sample will be collected when adipose tissue is exposed during the surgery after burn wound is excised. Burn wounds are excised down to the healthy adipose tissue to provide viable wound bed for skin grafting. The samples will be snap-frozen in liquid nitrogen and stored at -80°C until analysis. **Urine** will be collected either from the subject's urinary retention catheter (Bard Latex-Free Urinary Drainage Bag, C.R. Bard, Inc., Covington, GA) or directly from the subject in a urinal weekly, and/or at every surgery. The urine sample will be collected at approximately the same time each day that it is collected, and it will be stored at -20°C until analysis.

A **wound healing assessment** will also be taken upon admission and before the administration of vitamin E. We will use a variety of techniques, including photographs, planimetry and documenting information such as the number of days between surgeries and percentage of third degree burn. Additional techniques may be used to assess wounds, including non-invasive techniques using the spectrophotometer, durometer, and micrometer.

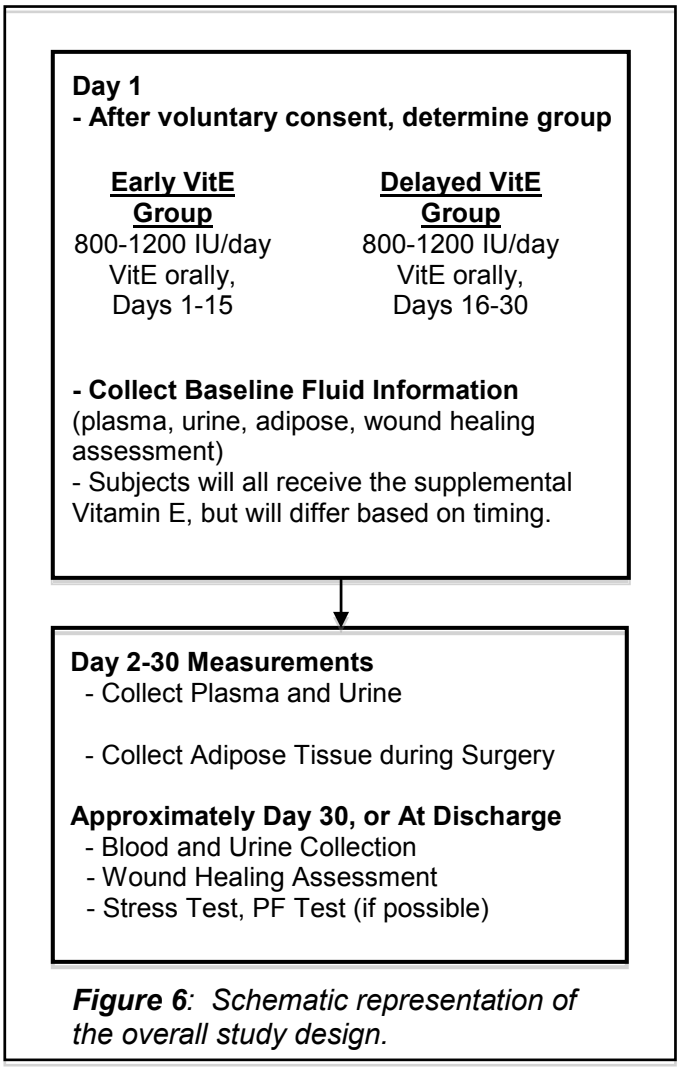


***Figure 6**: Schematic representation of the overall study design.*

The age of the subject will determine their dose of vitamin E [43-44]. It is based on the tolerable upper intake level (UL) of vitamin E, which is the highest level of intake daily that is likely to pose no adverse health risks in almost all individuals [44]. The doses have taken lowest-observed-adverse-effect-level (LOAEL), uncertainty factors (UF), and reference body weights into consideration. Ages 18-85 will receive 1200 IU/day. Vitamin E will be administered at roughly the same time every morning in a liquid or pill form. The dose of aqueous vitamin E will be given orally. When/If the subject is able to eat independently, the dose of vitamin E will be given in a pill form. The supplement of vitamin E will be given on a daily basis either on days 1-15 or days 16-30. It will be given with a meal since vitamin E is fat-soluble, and in the morning for consistency.

Day 2-30 Sample Collection. Samples of adipose, plasma, and urine will be taken from subjects in both the early and delayed vitamin E groups on a weekly basis, and/or at every surgery. Plasma and urine samples will be collected during the subject's normally scheduled surgeries, or during the following four times (1) between, and/or on days 2-7, (2) between days 8-14, (3) between days 15-21, and (4) days 22-30. Adipose tissue will be collected during the subject's reconstructive surgery. The surgery will occur at the surgeon's discretion and will be based on the needs of the subject. Depending on the analysis, the samples will either be shipped and stored at the Shriners Hospital and BBU, or they will be shipped on dry ice overnight to the Linus Pauling Institute at Oregon State University. **Table 3** illustrates the samples and the analyses that will be performed (a-T: alpha-tocopherol, g-T: gamma-tocopherol). **Table 4** specifies the details of how each tissue will be used. In the case of death only, lung, skin, and muscle tissue will be taken at autopsy with parental consent. Dr. Hal Hawkins, who is a pathologist and the Director of the Electron Microscopy Laboratory, will perform the autopsies of all subjects enrolled in this study who succumb to the burn injuries at SHC/BBU.

Other additional variables that will be documented include: weekly body surface area that is still open, weekly body weight, basal metabolic rate, diet history and food intake, and fluid balance. We will also document health of the subjects and problems that may occur such as acute respiratory distress syndrome (ARDS), pneumonia, and atelectasis.

Typically the patients are only on a ventilator for a limited amount of time during the beginning of their hospital stay, and the days on a ventilator vary per patient. If the subjects are on a ventilator, compliance, resistance, work of breathing, and the number of days ventilated will be documented. We will also document data related to pulmonary status such as spirometry, blood gas, and diffusion constant and pulmonary capillary surface area.

Table 3. *Sampling Schedule*

	a-T	g-T	MDA	Isoprostanes	Sample	Sampling
Plasma	x	X	x	X	~3.5 mL	Weekly/Surgery
Urine			x	X	10 mL	Weekly/Surgery
Adipose	x	X			~1 g	Weekly/Surgery
Lung	x	X	x	X	9 g	Autopsy
Skin	x	X	x	X	3 g	Autopsy
Muscle	x	X	x	X	4 g	Autopsy

Approximately Day 30, and/or at Discharge. Three additional groups of variables will be tested on approximately day 30 of the study, and/or at discharge: pulmonary function (PF), the cardiopulmonary stress test, and the wound healing assessment. Either Dr. Ronald Mlcak, who is the Director of Respiratory Care, or a member of his team will take the PF measurements at the Shriners Hospital in Galveston and BBU. The UTHealth has a cooperative agreement with BICU-MHH that will ensure these clinical research assessments take place and the data is properly reported. The test will be performed by certified professional employed by BICU-MHH and the results will be made available to the appropriate clinical researchers according to institutional guidelines. Either Dr. Kenneth Hawkins, the manager of Respiratory Care, or a member of his team with will take the PFT measurements at the PHHS-BU.

The PF study variables include forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), forced expiratory flow rate between 27-75% of the FVC (FEF_{25-75}), FEV_1/FVC ratio expressed as a percentage (FEV_1/FVC%), peak expiratory flow (PEF), vital capacity (VC), total lung capacity (TLC), residual volume (RV), functional residual capacity (FRC), diffusion (DLCO), and maximum voluntary ventilation (MVV). All spirometry measurements will be made with a PF testing system (Medical Graphics PF/DX, St. Paul, MN) with the subject in an upright position [45]. No spirograms will be documented until the subjects have practiced at least two forced maximal inhalations and exhalations, and at least three satisfactory spirograms will be documented for each subject. All expected values will be obtained using validated-general population equations [46].

The procedures will be performed in accordance with the guidelines from the American Thoracic Society [47]. The criteria that will be used to determine if the subjects performed the maximal maneuvers [45] include:

Table 4. *Minimum Sample Sizes*

Plasma	0.1 mL alpha- + gamma-tocopherols and cholesterol, 0.5 mL VitE metabolites, 1 mL MDA, 0.5 mL isoprostanes, 0.5 mL ADMA, 0.5 mL cytokines
Urine	0.5 mL VitE metabolites, 1 mL MDA, 1 mL isoprostanes, 3 mLs N_2 metabolites, 1 mL creatinine
Adipose	1 g alpha- + gamma-tocopherols
Lung	1 g alpha- + gamma-tocopherols, 1 g MDA, 1 g isoprostanes, 1 g hydroxyproline, 1 g western blots, 1 g arginase activity, 1 g NOS activity, 1 g amino acid analysis
Skin	1 g alpha- + gamma-tocopherols, 1 g MDA, 1 g isoprostanes,
Muscle	1 g alpha- + gamma-tocopherols, 1 g MDA, 1 g isoprostanes, 0.5 g western blots, 0.5 g mRNA

1. Appropriate curve shape
2. Lack of artifacts in results such as coughing, premature terminations, delayed onsets
3. Sustained expiration for at >3 seconds,
4. Satisfactory performance as observed by tester.

The cardiopulmonary stress test will be performed with the Horizon MMC 4400 that uses a breath-to breath technique [48]. Either Dr. Oscar Suman, who is the Director of Children's Wellness and Exercise Center at the Shriners Hospital in Galveston, or a member of his team will take the cardiopulmonary stress test variables. The UTHealth has a cooperative agreement with BICU-MHH that will ensure these clinical research assessments take place and the data is properly reported. The test will be performed by certified professional employed by BICU-MHH and the results will be made available to the appropriate clinical researchers according to institutional guidelines. Either Dr. BJ Karnath, who is the manager of Cardiographic Services at PHHS-BU, or a member of his team will take the cardiopulmonary stress test variables. The variables tested include: expired volume (VE), tidal volume (VT), respiratory rate (RR), tidal volume/dead space ratio

(VD/VT), work rate (WR), O_2 consumption (VO_2), respiratory quotient (R), heart rate (HR), oxygen pulse (O_2 pulse), expired volume/O_2 consumption ratio (VE/VO_2), and expired volume/maximum voluntary ventilation ratio (VD/MVV). The subjects will be asked to exercise on a treadmill as long as possible, and the Bruce protocol was used to increase the speed and grade every three minutes [49].

Expected Outcomes

The expected vitamin E-induced changes during days 1-15 or days 16-30 compared with the untreated days of the study are presented in detail under each specific aim and are summarized in **Table 5**.

Table 5. *Expected changes in response to vitamin E supplementation*

	Expected Results
Aim 1	↑ Alpha-tocopherol in Plasma and Adipose tissue (short- and long-term vitamin E status)
Aim 2	↓ MDA and Isoprostanes, ↑ Vitamin E Metabolites
Aim 3	↑ PFT and stress test readings, ↑ Wound Healing

Potential Problems and Alternative Strategies

- It will take approximately 1-1.5 years to gather subject data to account for dropouts.
  - On average, 300-360 patients are admitted annually to SHC/BBU. Approximately 110 patients are seen at the BBU alone each year. Roughly 70% of the patients who fit our inclusion criteria consent to various studies. We believe we will achieve a high percentage of consent because of the positive connotation associated with Vitamin E and nutritional supplements in general. Approximately 20-25 patients per year fit our inclusion criteria and will consent.
  - On average, 235 adult patients are admitted into BICU-MHH annually. Over the last three years, 31 patients have met the eligibility criteria for the study. Based upon previous clinical trials held at BICU-MHH, we anticipate that we will enroll and consent at least half of those patients (13-15 patients).
  - On average, 600 adult patients are admitted to PHHS-BU annually. Over the last three years, between 100-150 patients annually have met the eligibility criteria for the study.
- The proposed studies require that each subject be followed for at least 28 days while they are in the hospital and as an outpatient after their discharge. All patients stay after discharge as an outpatient for wound care and physical and occupational therapy. Some of the subjects may drop out or may be discharged from the hospital before completing the entire study. Based on our previous experience, we estimate that at least 40 of the 42 enrolled burn subjects will complete the entire study, which will allow us to perform the intended comparisons with enough statistical power (i.e., 80%, see *Power Analysis*). In some cases, the subjects may be discharged before 28 days but will stay as an outpatient and will be seen daily.
- The randomization of subjects represents an additional concern because of the wide range of ages and TBSA eligible for the study. The subjects will be matched for age, race, TBSA, and sex to avoid confounding from possible differences in vitamin E status. The presence of inhalation injury and sepsis will also be considered and matched. For example, the first subject with inhalation injury will enter the vitamin E group, while the second subject with inhalation injury will enter the second group. We will also document the health of the subjects and problems that may occur such as acute respiratory distress syndrome (ARDS), pneumonia, and atelectasis. The ability to enroll 42 subjects will aid in the ability to randomize the subjects with proper matching in each group.
- Pulmonary function and cardiopulmonary stress tests have been used in the past at outpatient follow-up visits and 6, 9, 12, 18, and 24 months post-burn [28, 50-52]. They may be difficult to complete in the acute phase of burn injury. The tests are effort-dependent, and burn patients are likely to perform sub-optimally in the first month after injury. Other methods such as the Bicore pulmonary monitor have been used to obtain reproducible respiratory data in the acute phase of hospitalization and may be needed in our studies [53]. The Bicore can continuously monitor airway pressure, airway flow, oesophageal pressure and inspired tidal volume at the bedside.
- Adipose tissue alpha-tocopherol concentrations have been used to assess long-term Vitamin E status [54]. We will collect adipose tissue when the subject enters surgery ideally on a weekly basis, and the amount of adipose tissue is dependent on each surgeon. Instructions will be distributed and discussed with all surgeons to ensure consistency in location and quantity of adipose tissue.
- Human studies have not shown any significant adverse effects associated with oral administration of dietary amounts of vitamin E [14]. The subjects in our study will take a vitamin E supplement orally up to the amount of 1200 IU *RRR*-alpha-tocopherol. Subjects will be consistently monitored 24 hours each day by a physician or nurse, and a Data Safety Monitoring Plan (described in the *Protection of Human Subjects* section of this grant) will ensure their protection and care. Vitamin E is an essential fat-soluble nutrient with a recommended daily intake of 15 mg. Supplements of vitamin E (100, 400, 800, 1000 IU; note that 1 IU is equivalent to 1 mg dl-alpha-tocopheryl acetate) are available for purchase over-the-counter in grocery stores,

drug stores, etc., and are considered to be safe for self-medication. The current authoritative statement for adverse effects of vitamin E limits daily intakes to 1500 IU (1000 mg *RRR*-alpha-tocopherol) [14]. The adverse effect of vitamin E supplements is an increased tendency to bleed [14]. Authors of a meta-analysis evaluating outcomes from 19 clinical trials have suggested that supplemental vitamin E may increase the risk of death from any cause [55]. These conclusions have been widely criticized for a variety of reasons, especially that similar meta-analysis models did report not find statistical significance of an effect of vitamin E supplements on all-cause mortality [56-66]. Moreover, the Women's Health Study of 39,876 women did not find an increase in mortality in women who took 600 IU vitamin E every other day for 10 years, however they did report an increase in nose bleeds [67], and a decreased risk of thromboembolism [68]. Close and consistent monitoring of our subjects by teams of highly trained physicians and nurses will prevent increases in the tendency to bleed. The administration of vitamins such as vitamin K will also prevent the tendency to bleed. In the TONIC trial, vitamin E supplementation to children with NAFLD for 96 week reported no adverse effects from increased daily intakes of vitamin E [7]. In an adult clinical trial by Sanyal *et.al.*, vitamin E attenuated nonalcoholic steatohepatitis in adults without diabetes compared to placebo [9].

- Gamma-tocopherol has the ability to actively scavenge both ROS and RNS, while alpha-tocopherol only scavenges ROS. However, much of ROS formation results from a reaction of superoxide and nitric oxide (NO), and alpha-tocopherol can effectively scavenge superoxide. Alpha-tocopherol reduces the formation of detrimental ROS such as peroxynitrite but sustains NO. NO has several anti-inflammatory properties such as stabilizing the Weibel-Palade body [69]. Thus, alpha-tocopherol accentuates the anti-inflammatory aspects of NO while reducing its inflammatory properties.

Future Directions

The findings of the proposed research will expand our knowledge of the therapeutic benefits of vitamin E supplementation. The results from the proposed studies will lay the foundation for future studies to test the following hypotheses: a) whether vitamin E supplementation increases wound healing in burn patients, b) whether the number of days a patient stays on a ventilator and in the hospital correlates with the supplementation of vitamin E, and c) whether vitamin E attenuates enzymes and substrates of the arginine metabolic pathway such as arginase that lead to the deposition of collagen in the lung in burn patients. This knowledge will ultimately lead to the development of hospital dietary changes and lifestyle interventions to attenuate burn injury.

Repeatedly at consensus conferences that statement is made that some burn patients will demonstrate bronchoscopic evidence of severe smoke inhalation yet will not require ventilatory support, while other burn patients demonstrate only minor bronchoscopic changes yet develop severe ARDS [70]. The relationship between the differences may be caused by variations in oxidation.

The primary difference between gamma- and alpha-tocopherol is their scavenging capability. Gamma-tocopherol scavenges and more effectively removes peroxynitrite-derived nitrating species than alpha-tocopherol by forming a stable adduct, 5-nitro-gramma-tocopherol (NGT) [71-74]. The stable adducts can be formed at the nucleophilic 5-position, which is blocked in alpha-tocopherol [20]. Alpha-tocopherol reacts with nitrogen dioxide to form a reactive intermediate tocopheroxide analogue, while gamma-tocopherol forms NO [75]. The use of gamma-tocopherol may more effectively attenuate patient post-burn outcomes than alpha-tocopherol, and we will examine its use in future studies.

- Future studies will analyze the enzymes and substrates of the arginine metabolic pathway such as arginase activity and collagen deposition in the lung. We will collect plasma samples weekly in our study, but plasma concentrations of variables such as alpha- and gamma-tocopherol and do not necessarily reflect their concentrations in the lung [25, 76].

Vitamin E deficiency in humans is manifested by a progressive, peripheral neuropathy, a symptom frequently observed in burn patients. We theorize that increased vitamin E supplementation may decrease neuropathy and improve this clinical problem. It should also be noted that studies of vitamin E supplementation to patients with a genetic defect in the alpha-tocopherol transfer protein have shown that vitamin E can slow or reverse the neurological symptoms, but only in the early stages of the disorder. Thus, it is important to prevent the occurrence of neurologic abnormalities, if indeed these symptoms in the burn patients are vitamin E-dependent.

Milestones

Forty-two subjects will participate in the study over 3 years. There are several aspects to the project, including the collection of data from subjects, sample and data analysis, and the dissemination of data. We will use the following milestones a guideline:

Completed

- We initially started the clinical trial in burn children (ages 6-17, ≥20% TBSA) at SHC. The protocol

and appropriate paperwork for the trial in children was approved on August 25, 2011 by the IRB at UTMB. This included the Research Consent Form, Parental Consent Form, Child Assent Form for Participating in Research, Data Safety Monitoring Plan, Application for Research Including Children as Research Subjects, and Application for Research Including Persons who are Mentally Incapacitated or Decisionally Impaired as Research Subjects Form.

- The SHC and BBU began enrolling subjects that were between 18-85 years old after IRB approval on November 29, 2011. Five subjects that fit our criteria are currently enrolled in the study. These subjects will be part of our current proposal.
- We have previously registered our clinical trial online at clinicaltrials.gov under the name 'VitE2011' on August 9, 2011.

Milestones

- CY12 Goals: Subject Enrollment, Screening, Weekly Sample Collection
  - Initiate the trial at BICU-MHH and PHHS-BU
    - Obtain IRB Approval
    - Thoroughly educate each site team about the trial
    - Ensure pharmacies at each site dispense same vitamin E dose and brand in liquid and pill forms
    - Confirm consistent sample collection and storage at each site
    - Train site members who will be consenting each eligible patient
    - Ensure each site is consistently performing the PFT and ETT for each subject at Day 30
  - Consistently collect and store samples between the three sites
    - Decode and properly mail samples to UTMB and OSU for analyses
- CY13 Goal: Sample Analyses
  - Continue enrolling subjects, sample collection, patient tests on day 30
  - Begin sample analyses at UTMB and OSU
  - Data interpretation
- CY14 Goal: Manuscripts and Reports
  - Collect final enrolled subject samples needed
  - Finalize data and sample analyses
  - Prepare abstracts, manuscripts, poster and oral presentations
  - Prepare progress and administrative reports

Military Significance

Vitamin E is commercially available and can be purchased and used by any member of the armed services. They can certainly be administered to patients in our burn units. If our hypothesis is correct, we would predict that the wounds would heal more rapidly, which reduces the opportunity for wound infection and sepsis and thus ICU and hospital stay. In the case of ongoing conflict, the individual could return to combat sooner and as a result of scar formation be more effective in combat. In those individuals who sustained concomitant lung injury, we hypothesize that there would be less ICU time on the ventilator, more rapid healing and less scaring and permanent disabling lung injury preventing the individuals from losing combat status. Thus the services regain a valuable combat experienced marine, sailor or soldier. At a time when the services are, seeking budgetary savings, vitamin treatment will result in reduction in costly critical care as well as the costs of recruiting and training a replacement for the wounded warrior. Certainly in the present conflicts, we have noted first degree burns (sun burns) on the exposed areas of their bodies. These burns are the result of tissue oxidation ultraviolet light. Lastly, the supplementation of a multi-vitamin with vitamin E is essential to maintain vitamin K status, which will prevent bleeding. The results of the preset proposal should indicate that consumption of vitamin E should reduce the tissue damage and pain associated with this burn.

Public Purpose

Many of the uses of vitamin E that will be seen in warriors will also be helpful for civilian casualties, including increased pulmonary function, increased wound healing, decreased oxidative stress, and fewer ventilator days.

Methods and Sample Analyses

Tocopherol (Plasma, Adipose, Lung, Skin, Muscle): Both alpha- and gamma-tocopherols will be measured following plasma or tissue saponification, extraction and analysis by high pressure liquid chromatography (HPLC) with electro-chemical detection, as described [77] Plasma and adipose alpha- and gamma-tocopherols will

be measured in all subjects, but only subjects who succumb to the burn injuries will have skin, muscle, and lung alpha- and gamma-tocopherols measured. For tissue samples, cholesterol in the hexane extract will be measured using an Amplex Red kit (Molecular Probes, Invitrogen, Carlsbad CA). Using the same saponified adipose tissue sample, triglycerides will be estimated from the fatty acids extracted from the acidified aqueous phase of the saponified samples using a spectrophotometric method, as described [78].

Malondialdehyde By HPLC-FL Detection (Plasma, Urine, Lung, Skin, Muscle): Malondialdehyde (MDA) is an end product of lipid peroxidation. Through the TBA reaction with heat and acid, MDA forms complex with TBA, called MDA-TBA adduct. In tissue, MDA-TBA adduct is usually measured by butanol extraction and absorbs at 532nm. The concentrations of MDA from tissue or plasma can be quantified based on the linear regression of MDA standards, which are generated from tetramethoxypropane (precursor of MDA). Measuring MDA with HPLC-fluorescence detection gives more sensitive and specific values for tissue and plasma MDA concentrations than spectrophotometric assay, which is a traditional method of lipid peroxidation. Plasma and urine MDA will be measured in all subjects, but only subjects who succumb to the burn injuries will have lung, skin, and muscle MDA measured.

F_2-Isoprostanes and their Metabolites (Plasma, Urine, Lung, Skin, Muscle): We developed techniques to measure plasma F_2-isoPs [79], as well as both urinary F_2-isoPs and their ß-oxidation products [80]. The latter methodology uses a single solid phase extraction (SPE) cartridge, separation by HPLC, and detection by negative mode selected reaction monitoring (SRM) mass spectrometry (MS). Our methods have been developed and validated, using PGF_{2a}; 8-iso-PGF_{2a}; 2,3-dinor-8-iso-$PGF_{1\alpha}$ and 2,3-dinor-8-iso-$PGF_{2\alpha}$ standards to identify specific chromatographic peaks. The paper describing these studies was published in Lipids 2008 [80]. Plasma and urine isoprostanes will be measured in all subjects, but only subjects who succumb to the burn injuries will have lung, skin, and muscle isoprostanes measured.

Hydroxyproline (Lung): The protocol to determine hydroxyproline content in lung tissue supernatant has previously been described in detail [81]. Only subjects who succumb to the burn injuries will have lung hydroxyproline measured. Briefly, lung supernatant will be incubated with trichloracetic acid, centrifuged, and resuspended in HCl. The pellets will be dried and reconstituted with water. The samples will be incubated at room temperature with chloramine-T solution, combined with Ehrlich's solution at 65°C, and optical density will be measured at 550 nm.

Arginase Activity (Lung): The protocol to determine arginase activity in lung tissue homogenate has previously been described in detail [81]. It is a colorimetric assay that uses α-isonitrosopropiophenone to assess urea formation. Protein levels for each sample will be determined using the Protein Quantification Kit (Dojindo Molecular Technologies, Rockville, MD) the according to manufacturer's instructions. All results will be expressed as μMol urea/μg protein. Only subjects who succumb to the burn injuries will have lung arginase activity measured.

Asymmetric Dimethylarginine Concentration (Plasma and Lung): ADMA concentration will be evaluated in plasma or lung homogenate supernatant by the liquid chromatography mass spectrometry technique (LC-MS). Plasma ADMA will be measured in all subjects, but only subjects who succumb to the burn injuries will have lung ADMA measured. Briefly, 25 μL of [2, 3, 3, 4, 4, 5, 5-$^2H_7$]-ADMA (25 mM/ml) will be added to 100 μL of homogenized lung or plasma. Selective ion monitoring of ADMA will be conducted using positive electrospray ionization. The samples will be analyzed by LC-MS performed with the Agilent Series 1100 LC-MSD System (Agilent Technologies, Livermore, CA) and the analytical Atlantis T3 column (Waters Corporation, Milford, MA), and ADMA is measured at (IS) m/z 210.1. A linear equation weighted by 1/x will be constructed to establish the relationship between concentrations and peak area ratio using the SPSS 11.0 statistical software. ADMA concentration will be calculated by interpolation of the linear equation.

Calculations and Statistical Analyses

Data Analysis. Analysis will be carried out with SAS version 9.2 (SAS Institute Inc., Carey, NC). All data sets will be checked for normality and constant error variance. Non-normal variables will be transformed for analysis and back-transformed for presentation as means and 95% confidence intervals. Results for the remaining parameters will be presented as means ± SE. Linear rates of disappearance of tocopherol concentrations will be calculated using individual concentrations versus days post-injury using Excel (Microsoft Excel 2008 for Mac version 12.2.5, Redmond, WA). Nonlinear fitting will be carried out using Prism 4 for Macintosh (Version 4.0b, GraphPad, La Jolla, CA). Data will be analyzed by using repeated-measures ANOVA with trial (within-subject) and gender (between-subject) as main factors (and initial observation as a covariate). Tukey-Kramer adjustments will be applied for post-hoc tests. Statistical significance will be set at two-tailed $p<0.05$.

Power Analysis. Based on previous findings, we expect differences in means between alpha- and gamma-tocopherol will reach or exceed 90 nmol/g and 26 nmol/g, respectively [3]. While the group variances will likely be heterskedastic, we expect the pooled standard deviations to be 91 nmol/g and 30 nmol/g, respectively. As a result, to achieve 80% power, we will need 17 subjects to test alpha-tocopherol and 22

subjects per group to test gamma-tocopherol. With 21 subjects per group, the power for testing a difference is 87% and 78%, respectively. Based on current clinical trial at Shriners and the BBU, we expect <2% dropouts. It is important to note that these calculations do not take into account potentially variance-reducing covariates such as initial conditions; thus, these values are somewhat conservative.

References

1. Nguyen TT, Cox CS, Traber DL, et al. Free radical activity and loss of plasma antioxidants, vitamin E, and sulfhydryl groups in patients with burns: the 1993 Moyer Award. J Burn Care Rehabil 1993;14:602-9.
2. Traber MG. Vitamin E regulatory mechanisms. Annu Rev Nutr 2007;27:347-62.
3. Traber MG, Leonard SW, Traber DL, et al. alpha-Tocopherol adipose tissue stores are depleted after burn injury in pediatric patients. Am J Clin Nutr 2010;92:1378-84.
4. Sokol RJ, Guggenheim MA, Heubi JE, et al. Frequency and clinical progression of the vitamin E deficiency neurologic disorder in children with prolonged neonatal cholestasis. Am J Dis Child 1985;139:1211-5.
5. Sokol RJ, Bove KE, Heubi JE, Iannaccone ST. Vitamin E deficiency during chronic childhood cholestasis: presence of sural nerve lesion prior to 2 1/2 years of age. J Pediatr 1983;103:197-204.
6. Kowalske K, Holavanahalli R, Helm P. Neuropathy after burn injury. J Burn Care Rehabil 2001;22:353-7.
7. Lavine JE, Schwimmer JB, Van Natta ML, et al. Effect of vitamin E or metformin for treatment of nonalcoholic fatty liver disease in children and adolescents: the TONIC randomized controlled trial. JAMA 2011;305:1659-68.
8. Foster T, Budoff MJ, Saab S, Ahmadi N, Gordon C, Guerci AD. Atorvastatin and antioxidants for the treatment of nonalcoholic fatty liver disease: the St Francis Heart Study randomized clinical trial. Am J Gastroenterol 2011;106:71-7.
9. Sanyal AJ, Chalasani N, Kowdley KV, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. N Engl J Med 2010;362:1675-85.
10. Nguyen TT, Cox CS, Traber DL, et al. Free radical activity and loss of plasma antioxidants, vitamin E, and sulfhydryl groups in patients with burns: the 1993 Moyer Award. J Burn Care Rehabil 1993;14:602-9.
11. Traber MG, Leonard SW, Traber DL, et al. {alpha}-Tocopherol adipose tissue stores are depleted after burn injury in pediatric patients. Am J Clin Nutr 2010.
12. Jeschke MG, Kulp GA, Kraft R, et al. Intensive Insulin Therapy in Severely Burned Pediatric Patients: A Prospective Randomized Trial. Am J Respir Crit Care Med 2010.
13. Handelman GJ, Epstein WL, Peerson J, Spiegelman D, Machlin LJ, Dratz EA. Human adipose alpha-tocopherol and gamma-tocopherol kinetics during and after 1 y of alpha-tocopherol supplementation. Am J Clin Nutr 1994;59:1025-32.
14. Food and Nutrition Board, Institute of Medicine. Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids. Washington: National Academy Press; 2000.
15. Bruno RS, Leonard SW, Park S-I, Zhao Y, Traber MG. Human vitamin E requirements assessed with the use of apples fortified with deuterium-labeled α-tocopheryl acetate. Am J Clin Nutr 2006;83:299-304.
16. Williams FN, Jeschke MG, Chinkes DL, Suman OE, Branski LK, Herndon DN. Modulation of the hypermetabolic response to trauma: temperature, nutrition, and drugs. J Am Coll Surg 2009;208:489-502.
17. Lavine JE, Schwimmer JB, Molleston JP, et al. Treatment of nonalcoholic fatty liver disease in children: TONIC trial design. Contemp Clin Trials 2010;31:62-70.
18. Sousse LE, Yamamoto Y, Enkhbaatar P, et al. Acute Lung Injury-Induced Collagen Deposition is Associated with Elevated Asymmetric Dimethylarginine and Arginase Activity. Shock 2011;35:282-8.
19. Burton GW, Traber MG. Vitamin E: antioxidant activity, biokinetics, and bioavailability. Annu Rev Nutr 1990;10:357-82.
20. Christen S, Woodall AA, Shigenaga MK, Southwell-Keely PT, Duncan MW, Ames BN. gamma-tocopherol traps mutagenic electrophiles such as NO(X) and complements alpha-tocopherol: physiological implications. Proc Natl Acad Sci U S A 1997;94:3217-22.
21. Colin C, Narbonne JF, Migaud ML, Grolier P, Cassand P, Pellissier MA. Lipid peroxidation and benzo[a]pyrene activation to mutagenic metabolites: in vivo influence of vitamins A, E and C and glutathione in both dietary vitamin A sufficiency and deficiency. Mutat Res 1991;246:159-68.
22. Buettner GR. The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. Arch Biochem Biophys 1993;300:535-43.
23. Munday R, Winterbourn CC. Reduced glutathione in combination with superoxide dismutase as an important biological antioxidant defence mechanism. Biochem Pharmacol 1989;38:4349-52.
24. Barbosa E, Faintuch J, Machado Moreira EA, et al. Supplementation of vitamin E, vitamin C, and zinc attenuates oxidative stress in burned children: a randomized, double-blind, placebo-controlled pilot study. J Burn Care Res 2009;30:859-66.
25. Traber MG, Shimoda K, Murakami K, et al. Burn and smoke inhalation injury in sheep depletes vitamin E: kinetic studies using deuterated tocopherols. Free Radic Biol Med 2007;42:1421-9.
26. Martin C, Papazian L, Payan MJ, Saux P, Gouin F. Pulmonary fibrosis correlates with outcome in adult respiratory distress syndrome. A study in mechanically ventilated patients. Chest 1995;107:196-200.

27. Nishimura N, Hiranuma N. Respiratory changes after major burn injury. Critical Care Medicine 1982;10:25-8.
28. Mlcak R, Desai MH, Robinson E, Nichols R, Herndon DN. Lung function following thermal injury in children--an 8-year follow up. Burns 1998;24:213-6.
29. Petroff PA, Hander EW, Clayton WH, Pruitt BA, Jr. Pulmonary function studies after smoke inhalation. Am J Surg 1976;132:346-51.
30. Selman M, Thannickal VJ, Pardo A, Zisman DA, Martinez FJ, Lynch JP, 3rd. Idiopathic pulmonary fibrosis: pathogenesis and therapeutic approaches. Drugs 2004;64:405-30.
31. Lamb NJ, Quinlan GJ, Westerman ST, Gutteridge JM, Evans TW. Nitration of proteins in bronchoalveolar lavage fluid from patients with acute respiratory distress syndrome receiving inhaled nitric oxide. Am J Respir Crit Care Med 1999;160:1031-4.
32. Sittipunt C, Steinberg KP, Ruzinski JT, et al. Nitric oxide and nitrotyrosine in the lungs of patients with acute respiratory distress syndrome. Am J Respir Crit Care Med 2001;163:503-10.
33. Cross CE, Frei B, Louie S. The adult respiratory distress syndrome (ARDS) and oxidative stress: therapeutic implications. Adv Exp Med Biol 1990;264:435-48.
34. Fridovich I. The biology of oxygen radicals. Science 1978;201:875-80.
35. Ogura Y, Yamazaki I. Steady-state kinetics of the catalase reaction in the presence of cyanide. J Biochem 1983;94:403-8.
36. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci U S A 1993;90:7915-22.
37. Aarabi S, Longaker MT, Gurtner GC. Hypertrophic scar formation following burns and trauma: new approaches to treatment. PLoS Med 2007;4:e234.
38. Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med 1999;341:738-46.
39. Havlik RJ. Vitamin E and wound healing. Plastic Surgery Educational Foundation DATA Committee. Plast Reconstr Surg 1997;100:1901-2.
40. Musalmah M, Fairuz AH, Gapor MT, Ngah WZ. Effect of vitamin E on plasma malondialdehyde, antioxidant enzyme levels and the rates of wound closures during wound healing in normal and diabetic rats. Asia Pac J Clin Nutr 2002;11 Suppl 7:S448-51.
41. Morita N, Shimoda K, Traber MG, et al. Vitamin E attenuates acute lung injury in sheep with burn and smoke inhalation injury. Redox Rep 2006;11:61-70.
42. Stylianos S, Eichelberger MR. Pediatric trauma. Prevention strategies. Pediatr Clin North Am 1993;40:1359-68.
43. Kappus H, Diplock AT. Tolerance and safety of vitamin E: a toxicological position report. Free Radic Biol Med 1992;13:55-74.
44. Compounds IoMPoDAaR. Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids. Washington D.C.: National Academies Press; 2000.
45. Suman OE, Mlcak RP, Herndon DN. Effects of exogenous growth hormone on resting pulmonary function in children with thermal injury. J Burn Care Rehabil 2004;25:287-93.
46. Knudson RJ, Slatin RC, Lebowitz MD, Burrows B. The maximal expiratory flow-volume curve. Normal standards, variability, and effects of age. Am Rev Respir Dis 1976;113:587-600.
47. Standardization of Spirometry, 1994 Update. American Thoracic Society. Am J Respir Crit Care Med 1995;152:1107-36.
48. Mlcak RP, Desai MH, Robinson E, McCauley RL, Richardson J. Increased physiological dead space/tidal volume ratio during exercise in burned children. Burns 1995;21:337-9.
49. Cumming GR, Everatt D, Hastman L. Bruce treadmill test in children: normal values in a clinic population. Am J Cardiol 1978;41:69-75.
50. Desai MH, Mlcak RP, Robinson E, et al. Does inhalation injury limit exercise endurance in children convalescing from thermal injury? Journal of Burn Care & Rehabilitation 1993;14:12-6.
51. Mlcak R, Cortiella J, Desai M, Herndon D. Lung compliance, airway resistance, and work of breathing in children after inhalation injury. J Burn Care Rehab 1997;18:531-4.
52. Mlcak RP, Desai MH, Robinson E, McCauley RL, Robson MC. Temperature changes during exercise stress testing in children with burns. Journal of Burn Care & Rehabilitation 1993;14:427-30.
53. Petros AJ, Lamond CT, Bennett D. The Bicore pulmonary monitor. A device to assess the work of breathing while weaning from mechanical ventilation. Anaesthesia 1993;48:985-8.
54. Kayden HJ, Hatam LJ, Traber MG. The measurement of nanograms of tocopherol from needle aspiration biopsies of adipose tissue: normal and abetalipoproteinemic subjects. J Lipid Res 1983;24:652-6.
55. Miller ER, 3rd, Paston-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. Ann Intern Med 2005;142:37-46.
56. Baggott JE. High-dosage vitamin E supplementation and all-cause mortality. Ann Intern Med 2005;143:155-6; author reply 6-8.

57. Blatt DH, Pryor WA. High-dosage vitamin E supplementation and all-cause mortality. Ann Intern Med 2005;143:150-1; author reply 6-8.
58. Carter T. High-dosage vitamin E supplementation and all-cause mortality. Ann Intern Med 2005;143:155; author reply 6-8.
59. DeZee KJ, Shimeall W, Douglas K, Jackson JL. High-dosage vitamin E supplementation and all-cause mortality. Ann Intern Med 2005;143:153-4; author reply 6-8.
60. Hemila H. High-dosage vitamin E supplementation and all-cause mortality. Ann Intern Med 2005;143:151-2; author reply 6-8.
61. Jialal I, Devaraj S. High-dosage vitamin E supplementation and all-cause mortality. Ann Intern Med 2005;143:155; author reply 6-8.
62. Krishnan K, Campbell S, Stone WL. High-dosage vitamin E supplementation and all-cause mortality. Ann Intern Med 2005;143:151; author reply 6-8.
63. Lim WS, Liscic R, Xiong C, Morris JC. High-dosage vitamin E supplementation and all-cause mortality. Ann Intern Med 2005;143:152; author reply 6-8.
64. Marras C, Lang AE, Oakes D, et al. High-dosage vitamin E supplementation and all-cause mortality. Ann Intern Med 2005;143:152-3; author reply 6-8.
65. Meydani SN, Lau J, Dallal GE, Meydani M. High-dosage vitamin E supplementation and all-cause mortality. Ann Intern Med 2005;143:153; author reply 6-8.
66. Possolo AM. High-dosage vitamin E supplementation and all-cause mortality. Ann Intern Med 2005;143:154; author reply 6-8.
67. Lee IM, Cook NR, Gaziano JM, et al. Vitamin E in the primary prevention of cardiovascular disease and cancer: the Women's Health Study: a randomized controlled trial. JAMA 2005;294:56-65.
68. Glynn RJ, Ridker PM, Goldhaber SZ, Zee RY, Buring JE. Effects of random allocation to vitamin E supplementation on the occurrence of venous thromboembolism: report from the Women's Health Study. Circulation 2007;116:1497-503.
69. Lowenstein CJ, Morrell CN, Yamakuchi M. Regulation of Weibel-Palade body exocytosis. Trends Cardiovasc Med 2005;15:302-8.
70. Palmieri TL. Inhalation injury consensus conference: conclusions. J Burn Care Res 2009;30:209-10.
71. Leonard SW, Bruno RS, Paterson E, et al. 5-nitro-gamma-tocopherol increases in human plasma exposed to cigarette smoke in vitro and in vivo. Free Radic Biol Med 2003;35:1560-7.
72. Jiang Q, Elson-Schwab I, Courtemanche C, Ames BN. gamma-tocopherol and its major metabolite, in contrast to alpha-tocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells. Proc Natl Acad Sci U S A 2000;97:11494-9.
73. Jiang Q, Christen S, Shigenaga MK, Ames BN. gamma-tocopherol, the major form of vitamin E in the US diet, deserves more attention. Am J Clin Nutr 2001;74:714-22.
74. Christen S, Jiang Q, Shigenaga MK, Ames BN. Analysis of plasma tocopherols alpha, gamma, and 5-nitro-gamma in rats with inflammation by HPLC coulometric detection. Journal of Lipid Research 2002;43:1978-85.
75. Cooney RV, Franke AA, Harwood PJ, Hatch-Pigott V, Custer LJ, Mordan LJ. Gamma-tocopherol detoxification of nitrogen dioxide: superiority to alpha-tocopherol. Proc Nat Acad Sci (USA) 1993;90:1771-175.
76. Shimoda K, Nakazawa H, Traber MG, Traber DL, Nozaki M. Plasma and tissue vitamin E depletion in sheep with burn and smoke inhalation injury. Burns 2008;34:1137 -41.
77. Podda M, Weber C, Traber MG, Packer L. Simultaneous determination of tissue tocopherols, tocotrienols, ubiquinols and ubiquinones. J Lipid Res 1996;37:893-901.
78. Kayden HJ, Hatam LJ, Traber MG. The measurement of nanograms of tocopherol from needle aspiration biopsies of adipose tissue: normal and abetalipoproteinemic subjects. J Lipid Res 1983; 24:652-6.
79. Taylor AW, Bruno RS, Frei B, Traber MG. Benefits of prolonged gradient separation for high-performance liquid chromatography-tandem mass spectrometry quantitation of plasma total 15-Series F2-isoprostanes. Anal Biochem 2006;350:41-51.
80. Taylor AW, Bruno RS, Traber MG. Women and smokers have elevated urinary F(2)-isoprostane metabolites: a novel extraction and LC-MS methodology. Lipids 2008;43:925-36.
81. Wells SM, Buford MC, Migliaccio CT, Holian A. Elevated Asymmetric Dimethylarginine Alters Lung Function and Induces Collagen Deposition in Mice. Am J Respir Cell Mol Biol 2008.

Protection of Human Subjects

Risks to the Subjects

a. Human Subjects Involvement, Characteristics, Design

Based on power calculations, we will need 40 subjects total (20 with vitamin E supplementation on days 1-15 and 20 with vitamin E supplementation on days 16-30) in order to perform comparisons between the two groups with a power of 80% and an overall significance level of 5%. Inclusion and exclusion criteria are listed on **Tables 1 and 2.**

Table 1. *Subject Inclusion Criteria*
• Age: 16 - 85 years
• ≥40% TBSA burn

Table 2. *Subject Exclusion Criteria*
• Bleeding disorders
• Pregnancy
• Positive hepatitis or HIV screens

We plan to study the effect of Vitamin E supplementation on burn subjects (n=42 total, 18-85 years) with ≥40% TSBA for 30 days. Twenty-one subjects will receive vitamin E supplementation (800-1200 IU RRR alpha-tocopherol daily) orally in a liquid or pill form on days 1-15 of the study, while 21 subjects matched for gender, age, race, and TBSA will receive supplemental vitamin E on days 16-30. TBSA will be determined using either the Lund and Browder chart or the 'rule of nines' method during excisional surgery [42]. Plasma, urine, and adipose will be collected on a weekly basis and/or at every surgery from both groups, and all subjects will receive similar standard medical care and treatment from the time of emergency admission until their discharge.

Table 1 describes the inclusion criteria. We are evaluating subjects with a ≥40% TBSA because (1) vitamin E status will be altered with higher percentages of burns, and (2) to obtain an adequate enrollment of subject samples for statistical analysis.

Table 2 describes the exclusion criteria for our study. We plan to exclude subjects with bleeding disorders because of the increased tendency to bleed from excess vitamin E. Also, we plan to draw approximately 35 mL blood from the subjects during the 4-week study and will enroll only subjects with normal levels of hemoglobin. Individuals with positive immune status for HIV and hepatitis virus will be excluded, as these conditions can confound the study results. For the same reason, we will exclude pregnant women. The study physicians and research nurses will be responsible for informing volunteers with abnormal screening results and encourage them to seek further medical care. All the results of the initial screening will be part of the hospital records. Subjects will not be excluded based on gender or ethnicity, and no particular subpopulation will be excluded.

Assignment to a Study Group, Dose

Subjects will be randomly assigned to the early or delayed vitamin E treated group and will be matched for age, race, TBSA, and sex to avoid confounding from possible differences in vitamin E status. The presence of inhalation injury and sepsis will be considered and matched.

The age of the subject will determine their dose of vitamin E [43, 44]. It is based on the tolerable upper intake level (UL) of vitamin E, which is the highest level of intake daily that is likely to pose no adverse health risks in almost all individuals [44]. The doses have taken lowest-observed-adverse-effect-level (LOAEL), uncertainty factors (UF), and reference body weights into consideration. Ages 18-85 will receive 1200 IU/day. Vitamin E will be administered at the same time every morning in a liquid or pill form. The dose of aqueous vitamin E will be given orally. When/If the subject is able to eat independently, the dose of vitamin E will be given in a pill form. The supplement of vitamin E will be given on a daily basis each morning on either days 1-15 or 16-30, depending on the subject group.

Collaborating Sites

All samples (plasma, tissue, urine, and autopsy tissues) will only be identified by code so that patient confidentiality is maintained. Dr. Maret Traber (Linus Pauling Institute, Oregon State University) will analyze the collected samples for Specific Aims 1 and 2. Her laboratory will measure vitamin E and biomarkers of vitamin E metabolism and oxidative stress.

The samples will be sent by overnight mail on dry ice to Dr. M Traber's lab. All research records will be handled confidentially. No individual identities will be used in any reports or publications resulting from this study.

b) Sources of Materials

Research material obtained from the subjects will consist of (1) plasma, (2) adipose tissue, (3) urine, (4) data resulting from their analysis, (5) results from the wound healing assessment and the pulmonary function and cardiopulmonary stress tests, and (5) related clinical data. Samples of plasma, adipose tissue, and urine will be collected on the first day of the study prior to vitamin E supplementation and on a weekly basis and/or every surgery. All research material will be used explicitly for the purpose of research. **Blood samples** will be collected in chilled tubes containing EDTA to measure substrate concentrations. The samples will be placed on ice, and plasma will be separated by centrifugation within 30 min of collection. The plasma samples will be stored at -80ºC until analyses are performed. **Adipose tissue** will be obtained from the subject during their weekly surgery to remove the eschar until the wounds are healed. The surgeons will collect it from live tissue exposed during the surgery that was neither involved in the wound nor thermally injured, and the sample site will be recorded. The burn wounds are typically excised down to adipose, where the samples are collected. One part of the sample will be snap-frozen in liquid nitrogen and stored at -80°C until analysis and a second part will be fixed in buffered 10% formalin solution for histology and immunohistochemistry measurements. **Urine** will be collected either from the subject's urinary retention catheter or directly from the subject in a urinal weekly and/or at every surgery. The urine sample will be collected at approximately the same time each day that it is collected, and it will be stored at -20°C until analysis. In the case of death only, lung, skin, and muscle tissue will be taken at autopsy with parental/guardian consent.

Three additional groups of variables will be tested on the final day of the study: wound healing assessment, pulmonary function (PF), and cardiopulmonary stress test. Either Dr. Ronald Mlcak, who is the Director of Respiratory Care, or a member of his team will take the PF measurements at the Shriners Hospital in Galveston and BBU. The UTHealth has a cooperative agreement with BICU-MHH that will ensure these clinical research assessments take place and the data is properly reported. The test will be performed by certified professional employed by BICU-MHH and the results will be made available to the appropriate clinical researchers according to institutional guidelines. Either Dr. Kenneth Hawkins, the manager of Respiratory Care, or a member of his team with will take the PFT measurements at the PHHS-BU. The PF study variables include forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), forced expiratory flow rate between 27-75% of the FVC (FEF_{25-75}), FEV_1/FVC ratio expressed as a percentage (FEV_1/FVC%), peak expiratory flow (PEF), vital capacity (VC), total lung capacity (TLC), residual volume (RV), functional residual capacity (FRC), diffusion (DLCO), and maximum voluntary ventilation (MVV). All spirometry measurements will be made with a PF testing system (Medical Graphics PF/DX, St. Paul, MN) with the subject in an upright position [45].

The **cardiopulmonary stress test** will be performed with the Horizon MMC 4400 that uses a breath-to breath technique [48]. Either Dr. Oscar Suman, who is the Director of Children's Wellness and Exercise Center at the Shriners Hospital in Galveston, or a member of his team will take the cardiopulmonary stress test variables. The UTHealth has a cooperative agreement with BICU-MHH that will ensure these clinical research assessments take place and the data is properly reported. The test will be performed by certified professional employed by BICU-MHH and the results will be made available to the appropriate clinical researchers according to institutional guidelines. Either Dr. BJ Karnath, who is the manager of Cardiographic Services at PHHS-BU, or a member of his team will take the cardiopulmonary stress test variables. The variables tested include: expired volume (VE), tidal volume (VT), respiratory rate (RR), tidal volume/dead space ratio (VD/VT), work rate (WR), O_2 consumption (VO_2), respiratory quotient (R), heart rate (HR), oxygen pulse (O_2 pulse), expired volume/O_2 consumption ratio (VE/VO_2), and expired volume/maximum voluntary ventilation ratio (VD/MVV). The subjects will be asked to exercise on a treadmill as long as possible, and the Bruce protocol was used to increase the speed and grade every three minutes [49].

A **wound healing assessment** will also be taken upon admission and before the administration of vitamin E. We will use a variety of techniques, including photographs and planimetry and documenting information such as the number of days between surgeries and percentage of third degree burn. Additional techniques may be used to assess wounds, including non-invasive techniques using the spectrophotometer, durometer, and micrometer.

Other additional variables that will be documented include: weekly body surface area that is still open, weekly body weight, basal metabolic rate, diet history and food intake, and fluid balance. We will also document health of the subjects and problems that may occur such as acute respiratory distress syndrome (ARDS), pneumonia, and atelectasis. Typically the patients are only on a ventilator for a limited amount of time during the beginning of their hospital stay, and the days on a ventilator vary per patient. If the subjects are on a ventilator, compliance, resistance, work of breathing, and the number of days ventilated will be

documented. We will also document data related to pulmonary status such as spirometry, blood gas, and diffusion constant and pulmonary capillary surface area.

Due to the design of the study, the Date Computer Core (DCC) has ensured the confidentiality of subject information. The co-investigators and their staff will not have access to any specific subject identifiers such as hospital number, name, initials, address, etc. When the samples are collected, they will be labeled with a unique coded study number identifying the subject. Only the PI and study coordinator will have access to the subject identifications and codes. The samples will then be entered into the database and the investigators can download relevant clinical information related to the samples in their possession. All data will be kept on computers or paper forms and will be protected with locked passwords and/or locked cabinet file rooms. Name, social security number, address, telephone number or any other direct personal identifier will not identify the subjects outside of SHC/BBU, the BICU-MHH, and the PHHS-BU except when required by law. The subjects will be assigned a unique code number for records disclosed outside of SHC/BBU, BICU-MHH, and PHHS-BU, and the key to the code will be kept in a locked file in the office of Drs. David Herndon, James Cross, and Steven Wolf, who are the Chief of Staffs at SHC/BBU, BICU-MHH, and PHHS-BU, respectively.

c) Potential Risks

Potential risks from participation in the study include those associated with vitamin E supplementation, blood drawing, fat biopsy procedures during the weekly surgery, and confidentiality:

Vitamin E Supplementation. Possible side effects include blood coagulation abnormalities and bleeding. However, human studies have not shown any significant adverse effects associated with oral administration of dietary amounts of vitamin E [14]. The volunteers will take a single vitamin E supplement orally up to the amount of 1200 IU *RRR*-a-tocopherol. Vitamin E is an essential fat-soluble nutrient with a recommended daily intake of 15 mg. Supplements of vitamin E (100, 400, 800, 1000 IU; note that 1 IU is equivalent to 1 mg dl-alpha-tocopheryl acetate) are available for purchase over-the-counter in grocery stores, drug stores, etc., and are considered to be safe for self-medication. The current authoritative statement for adverse effects of vitamin E limits daily intakes to 1500 IU (1000 mg *RRR*-a-tocopherol) [14]. The adverse effect of vitamin E supplements is an increased tendency to bleed [14]. Authors of a meta-analysis evaluating outcomes from 19 clinical trials have suggested that supplemental vitamin E may increase the risk of death from any cause [55]. These conclusions have been widely criticized for a variety of reasons, especially that similar meta-analysis models did report not find statistical significance of an effect of vitamin E supplements on all-cause mortality [56-66]. Moreover, the Women's Health Study of 39,876 women did not find an increase in mortality in women who took 600 IU vitamin E every other day for 10 years, however they did report an increase in nose bleeds [67], and a decreased risk of thromboembolism [68].

Blood Draws. Blood samples will be collected solely for the purpose of experimentation. The risks of phlebotomy are pain and infection if sterile procedure is not used. We will draw approximately 7 mLs of blood weekly and/or at surgery, and 7 mls during normally scheduled surgeries for up to one year after admission.

Tissue Biopsies. Tissue biopsies of adipose tissue will be obtained from the subject during their weekly surgery for removal of eschar and skin grafting until the wounds are healed. Surgeons will collect samples when adipose tissue is exposed during the surgery after burn wound is excised.

Confidentiality. All samples (plasma, tissue, urine, and autopsy tissues) will only be identified by code so that subject confidentiality is maintained. Dr. Maret Traber (Linus Pauling Institute, Oregon State University) will analyze the collected samples for Specific Aims 1 and 2. Her laboratory will measure vitamin E and biomarkers of vitamin E metabolism and oxidative stress. The samples will be sent by overnight mail on dry ice with confirmation from Dr. M Traber's lab that an individual will be there to pick up the package the following day. The lung function and wound healing assessment data in Specific Aim 3 will be analyzed by Dr. Linda Sousse at UTMB. All research records will be handled confidentially. No individual identities will be used in any reports or publications resulting from this study.

Research Ethics

Concerns or complaints can be directed to the hospital/university ethics committee, the study physician, or the research nurses. Concerns voiced from subjects or families regarding ethics, rights or safety are dealt with

immediately. A participant of this project will immediately seek out the subject and family to discuss their concerns and provides intervention and/or education as necessary.

Adequacy of Protection Against Risks

a. Recruitment and Informed Consent

Any burn patient between 18 and 85 years with ≥40% TBSA with full-thickness (third degree) burns and admitted within one week of the burn injury will be eligible to participate in the study as long as they fall within the parameters set by the inclusion and exclusion criteria. All demographic groups are eligible and will be invited to participate in our study. All study subjects will receive care in the intensive care units of the the SHC/BBU, the BICU-MHH, and the PHHS-BU. These units are especially designed for the care and monitoring of burn patients. We will ensure equitable subject selection by specific definitions of the study population (age range, surface area of burn), Also, if a subject is eligible for several trials, the information will be given to the subject and/or their legal representative. A decision about the trials will be made after consensus among the subject and/or their legal representative, the PI and the attending burn surgeon. The study design will reflect appropriate scientific, clinical, and ethical norms in the recruitment and negotiation with potential subjects, including norms of nondiscrimination.

Volunteers will be enrolled from the Blocker Burn Unit (BBU) at the University of Texas Medical Branch (UTMB) in Galveston, the Burn Intensive Care Unit at Memorial Hermann Hospital (BICU-MHH) in Houston, and the Parkland Health and Hospital System Burn Unit (PHHS-BU) in Dallas. Collectively, the three burn centers admit a large number of burn patients. The Human Subjects Core will enroll all burn subjects. No subject will be admitted to a study without written informed consent from the subject and the subject's family. The consents allow collection of clinical data, tissues, blood, and urine as well as generation of chemical analysis from these samples. Because of the nature of this research project, an informed consent/assent must be obtained within the first week of admission. The approach and enrollment of eligible subjects will be sought after patients and/or families have been admitted. However, many patients and their families are likely to still be in shock psychologically for several days after a traumatic burn injury, and their enrollment will occur with when/if appropriate.

Recruitment will occur in the patient room or in a separate consultation room. Research nurses will speak with the subjects and their families/legal guardians to see if they have any questions and to ensure that they are completely informed about the details of the study. The details of the study are documented on the research consent, parental consent, and child assent forms that are provided to the subjects and their families/legal guardians. They also explain the research project again several times throughout their hospitalization and convalescence.

The assent of the child will be documented as well as parental consent. Trained, certified translators have translated all of the consent forms into the appropriate language, particularly into Spanish. In addition, research nurses that speak native Spanish explain the study design, and translators will be included in all consent negotiations if needed. The study coordinator or any of the members of the research team, which includes individuals who are fully knowledgeable about the project such as physicians, physician's assistants, clinical investigators, nursing personnel, and PhDs, will be responsible to answer questions that potential subjects may ask about the project and/or procedures performed as part of the project may recruit/enroll/consent subjects. There will be a comprehensive explanation of the study purposes and the associated risks and benefits to the subject and/ or their parents and/or guardians, and all the questions will be answered to their satisfaction. After consent, the subject is enrolled into the study and is entered into a randomization list to determine their study group. A research chart and the subject's demographic data are then entered into a research database. All subject testing is coordinated eliminating possible duplication between projects and unnecessary additional sampling.

Study Hold or Termination Criteria

A subject will be discontinued if PI, Staff Physician, and/or the Data and Safety Monitoring Board (DSMB) believe the subject's health or integrity of the data may be compromised by continued participation in the study. A subject will also be discontinued from the study at the subject's request to no longer participate in the study.

b) Protection against Risks

All faculty and collaborators associated with the proposed research have completed the NIH mandated online training and successfully passed the accompanying exams. The course is offered through the Collaborative Institutional Training Initiative (CITI, http://www.citiprogram.org). All investigators and personnel associated with this study have already completed the online instructional courses and have printed

their certificates of successful completion to Dr. Enkhbaatar for his use in preparing the letter certifying completion of this educational activity. Also, Dr. Enkhbaatar has successfully completed the "Navigating the IRB and Investigator Responsibilities" and the ITS-CRC "Data & Safety Monitoring Planning: Internet Course." Our collaborator at Oregon State University (OSU), Dr. Maret Traber, is the Vice Chair of the IRB at OSU and is well aware of the regulations concerning human subject research.

All inpatient acute burn study subjects are cared for in intensive care units specifically designed for the care and monitoring of burn patients. Drs. Jong Lee and Herndon, Cross, Wolf, the faculty physicians, and the research nurses will directly supervise the care of the enrolled subjects. While subjects are in the ICU setting, they will be monitored 24 hours a day. Blood pressure, heart rate, and arterial O_2 saturation are monitored electronically at all times. Blood tests will be taken regularly and will be reviewed and recorded by the medical staff for clinical and research purposes.

Research nurses and technicians will attend clinical rounds daily, and they will monitor and record clinical and research adverse events (AE) for all subjects. Attending physicians, research nurses, and technicians will promptly report any abnormal lab values and adverse events directly to Drs. Lee and Herndon, Cross, and Wolf. A mechanism has been developed that provides a flow of communication concerning any adverse events identified in research to all appropriate individuals and groups. It includes reporting to the PI, clinical physician, the IRB, the Data and Safety Monitoring Board, and the morbidity/mortality conferences. Dr. Enkhbaatar and the Co-I's are responsible for informing the DCC and Data Safety Monitoring Board (DSMB). In case of a mild AE, the research nurse or technician will inform Drs. Lee and Herndon, Cross, and Wolf, local IRB and DCC, and it will be reviewed by the DSMB every 6 months. In case of a severe or life threatening AE, the event must be reported within 24 hours to the Drs. Lee and Herndon, Cross, and Wolf and the local IRB and DMSB.

Screening tests will allow us to exclude subjects with potentially higher risk of developing complications. Protections against risks for all subjects will be the following:

Vitamin E Supplementation. Although the administered dose of vitamin E dose will be less than the determined upper tolerable dose (800 IU for subjects 14-17 years and 1200 IU for subjects 18-70 years versus the UL 1500 IU RRR-alpha-tocopherol), participation in this study potentially increased the risk of bleeding. Close and consistent 24-hour monitoring of our subjects by teams of highly trained physicians and nurses will prevent increases in the tendency to bleed. The administration of vitamins such as vitamin K will also prevent the tendency to bleed. In the TONIC trial, vitamin E supplementation to children with NAFLD for 96 week reported no adverse effects from increased daily intakes of vitamin E [7]. Any adverse effects will be immediately addressed with faculty staff assessing whether the administration of vitamin E will be stopped. The project will be discontinued if the PI, Independent Research Monitors, and/or the Drug and Data safety and Monitoring Board believe the subjects health or integrity of the data would be compromised by continued participation in the study.

Blood Draws. To minimize pain, we will apply an anesthetic cream (Emla) one hour prior to phlebotomy if needed, and sterile procedure will be used to minimize the risk of infection. The amount of blood to be drawn from each subject will be the least possible (7 mLs per week and/or at surgery for 4 weeks and 7 mLs at every surgery after the initial 30 days). This amount is not adequate to increase the risk of anemia or to significantly affect levels of hemoglobin (Hg). In case of abnormal Hg values, subjects will be dropped from the study.

Confidentiality. Due to the design of the study, the Date Computer Core (DCC) has ensured the confidentiality of subject information. The co-investigators and their staff will not have access to any specific subject identifiers such as hospital number, name, initials, address, etc. When the samples are collected, they will be labeled with a unique coded study number identifying the subject. Only the PI and study coordinator will have access to the subject identifications and codes. The samples will then be entered into the database and the investigators can download relevant clinical information related to the samples in their possession. All data will be kept on computers or paper forms and will be protected with locked passwords and/or locked cabinet file rooms. Name, social security number, address, telephone number or any other direct personal identifier will not identify the subjects outside of SHC/BBU, the BICU-MHH, and the PHHS-BU except when required by law. The subjects will be assigned a unique code number for records disclosed outside of SHC/BBU, BICU-MHH, and PHHS-BU, and the key to the code will be kept in a locked file in the office of Drs. Herndon, Cross, and Wolf, who are the Chief of Staffs at SHC/BBU, BICU-MHH, and PHHS-BU, respectively.

Potential Benefits of the Proposed Research to Human Subjects and Others

A dietary antioxidant, alpha-tocopherol, will be used to potentially attenuate Vitamin E short- and long-term losses in plasma and adipose, reverse the oxidative stress of burn injury and, in the process, decrease the secondary consequences of burn injury, including lung injury and impaired wound healing. This may improve

the quality of life of the burn patient by preventing pathophysiology that may result from oxidative stress and may reduce hospital stay. Our research will lay the foundation for the future development of effective, safe, and economic therapeutic interventions to treat burn injury-associated metabolic abnormalities. Also, it will provide the basis for the development of supplemental regulations and pharmacotherapy to treat burn patients with vitamin E. The risks are very reasonable in relation to the anticipated benefits to our subjects because a) vitamin E is a simple vitamin that is abundant and approved for clinical use, and b) the subjects will be monitored consistently for the minimal increased tendency to bleed.

Importance of Knowledge to be Gained

We anticipate that this research will yield the following outcomes: a) improve our understanding of the metabolism of alpha-tocopherol in thermally injured patients, and b) demonstrate the mechanism by which vitamin E administration reverses the oxidative stress induced by burn injury. We will explore whether attenuation of vitamin E depletion by the administration of supplemental vitamin E will be beneficial in terms of post-burn outcomes, including lung dysfunction and impaired wound healing. The risks are reasonable in relation to the importance of the knowledge that are expected because vitamin E is approved for clinical use and can be purchased easily, and the subjects will be closely monitored for any AE. Ultimately, this will provide the basis for the development of supplemental regulations and pharmacotherapy to treat burn patients.

Data Safety Monitoring Plan

The following Data and Safety Monitoring Plan will describe the oversight and monitoring of our subjects to ensure their safety. Careful monitoring of the recruitment, enrollment, retention, informed consent process, AE and study procedures will help to protect the safety of study subjects, the quality of data, and the integrity of the study. The plan was been approved through the Internal Review Board (IRB) at UTMB on August 26, 2010 and is pending approval at BICU-MHH and PHHS-BU.

The Data and Safety Monitoring Board (DSMB) will monitor the side effects and adverse events associated with the post-burn use of supplemental vitamin E. The DMSB for this grant will consist of three established burn investigators who are tenured full professors and a senior statistician. Once the trial is funded, we will select the DSMB with the approval of the NIGMS liaison. They will monitor a number of different clinical parameters (mild AE) on a regular basis and severe AE whenever they occur. They will meet twice per year to review the data collected, and all findings will be reported to Dr. Enkhbaatar. The clinical trial will also be monitored by the IRBs at each participating site.

All procedures for sample analysis and vitamin E supplementation will be performed by qualified individuals and under the direct supervision of a physician at SHC/BBU, BICU-MHH, and PHHS-BU. A physician will be available and on call twenty-four hours a day at the sites and will provide additional monitoring of study procedures, laboratory values, and clinical performance. A faculty physician will round on each subject on a daily basis. Research nurses and technicians will attend clinical rounds daily, and they will monitor and record clinical and research AE for all subjects.

<u>Plan for Monitoring and Safety Review</u>

A. All study subjects will be cared for in an ICU specifically designed for the care and monitoring of burn subjects. Subjects will have continuous hemodynamic and respiratory monitoring and frequent analysis of electrolytes and blood gases.

B. Physical risks will be minimized by monitoring the subject daily during rounds to observe any complications in bleeding. In addition, a trained intensive care nurse will attend each subject 24 hours each day, and a physician is present on the units at all times. All nursing personnel and physicians are familiar with the research treatment protocols, which are reviewed daily in a specific meeting to discuss research on the units.

C. The Independent Research Monitor (Medical Monitor, Research Monitor), Dr. Michael Kinsky, will independently oversee the study. Dr. Kinsky has the authority to stop the research protocol in progress, remove individual human subjects from a research protocol, and take whatever steps are necessary to protect the safety and well being of human subjects until the IRB assesses the Dr. Kinsky's report.

1. The Independent Research Monitor, Dr. Kinsky, will support the PI, Dr. Enkhbaatar, in meeting the Human Research Protection Office (HRPO) reporting requirements for unanticipated problems involving risk to subjects or others. Dr. Kinsky will be required to review all unanticipated problems involving risk to subjects or others, serious adverse events and all subject deaths associated with the protocol and provide an unbiased written report of the event. At a minimum, the he must comment on the outcomes of the event or problem and in case of a serious adverse event or death, comment on the relationship to participation in the study.
2. The Independent Research Monitor may also perform oversight functions and may discuss the research protocol with the investigators, interview human subjects, and consult with others outside of the study about the research. Examples of oversight functions include: observe recruitment, enrollment procedures, and the consent process for individuals, groups or units; oversee study interventions and interactions; review monitoring plans; and oversee data matching, data collection, and analysis. Research monitors shall have the responsibility to promptly report their observations and findings to the IRB and to HRPO.
3. Dr. Kinsky must also indicate whether he concurs with the details of the report provided by Dr. Enkhbaatar. Reports for events determined by either the investigator or independent research monitor to be possibly or definitely related to participation and reports of events resulting in death must be promptly forwarded to the United States Army Medical Research and Materiel Command (USAMRMC)

Office of Research Protection (ORP) HRPO.

D. Any AE will be immediately addressed with the physicians to assess whether the administration of vitamin E will be stopped. Any changes in the health status of the subjects will be reported immediately to Drs. Lee and Herndon, Cross, and Wolf.

E. The project will be discontinued if the PI, Independent Research Monitor, and/or the DMSB believe the subject's health or integrity of the data would be compromised by continued participation in the study.

Plan for Data Management

A. Subject records include consent forms, case report forms, flow of data forms, laboratory specimen records, inclusion/exclusion forms, cumulative toxicities and/or adverse event logs, and medical charts. All records and data for the study will be collected by the research team and recorded on data flow sheets. To preserve confidentiality, immediately after enrollment, each subject will be assigned a numerical code by which each research sample will be identified for further analyses to avoid identification by non-qualified individuals. Only the PI and study coordinator will have access to the uncoded information. Dr. Enkhbaatar, the PI, is the individual responsible for storage of data. Recognizable personal subject information will be stored in a locked file cabinet in a secure area of the office of Drs. Herndon, Cross, and Wolf. All the information obtained in connection with these studies will remain confidential as far as possible within state and federal law. The results of these studies will be published in scientific journals without identifying the subjects by name.

B. The PI and the research team will review data weekly. The PI will review individual study subject records to ensure that appropriate mechanisms to protect the safety of study participants are being followed, that protocol requirements are being adhered to, and that data is accurate, complete, and secure.

Plan for Adverse Event Reporting

A. Dr. Enkhbaatar, as PI, is the individual responsible for reporting the occurrence of adverse events to entities such as the IRB. Throughout the study, Drs. Lee and Herndon, Cross, and Wolf will be responsible for direct clinical care of the subjects. The subjects will be monitored 24/7 throughout the study and at a follow up visits after the study.

B. Anticipated AE discussed in the Informed Consent Form for this study include bleeding.

C. All AEs are reported to the IRB. The research team will follow the IRB Adverse Event Policy on mandatory reporting of serious and unexpected AE, and will report them immediately both orally and in writing within 24 hours of occurrence or recognition.

D. If an adverse event occurs, Dr. Enkhbaatar will be immediately notified and a note will be entered into the subject's medical chart using the grading scale listed below. Once entered into the data system, the events will automatically be forwarded to the Medical Director and the Research Nurse, allowing for immediate review and rapid follow up when necessary.

Adverse Event Grading Scale (UTMB ITS-CRC Scale)

0 = No Adverse Event: Within normal limits.

1 = Mild Severity: Transient laboratory test alterations; discomforts noted but no disruption of daily activities; no therapy, or only symptomatic therapy required.

2 = Moderate Severity: Laboratory test alterations indicating injury without long-term risk; discomfort sufficient to modify normal daily activity; specific therapy required (i.e., more than symptomatic).

3 = Serious Severity: Laboratory test indicating a serious health threat or permanent injury; incapacity, inability to work, inability to perform normal daily activity; hospitalization required or prolonged; emergency treatment required; life-threatening events; death.

The attribution scale assesses the relation of the event to the study procedures. Drs. Lee and Herndon, Cross, and Wolf and the DMSB will judge whether or not an adverse event is: a) not related; b) possibly

related; c) probably related; d) definitely related to the study procedures and interventions. The DSMB members will review each AE with Dr. Enkhbaatar to determine its attribution and impact.

E. All unanticipated, severe, fatal and/or life-threatening adverse events will be reported to the IRB, PI, and DSMB within 24 hours of occurrence. If a determination is made that participants are found to be exposed to excessive risks in relation to anticipated benefits, the study will be immediately suspended. Studies will not resume until modifications are made that are deemed to result in an acceptable risk/benefit ratio by the PI and IRB. The PI and IRB are responsible for determining whether modifications to the protocol and consent form are required. Aggregate reports of adverse events will be prepared on a bi-annual basis and forwarded to the DSMB bi-annual review.

F. Dr. Enkhbaatar and the DSMB will review study subject records to ensure that appropriate mechanisms to protect the safety of study participants are being followed, that protocol requirements are being adhered to, and that data is accurate, complete, and secure. A bi-annual Data and Safety Report of this assessment will be forwarded to the IRB. The DMSB and the IRB will have reports of all AE from the PI.