CHAPTER I
INTRODUCTION

Parenteral nutrition is commonly administered to critically ill small animal patients to prevent malnutrition and enhance recovery from illness. Although the most physiological route of nutritional support is enteral feeding, parenteral nutrition is indicated when the gastrointestinal tract is dysfunctional, or the patient is unable to take nutrients orally.

Parenteral admixture is commonly delivered either via a central or peripheral catheter. Published reports of parenteral nutrition in small animal patients show that placement and maintenance of a central catheter is more difficult, complication rates and cost are higher, compared to a peripheral catheter (1-3). Therefore, peripheral parenteral nutrition has become a practical alternative to the central vein route for delivery of nutrients in small animal patients.

Formulation of PA for peripheral administration requires a lower osmolarity (<700 mOsm/L) to prevent thrombophlebitis. Reduction of osmolarity is achieved by increasing the proportion of calories from low-osmolarity lipids while decreasing the proportion of high-osmolarity carbohydrate solution. However, lipid emulsions contain high concentrations of polyunsaturated fatty acids (PUFA) which can degrade and form lipid hydroperoxides.

The most commonly used lipid emulsion (20% Intralipid) is protected from excessive oxidation by vitamin E, as a mixture of tocopherols, originating from soybean oil. However, lipid emulsion undergoes peroxidation during storage and before administration despite the inherent amount of tocopherols (4-6). This is suggestive of a relative deficiency of protective antioxidants.

Vitamin E has been recognized as an essential nutrient for people and animals (7). One of its widely accepted biological functions is as an antioxidant. Vitamin E is a fat-soluble chain-breaking antioxidant, incorporated into biological membranes and lipoprotein surfaces, and thus protects them against damage from reactive oxygen species.

Lipid hydroperoxides have been implicated to alter human and animal oxidative status. Products of lipid peroxidation can be detrimental to cell viability, disrupt biological membranes, and damage proteins and DNA (8). Many disease states, including obesity, are associated with imbalance of antioxidant defenses. Furthermore, intravenous administration of oxidized lipids, as a form of nutritional support, can contribute to existing oxidative stress (9, 10). Therefore, protecting parenteral admixtures from oxidation before administration may minimize oxidative cell damage in small animal patients.

The goals of this research project were to investigate the antioxidative function of α-tocopherol in a HL:LD PA and its effect on oxidative status of an obese animal model during intravenous administration. We hypothesized that α-tocopherol is a potent lipid-soluble antioxidant *in vitro* and *in vivo,* and has potential to prevent lipid peroxidation in the HL:LD PA, and in patients receiving parenteral nutrition.

<u>Specific Aims</u>

The objectives of this research were:

Phase I (*in vitro*)

1. To quantify hydroperoxide formation in HL:LD PA.

2. To evaluate the effect of d-α-tocopherol supplementation on hydroperoxide formation in HL:LD PA.

3. To determine the d-α-tocopherol concentration required in an HL:LD PA to minimize hydroperoxide formation.

Phase II (*in vivo*)

1. To quantify the degree of lipid peroxidation in extremely obese cats receiving HL:LD PA.

2. To evaluate the effect of d-α-tocopherol on lipid peroxidation in extremely obese cats receiving HL:LD PA.

CHAPTER II

LITERATURE REVIEW

PHASE I (*IN VITRO*)

Lipid hydroperoxide formation in lipid emulsions utilized for parenteral admixtures

Lipid emulsions represent a major source of calories for small animal patients receiving parenteral nutrition. The most commonly utilized lipid solution for PA is a 20% Intralipid emulsion. The lipid component of the 20% Intralipid emulsion is soybean oil. Soybean oil is a mixture of triglycerides, predominantly (73%) linoleic and linolenic acids. This high concentration of PUFA provides substrate for autoxidation of unsaturated double bonds and formation of degradation products, such as lipid hydroperoxides. Chemical degradation of oils has been known as rancidity and is characterized by changes in the organoleptic properties of oil-containing products, making them undesirable for consumption. This chemical reaction can be induced by exposure to oxygen, light, high temperatures, and prooxidants. During Intralipid emulsion manufacturing, the concentration of oxygen is minimized by saturating the product with nitrogen. The manufacturer recommends storing the glass or polyvinylchloride containers containing lipid emulsion below 25°C to avoid heat induced decomposition. Although containers are transparent, light protection during storage is not required. It has been speculated that manufacturing and storage conditions of Intralipid may not be sufficient to prevent peroxidation of the product prior to administration to the patient.

The degree of chemical degradation of lipid emulsions used in PA has been evaluated by several researchers who demonstrated that soybean lipid emulsions undergo oxidation during storage and during the hang time prior to administration. Neuzil et al performed a study simulating clinical settings by attaching syringes filled with 20% Intralipid to infusion tubes (11). These tubes were exposed to ambient room light and temperature (25°C) in an intensive care unit setting for 24 hours. Measurable concentrations of lipid hydroperoxides were detected in the fresh 20% Intralipid emulsion. Concentration of lipid hydroperoxides increased in the syringe three-fold (P < 0.0005) and eight-fold in the

infusion (P < 0.0005) after 24 hours. These investigators concluded that 20% Intralipid was highly susceptible to oxidation and that elevated levels of oxidized lipids can form during clinical use. Steger et al (12) exposed ethylvinyl acetate bags, filled with 20% Intralipid, to 11 hours/day of daylight at room temperature (20-27°C) for 29 days. The 20% Intralipid in commercial glass bottles, sealed under nitrogen, served as a control. Investigators reported a measurable amount of hydroperoxides in all sealed bottles. Significant and time-dependent peroxide formation was measured in ethylvinyl acetate bags in contrast to lipids stored in the closed glass bottles. A later study by Steger et al (5) demonstrated an increased rate (r > 0.999) of hydroperoxide formation in heat stressed (40 ± 0.5^{o}C) 20% Intralipid emulsion after 41 days of storage in light protected ethylvinyl acetate bags. Those results suggest that the manufacturing process and storage conditions of Intralipid did not prevent peroxidation of lipid emulsions. The risk of lipid peroxidation increased with time, high temperature, and light exposure.

Few investigators have measured peroxidation potential of lipid emulsions within the PA. Pironi et al (13) compared three different lipid emulsions: 20% soybean oil (Intralipid); 20% soybean oil + medium-chain triglycerides (MCT) (50:50 proportion); and 20% olive oil. The PA was compounded from 50% dextrose, 10% amino acid solution, electrolytes, trace metals, and one of the three lipid emulsions. Bags with PA were maintained at room temperature (20°C) and protected from the light. All bottles containing lipids were sampled immediately after opening. The bags with PA were sampled immediately after compounding and again after 24 hours. At baseline, the soybean lipid emulsion contained the highest concentration of lipid hydroperoxides which was 17-fold higher than in soybean oil + MCT emulsion and seven-fold higher than in the olive oil emulsion. Twenty-four hours after compounding, the concentration of lipid hydroperoxides increased for all lipid emulsions and was the highest for the soybean oil emulsion. However, these differences were not statistically significant from baseline values. Dupont et al performed an experiment with different fractions of 20% Intralipid emulsion (14). The emulsion was separated into triglyceride-rich particles and phospholipid-rich particles by ultracentrifugation. Oxidation of both fractions and of the

whole emulsion was initiated at 37°C by addition of 2.2'-azobis (amidinopropane) dihydrochloride and was maintained for 120 minutes. The reaction revealed that the major contributor of hydroperoxides in 20% Intralipid was the triglyceride-rich fraction. These results suggest that 20% Intralipid may be an important source of hydroperoxides due to the amount of PUFA from soybean oil. The chemical reaction causing degradation of PUFA in vegetable oils was unknown until the early 1940's, when described as an autoxidative free-radical chain reaction.

<u>Chemistry of polyunsaturated fatty acid peroxidation</u>

Lipid peroxidation *in vitro* (autoxidation) is a non-enzymatic free-radical mediated autocatalytic breakdown of PUFA that become oxidized into lipid hydroperoxides (LOOH). PUFA contain a high number of unsaturated double bonds, making them sensitive to oxidation. The oxidative process is induced and propagated by atmospheric oxygen (15). Many catalytic systems can oxidize lipids. Among these are light, temperature, enzymes, metals, metalloproteins, and microorganisms. Most of these reactions involve some type of free radical species. The lipid autoxidation reaction is initiated by the attack of a fatty acid with any chemical species that has sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the fatty acid side chain. Examples of these chemical species include: hydroxyl radical (OH$^{\bullet}$), alcoxyl radical (RO$^{\bullet}$), and peroxyl radical (ROO$^{\bullet}$) (16) (Figure 1). This abstraction initiates a self-perpetuating cascade reaction that gives rise to a reactive oxygen species (ROS) such as lipid peroxyl radical (LOO$^{\bullet}$), and LOOH. End products of this chain reaction are a variety of hydroperoxides and cyclic peroxides. The rate of oxidation of PUFA increases with the degree of unsaturation (14). In the presence of reduced metals (e.g. Cu$^+$, Fe^{2+}), the decomposition of lipid peroxides leads to alcoxyl radicals (LO$^{\bullet}$). The ROO$^{\bullet}$ and RO$^{\bullet}$ radicals are able to abstract a hydrogen atom from another fatty acid which perpetuates this chain reaction (17). Decomposition of hydroperoxides generates a complex mixture of secondary lipid peroxidation products, hydrocarbon gases (e.g. ethane and penthane) and aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE). Several

other reagents are capable of initiating and perpetuating autoxidation, including radicals generated by photolysis or high-energy radiation (17). The rate of this chemical deterioration increases in the presence of atmospheric oxygen, light, and high temperatures and is influenced by a variety of factors including container material, vitamin-mineral supplements, and concentration of chain-breaking antioxidants (6, 18, 19). Therefore, the handling and storing of lipid emulsions likely alters the rate of lipid peroxidation. As the oxidation of unsaturated double bonds relies largely on the presence of atmospheric oxygen, it is possible the various methods of PA compounding allows different degrees of oxygenation and influence the rate of PUFA decomposition.

<center>Parenteral admixture compounding issues</center>

PA can be compounded by one of the following three methods: 1) computerized flow, 2) gravity flow, or 3) syringe (20). Computerized flow, used in large practices, is a high-speed, closed-circuit fluid system that pumps all solutions directly into one bag within 60 seconds. Using a computerized compounder is safe, fast, accurate, and efficient. This system minimizes the risk of contamination and likely decreases oxygenation of the solution. Gravity flow is also a closed-circuit fluid system. In this method a bag for PA comes with an attached three-lead transfer set. Each lead is inserted directly into the individual nutrient solution. The solutions are transferred to the bag by gravity flow. For the last method, a syringe is used to transfer nutrient solutions into a sterile fluid bag and compounding is done under a laminar flow hood. The later two methods are economical when only a few patients require parenteral nutrition, such as in many small animal hospitals. The syringe method, when compared with computerized compounding, is more time consuming and requires multiple transfers of solutions into the fluid bag. With this method, prolonged compounding, use of partially opened nutrient bottles, and exposure to oxygen may increase oxidation of nutrients prior to administration to the patient.

Aspects of parenteral nutrition administration in small animal patients

Nutritional support is an important component in the management of critically ill small animal patients. Onset of acute illness results in a catabolic and hypermetabolic state that promotes an acceleration of protein breakdown and loss of lean body mass (21). The provision of adequate calories and protein is crucial to minimize this catabolic state, prevent malnutrition, promote wound healing and shorten recovery time.

Anorexia, dysphagia or other deterrents to voluntary nutrient intake in patients are addressed by assisted feeding either enterally or parenterally. The most physiologic, safest and least expensive route of nutrient delivery is assisted enteral feeding. This can be used in patients that are unable to take nutrients by mouth but have normal intestinal function. Parenteral nutrition remains the primary route of nutritional support in patients with a dysfunctional gastrointestinal tract or clinical conditions in which enteral feeding is contraindicated.

PA is formulated to meet the patient's energy requirements and protein needs by using lipid, dextrose and amino acid solutions. The total required fluid volume is adjusted with crystalloid solution. Electrolytes, vitamins and minerals are added when necessary (22). Historically, parenteral nutrition has been administered via the central venous route because of the development of thrombophlebitis when high osmolarity PA was administered into the peripheral veins (23). However, insertion and use of a central venous catheter can be associated with a high incidence of mechanical complications, increased risk of central venous thrombus, and catheter-associated sepsis (23). Reported rates of mechanical and septic complications in animal patients given parenteral nutrition through a central catheter range from 21% to 46% and from 6% to 42%, respectively (1-3). Advantages associated with use of peripheral venous catheter for parenteral feeding, include lower cost, ease of catheter placement and maintenance of the catheter site, and availability of safe fat emulsions of low osmolarity (24). In humans, complication rates from peripheral nutrition delivery range from 2.3% to 70% (23). Recently published complication rates, resulting from use of peripheral vein catheter in 127 small animal patients, revealed mechanical complications in 19.7%, and septic complications in 3% of

animals (25). Nevertheless, in critically ill small animal patients, peripheral vein delivery of PA has become a practical alternative to central venous administration. Factors that contribute to the development of peripheral catheter-associated complications in small animal patients include catheter type, infusion rate, and osmolarity of PA. Therefore, lower-osmolarity admixtures (< 750 mOsm/l) delivered via the peripheral route may prevent complications (25). To reduce the osmolarity, a HL:LD PA in which more than 75% of energy requirements come from lipids and less than 25% of energy comes from carbohydrate is required. Lipids are a more concentrated energy source than glucose and thus reduce the osmolarity of PA. The proportion of lipids and carbohydrates to achieve the desired osmolarity (< 750 mOsm/l) was calculated for purposes of this project and can be used in small animals above 2 kg of body weight that receive volume of PA equivalent to their daily maintenance requirements. This type of formulation is suitable for peripheral vein administration and provides most of its calories as lipids.

Lipid emulsions are a common component of parenteral nutrition regimens. Intralipid not only provides energy but also delivers essential fatty acids (linoleic and linolenic acids) to the patient. Linoleic acid is a n-6 fatty acid and linolenic acid a n-3 fatty acid. These fatty acids cannot be synthesized de novo in mammals and must be provided in the diet. Linoleic acid is required for growth and reproduction while linolenic acid supports brain and retinal function. Both fatty acids are precursors for eicosanoid and prostaglandin synthesis, and also contribute to cell membrane fluidity. The remaining fatty acids in 20% Intralipid are non-essential saturated (palmitic) and monounsaturated (oleic) fatty acids.

High-fat parenteral formulations, compared with high-dextrose PA, have been shown to better control hyperglycemia in dogs and cats (3, 25). Lipids have been safely used in human patients with hyperlipidemia with serum triglycerides below 400 mg/dl (26). Similarly, dogs and cats at risk of hypertriglyceridemia received a high-fat PA without complications (27). The use of a high-lipid PA in dogs and cats appears to be well tolerated with only a few reports in veterinary literature to contradict this approach.

Despite the benefits of lipid emulsions as part of parenteral regimens, human researchers in 1990's began to raise concerns about possible adverse effects of lipid hydroperoxides on the health of human premature neonates (9, 11, 28). In these neonates, the infusion of lipids has been associated with decreased oxygenation, accumulation of lipids in pulmonary vessels and increased incidence of retinopathies. The suggested oxidative instability of parenteral lipid emulsions led researchers to identify the risk factors leading to oxidation and to identify some protection of PA against this chemical deterioration.

<u>Protection of parenteral admixtures from peroxidation</u>

The concept of hydroperoxide formation and protection of lipid emulsion in PA from peroxidation has been evaluated over the past two decades. Initial studies focused on multivitamin sources as a protection against lipid peroxidation. Results of those studies were contradictory. Lavoie et al (18) conducted an experiment evaluating the role of a multivitamin preparation containing ascorbate, tocopherol, vitamin A, mannitol and butylhydroxytoluene on the peroxidation of PA. PA were maintained in daylight for 6 hours. The hydroperoxide content rose significantly ($P < 0.001$) over time in the PA supplemented with multivitamins, which was suggestive of a pro-oxidative effect of multivitamins in the PA. Different results were published by Laborie et al (29) who detected peroxide formation induced by riboflavin and a possible protective effect of ascorbate on peroxide generation in PA. Conversely, Silvers et al observed a protective effect of multivitamin solution (ascorbate, sodium riboflavin, α-tocopherol acetate) against peroxide formation in Intralipid (30). In this study, 20% Intralipid with or without multivitamin solution was incubated at room temperature ($25^{o}C$) in 2 ml plastic syringes under ambient room light for 24 hours. The total peroxide concentrations increased over a 24-hour period and the multivitamin solution significantly protected 20% Intralipid against peroxidation. Similarly, Neuzil et al reported a protective effect of ascorbate in lipid emulsion but the final ascorbate concentration was too high (1 mMol/L) for use in human patients (11).

Earlier studies utilized vitamin E supplementation of lipid emulsion to address a low vitamin E status in human patients during total parenteral nutrition (31). In later studies, the role of vitamin E as an antioxidant in lipid emulsions was investigated. Steger et al subjected 20% Intralipid emulsions to heat stress conditions (40°C) in light-protected ethylvinyl acetate bags for 41 days (5). Over this time period, tocopherols were significantly oxidized while peroxidation of emulsion linearly increased. α-Tocopherol concentration was reduced by 38.4%, γ-tocopherol by 13.5%, and δ-tocopherol by 11.1%. The order of decomposition of the tocopherols in that experiment was alpha > gamma > delta. These decomposition rates of the tocopherol isomers agree with their presumed activities as chain-braking antioxidants (32). Dupont et al reported degradation of α-tocopherol in lipid emulsion mixed from MCT (50%), soybean long-chain triglycerides (LCT) (40%), and fish oil (10%). This mixed lipid emulsion was manufactured with three different concentrations of α-tocopherol: 50 mg/L, 100 mg/L, and 200 mg/L. Mixed emulsions were subjected to oxidative stress in the presence of 2.2'-azobis (amidinopropane) dihydrochloride for 120 minutes. A markedly decreased peroxidation was observed in emulsions containing α-tocopherol concentrations of 100 and 200 mg/L when compared to those containing 50 mg/L. Concentrations of 100 and 200 mg/L were equally effective. The α-tocopherol consumption was more rapid for emulsions containing the lowest concentration 50 mg/L. Both studies revealed that tocopherols are degraded and loose their antioxidative function during the peroxidation process. As the concentration of tocopherol decreases, the rate of peroxidation increases. While studies using multivitamins as antioxidants yielded conflicting results, α-tocopherol showed concentration depended antioxidative function in lipid emulsions in the last two reports.

Chain-breaking antioxidant – vitamin E

Vitamin E, nature's most potent lipid-soluble antioxidant, has a potential to inhibit chain peroxidation reactions (33). The term vitamin E is used for a family of eight molecules of related structure (Figure 2). The four tocopherols consist of a chromanol ring with a different substitution pattern of methyl groups at positions 5, 7, and 8 of the

head group (α-, β-, δ, and γ), and 16-carbon saturated phytyl side chain. Tocopherols have three chiral centers at carbons 2, 4', and 8', and the naturally occurring isomers have the R-configuration at all three positions. Tocotrienols have the same substitution pattern on the chromanol ring but an unsaturated C_{16} isoprenoid side chain with double bonds in the positions 3', 7', and 11'. All of these molecules possess antioxidant activity although α-tocopherol is considered chemically and biologically the most active (33).

Alpha-tocopherol is an antioxidant whose primary function is to protect PUFA against peroxidation by scavenging the reactive oxygen species derived from double bonds. The antioxidant reaction of α-tocopherol is not a direct reaction with oxygen but an interruption of the autoxidation radical chain process which is perpetuated by $LOO^{\bullet}$. Alpha-tocopherol reacts with $LOO^{\bullet}$, the products of lipid peroxidation, which interrupts the chain reaction (34). This reaction is extremely efficient and fast, and prevents further radical reaction of oxidizing fatty acid. After reacting with a free radical, α-tocopherol is transformed into a fairly stable tocopheroxyl radical (α-$TO^{\bullet}$). This radical will only react with another radical, either α-$TO^{\bullet}$ or $LOO^{\bullet}$, to form a stable, nonradical product (33). This reaction leads to destruction of the α-tocopherol as an antioxidant. Alpha-tocopherol can be restored by reduction of α-$TO^{\bullet}$ with redox-active reagents like vitamin C (ascorbate) or ubiquinol (35).
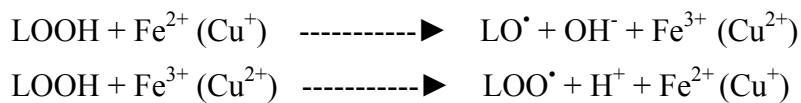
The most prevalent isoform of vitamin E in plant seeds and in products derived from them is γ-tocopherol. Vegetable oils such as soybean, corn, sesame, and nuts are rich sources of this vitamin E isoform. The 20% Intralipid emulsion is derived from soybean oil and thus contains a mixture of natural tocopherols, predominantly γ-tocopherol. The proportion of tocopherol isoforms in Intralipid is γ-tocopherol (65%); α-tocopherol, the most biologically active isoform (9%); and β- and δ-tocopherols (26%). The relative *in vitro* antioxidant activity (%) of tocopherols with regard to peroxyl radical scavenging is alpha (100%) > beta (71%) > gamma (68%) > delta (28%) (33). What makes α-tocopherol the most active from of vitamin E is its chemical structure. The substitution pattern of methyl groups on the chromanol ring makes the hydrogen of the C-6 hydroxy group especially active, facilitating the transfer of the hydrogen to a peroxyl

radical (34). Under the conditions of a homogenous lipid phase, α-tocopherol has a greater rate of hydrogen atom transfer to LOO$^{\bullet}$ than any other lipid-soluble antioxidant (3 x 10^6 M^{-1} s^{-1}) (34). The lower antioxidant potency of γ-tocopherol is attributed to the lack of one electron-donating methyl group on the chromanol ring. Therefore α-tocopherol rather than γ-tocopherol is considered to be a more potent chain-breaking antioxidant for inhibiting lipid peroxidation (36). The lower antioxidative activity of γ-tocopherol compared with α-tocopherol may explain the relative oxidative instability of γ-tocopherol enriched intravenous lipid emulsions.

PHASE II (*IN VIVO*)

Lipid peroxidation *in vivo*

Lipid peroxidation i*n vivo* is one of the processes involved in free radical damage to the cells. The disruption of cell membranes is perpetuated by the products of lipid peroxidation that can damage cellular proteins and DNA (8, 37). These processes can be detrimental to cell function and viability. Biological membranes contain PUFA, substrates for this damaging process. Susceptibility to lipid peroxidation increases with the number of double bonds. While fatty acids with high numbers of unsaturated double bonds such as linoleic acid (18:2), alpha-linolenic acid (18:3), arachidonic acid (20:4), eicosapentaenoic acid (20:5), and docosahexaenoic acid (20:6) are highly susceptible to oxidation, mono-unsaturated fatty acids such as oleic acid (18:1) are relatively resistant (38). Saturated fatty acids will only oxidize under extreme conditions. The primary products of cell membrane lipid peroxidation are LOO$^{\bullet}$ and LOOH. LOO$^{\bullet}$ are capable of abstracting hydrogen atoms from other PUFA within the cell membrane and thus initiating a new chain reaction of oxidation. This reaction yields LOOH that can readily diffuse across the cell membrane and become substrates for cytosolic glutathione peroxidase (GPx) enzyme. However, LOOH are particularly unstable in the presence of transitional metals such as iron or copper, and generate LO$^{\bullet}$ and LOO$^{\bullet}$ that perpetuate cell damage:

$$LOOH + Fe^{2+} (Cu^+) \quad \text{-----------►} \quad LO^\bullet + OH^- + Fe^{3+} (Cu^{2+})$$

$$LOOH + Fe^{3+} (Cu^{2+}) \quad \text{-----------►} \quad LOO^\bullet + H^+ + Fe^{2+} (Cu^+)$$

Researchers suggest that the most likely source of iron for biological lipid peroxidation is the iron storage protein, ferritin (39). Additionally, Gutteridge et al have shown that iron can be released from hemoglobin presumably catalyzed by hydrogen peroxide (H_2O_2) (40).

Another decomposition pathway of lipid hydroperoxides is a radical-induced mechanism. A radical ($R^\bullet$) abstracts a hydrogen atom from the hydroperoxy group, leading to the formation of a lipid peroxyl radical ($LOO^\bullet$):

$$LOOH + R^\bullet \quad \text{---------►} \quad LOO^\bullet + RH$$
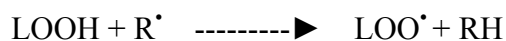
The free-radical chain reaction of lipid peroxidation propagates until two free radicals interact and terminate the chain reaction by forming cyclic peroxide (LOOL)(41):

$$LOO^\bullet + LOO^\bullet \text{----------►} \quad LOOL + O_2$$

$$LOO^\bullet + L^\bullet \quad \text{----------►} \quad LOOL$$
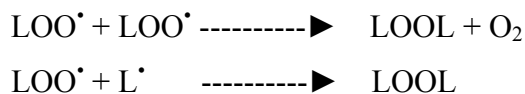
Lipid peroxidation in biological membranes causes a loss of membrane fluidity, a fall in membrane potential, increases permeability to H^+ and other ions, and ultimately results in cellular rupture leading to the release of intracellular contents and organelles (17).

The secondary products of peroxide breakdown include a wide range of aldehydes, many of which are biologically active and act as mutagens and/or carcinogens (42, 43). The products typically found in extracts of oxidized biological samples include: n-alkanals; 2-alkenals; 2,4-alkadienals; 4-hydroxyalkenals; and several carbonyl compounds including MDA (38). The 4-hydroxyalkenals, especially HNE, the product of arachidonic acid oxidation, are significant since they are produced in large amounts and

are very reactive (44). HNE reacts with amino acids and thiol groups of enzymes, thus inhibiting DNA-, RNA- and protein-synthesis and causing damage of membrane-bound enzymes such as cytochrome P-450. The reaction of HNE with reduced glutathione (GSH) provides a route of detoxification for HNE. However, at high concentrations HNE causes depletion of GSH, loss of calcium homeostasis, inhibition of mitochondrial respiration and synthesis of nucleic acids and proteins (38).

The consequences of lipid peroxidation in living organisms are very complex. The tissue damage inactivates antioxidative pathways and results in the release of metal ions from storage sites and from metalloproteins hydrolyzed by enzymes released from disrupted lysosomes. These processes perpetuate the cycle of oxidative stress, cell damage, and increased lipid peroxidation. Lipid emulsions rich in PUFA used for intravenous administration lead to excessive accumulation of PUFA in cell membranes as phospholipids, which can increase their sensitivity to oxidation. Additionally, 20% Intralipid was found to be oxidized during storage and prior administration to the patient (11). Despite nutritional advantages of parenteral feeding, the infusion of oxidized lipid emulsions may represent an unnecessary load of oxidants to the patient and could contribute to complications during recovery from illness.

The effect of lipid hydroperoxides on *in vivo* oxidative status

Several studies in human patients assessed the oxidative status *in vivo* after intravenous lipid administration. The results indicate that intravenous administration of lipids enhanced *in vivo* peroxidation. Pitkänen et al (4) demonstrated increased excretion of expired pentane in eight premature human infants receiving intravenous 10% Intralipid emulsion. This alkane is produced during peroxidation of linoleic acid, the main constituent of Intralipid emulsion. The pentane excretion exceeded baseline values by 28-fold (P < 0.001) and declined 2 hours after cessation of Intralipid infusion, suggesting that *in vivo* peroxidation was related to the lipid emulsion administration. Similar results were reported in adult human patients. Van Gossum et al (45) studied the effect of single, 30 minute long administration of intravenous 10% lipid emulsion on lipid peroxidation in

ten adult human patients (mean age 39.2 years) with gastrointestinal diseases and ten healthy adult control subjects (mean age 34.2 years). All diseased-patients included in the study were receiving long term (mean duration 57 months) home parenteral nutrition (HPN). The lipid peroxidation in both groups was measured as levels of exhaled breath pentane prior to, and immediately after a 30 minute intravenous lipid infusion. Exhaled breath pentane output increased in both HPN patients and healthy controls after lipid administration. The exhaled breath pentane output was significantly ($P < 0.001$) higher in HPN patients compared to controls at baseline and post-infusion. Additionally, Pironi et al (10) assessed lipid peroxidation in 12 human adult patients (mean age 42.1 years) given HPN containing 20% Intralipid emulsion. Serum MDA concentrations in HPN patients were compared to 25 age- and sex-matched, healthy volunteers (mean age 43.4 years) not given PA. HPN patients had numerically higher concentrations of serum MDA than their controls, 2.19 ± 0.82 µMol/L and 1.75 ± 0.41 µMol/L, respectively. Data obtained from those studies showed that administration of lipid emulsion may induce lipid peroxidation *in vivo* as assessed by exhaled breath pentane excretion as well as by serum MDA.

Healthy subjects likely possess sufficient body stores of antioxidants. Conversely, patients requiring PA may have depleted antioxidant stores due to chronic malnutrition. In addition, long term administration of lipid emulsion to patients has been shown to decrease antioxidant status (46, 47). This effect of lipid emulsion administration on oxidative status was evaluated by Linseisen et al (46). In this study, 33 adult human diseased-patients underwent abdominal surgery. Surgery was followed by five days of intravenous administration of PA containing 20% lipid emulsion. There was a trend towards decreased plasma concentrations of ascorbate, carotenoids and selenium during PA administration when compared to baseline values. Thurlow et al (47) evaluated two groups of human adult patients for development of vitamin E deficiency prior to and during parenteral nutrition. Forty-three patients with various underlying diseases were divided between two groups. One group received fat-free PA supplemented with synthetic *all-rac*-α-tocopherol, and the other group received PA supplemented with 10%

Intralipid emulsion. Vitamin E status was evaluated by measuring plasma vitamin E concentration, presence of abnormal platelet aggregation, and increased *in vitro* peroxide-induced hemolysis. Regardless of the group assignment, half of the patients enrolled in the study had plasma vitamin E below the reference interval at baseline. Ninety-five percent of these vitamin E deficient patients had abnormal platelet aggregation, and 52% showed abnormal peroxide-induced hemolysis. The group receiving PA supplemented with *all-rac*-α-tocopherol had significantly (P < 0.001) increased plasma vitamin E levels, corrected abnormal platelet aggregation and peroxide-induced hemolysis, while the group receiving PA supplemented with 10% Intralipid did not demonstrate any changes from baseline. Lespine et al (48) designed a study to compare the effect of lipid containing PA on liver oxidative metabolism and antioxidant defenses. Seven-week-old rats (n = 28) received either intravenous PA containing 20% lipid emulsion or the same equivalent of lipids orally for 6 days. Lipids in both diets contributed 12% of total energy requirements. Lipid delivery via PA, as compared to the oral route, resulted in decreased liver superoxide dismutase (SOD), catalase, and GPx (26%, 55% and 26%, respectively). In contrast, glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD) activities remained unchanged during parenteral feeding as compared to oral feeding. The rats receiving PA had significantly lower liver cytochrome P-450 level. There was no difference in vitamin A and dl-α-tocopherol concentration in the liver between the groups. However, the concentration of GSH was decreased in rats receiving PA (P < 0.004). These authors concluded that PA decreased liver oxidative metabolism and enzymatic antioxidant defenses. Finally, Vandewoude et al (49) measured plasma tocopherol status in 16 adult diseased-human patients receiving PA with 20% Intralipid for 2 weeks. This group was compared to the 16 age-and sex-matched healthy volunteers, not receiving PA. Plasma α-tocopherol concentrations in patients at baseline were significantly lower (P < 0.05) as compared to their healthy controls. However, during the course of PA administration, plasma α-tocopherol levels in patients tended to decrease even further (P = 0.07). These observations demonstrate that disease states are associated

with altered antioxidative status. Additionally, administration of PA containing Intralipid emulsion tends to lower antioxidant stores in diseased-human patients.

There is increasing evidence that peroxides derived from lipids can cause oxidative cell damage in cell cultures (4, 37). In addition, hydroperoxides have been implicated to play a role in various toxic tissue injuries and diseases. Many studies have linked oxidative cell damage with pathogenesis of multiple disease states including obesity (50), ischemia reperfusion injury (8), hemolytic anemia (8), neurodegenerative diseases (51), inflammatory diseases (52), septic shock (53) diabetes mellitus (54), cancer (8), aging (55), and cognitive disorders (51). Any of those diseases may therefore serve as an appropriate model to study oxidative stress.

Obesity as a model of oxidative stress

Obesity is characterized by increased fat storage and excessive intracellular triglycerides. In human and rat studies, obesity is associated with imbalance between tissue oxidants and antioxidants, and an increase in biomarkers of oxidative stress (56). This state of oxidative stress in obese human subjects has been suggested as the underlying mechanism for obesity-associated diseases: diabetes mellitus, cardiovascular disease, insulin resistance, chronic inflammation and hypertension (56).

There are several possible contributors to oxidative stress in obesity, including hyperglycemia, increased muscle activity, elevated tissue lipid levels, inadequate antioxidant defenses, chronic inflammation, and hyperleptinemia (56). Hyperglycemia, as a result of insulin resistance in obesity, activates several oxidative pathways such as the polyol pathway, the advanced glycation end-products pathway and glucose auto-oxidation (56). Increased muscle activity to carry excessive body weight can also activate metabolic pathways that form free radicals. Active skeletal muscles during aerobic metabolism have higher oxygen demands. The increased respiration may cause rapid electron transfer and some electrons can leak from the electron transport chain and reduce oxygen to the superoxide radical ($O_2^{\bullet-}$). Additionally, compared to non-obese subjects, obese humans are found to have lower levels of antioxidants, such as β-

carotene, vitamins E and C, cofactors of antioxidant enzymes - zinc and selenium (57, 58). Oxidative stress may also be due to the metabolic impact of intracellular triglycerides. It has been suggested that accumulation of intracellular triglycerides increases $O_2^{\bullet-}$ production within the electron transport chain by inhibiting the mitochondrial adenosine nucleotide transporter. This leads to a decrease in intramitochondrial adenosine diphosphate (ADP) and electrons accumulate within the electron transport chain, which can then reduce O_2 to form superoxide $O_2^{\bullet-}$ (59). Alternatively, the increased amount of lipid molecules in obese subjects may represent a target for reactive oxygen species. Adipose tissue is not only a depot for energy but is also an active endocrine organ. Adipocytes express pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). Thus expansion of the adipose cell pool may increase IL-6 and TNF-α levels which activate the production of C-reactive protein by the liver (60) and stimulate production of reactive oxygen/nitrogen species (ROS/RNS) by macrophages and monocytes. Specifically, IL-6 and TNF-α can up-regulate the activity of oxidant-generating enzymes, such as NAD(P)H-oxidase, inducible NOS (iNOS), and myeloperoxidase (61, 62) (Figure 3). The final hypothesis suggests that excessive adipose tissue produces high concentrations of leptin, a regulator of food intake and energy expenditure. Leptin is a proinflammatory substance that stimulates the proliferation of monocytes, macrophages and the production of inflammatory cytokines (IL-6, TNF-α) (56) and thus up-regulates oxidant-generating enzymes.

Evidence of obesity-induced oxidative stress has been accumulating over the past decade. Skrha et al (63) measured oxidative stress in obese diabetic and non-obese healthy men and women (n = 20, ages 30-58 years). The obese diabetic patients had significantly higher plasma MDA concentrations as compared with healthy subjects. Plasma MDA concentrations were 65% higher and plasma vitamin E concentrations were 53% lower in the obese compared to the non-obese group. Keaney et al (64) published a study where the Framingham Offspring cohort (n = 2828, ages 33-88 years) underwent a series of laboratory assessments, obesity measures and oxidative stress measures. When

adjusted by gender and age, there were significant positive correlations between body mass index (BMI) and urinary 8-epi-PGF$_{2\alpha}$ isoprostanes, a product of arachidonic acid oxidation. BMI was a strong predictor of creatinine-indexed 8-epi-PGF$_{2\alpha}$. Every 5 kg/m^2 increase in BMI was associated with a 9.9% increase in 8-epi-PGF$_{2\alpha}$. These data suggest that obesity is an independent predictor of oxidative stress. More recently, Furukawa et al (65) examined the relationship between lipid peroxidation, measured as thiobarbituric acid reactive substances (TBARS) and 8-epi-PGF$_{2\alpha}$, and obesity in humans with metabolic syndrome. Both BMI and waist circumference were directly correlated with plasma TBARS and urinary 8-epi-PGF$_{2\alpha}$. Based on these findings, the authors concluded that excessive accumulation of fat leads to enhanced production of ROS in adipocytes and systemic tissues. These results imply that antioxidative capacity of individual may be insufficient to lower obesity related oxidative stress. The potential intervention to minimize oxidative stress can be administration of antioxidants.

<u>Antioxidative defenses *in vivo*</u>

Living organisms have acquired inherent antioxidative defenses against free radical and lipid peroxidation damage. Tissue antioxidant defenses work in several ways: 1) reduce the energy of the free radical, 2) stop the free radical from forming, 3) or interrupt an oxidizing chain reaction to minimize the free radical damage (17). Thus antioxidants have the potential to prevent and inhibit propagation of the lipid peroxidation chain. The hydrophilic antioxidants are found in cytosolic, mitochondrial and nuclear aqueous compartments. They include nonenzymatic defenses, such as ascorbate, GSH, and enzymes SOD, catalase and GPx. Functions of the molecules vary but are often interrelated. SOD and catalase catalyze the following reactions:

$$O_2^{\bullet-} + O_2^{\bullet-} \xrightarrow{\quad SOD \quad} H_2O_2 + O_2$$

$$2H_2O_2 \xrightarrow{\quad catalase \quad} O_2 + 2H_2O$$
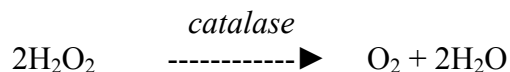
Glutathione peroxidase catalyzes the reduction of H_2O_2 water and organic peroxides (e.g. LOOH) to the corresponding stable alcohols (LOH) using GSH as a source of reducing equivalents:

$$H_2O_2 + 2\ GSH \xrightarrow{\ GPx\ } GSSG + 2H_2O$$

$$LOOH + 2GSH \xrightarrow{\ GPx\ } LOH + GSSG + H_2O$$
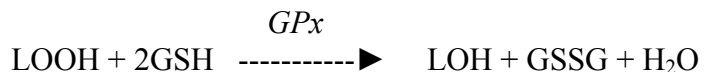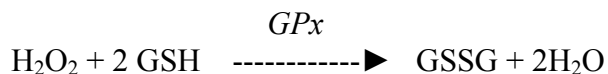
(GSSG; glutathione disulfide, oxidized glutathione)

The lipophilic scavengers are found in the cell membranes and lipoproteins. These include carotenoids, vitamin E and coenzyme Q (17). Beta-carotene is an effective free radical scavenger and protects against both lipid peroxidation and DNA oxidation (56). Alpha-tocopherol is the best known isoform of vitamin E and has the ability to terminate the lipid peroxidation process while γ-tocopherol isoform is less potent in donating electrons (32). Although α-tocopherol is considered to be the more potent antioxidant, the unsubstituted C-5 position of γ-tocopherol appears to increase ability to trap RNS (32). One of the important features of α-tocopherol is the ability to be renewed. The regeneration in membranes is coupled to ascorbate and glutathione. The enzyme glutaredoxin regenerates ascorbate from dehydroascorbate at the expense of GSH. The α-TO⋅ that are produced by the reaction of α-tocopherol with LOO⋅ are efficiently scavenged by ascorbate at the water/membrane interface (66) (Figure 4).

Biologically, α-tocopherol is considered the most active isomer of vitamin E because it is preferentially metabolized in the liver (32). Although the other naturally occurring isoforms, such as β-, δ, and γ-tocopherols and tocotrienols, are absorbed without discrimination they are not well recognized by the α-tocopherol transfer protein (α-TTP) in the liver. It was estimated in humans that 50% of γ-tocopherol is converted to the water-soluble metabolite 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (γ-CEHC) molecule which is excreted in the urine. Jiang et al summarized the current knowledge of the absorption and metabolism of α- and γ-tocopherol (32) (Figure 5).

Vitamin E is taken up as the free alcohol form (i.e., 6-hydroxyl) by the intestine without discrimination of the individual isomers. Both α- and γ-tocopherols are secreted in chylomicron particles together with triacylglycerol and cholesterol. The chylomicron particles are subsequently catabolized by lipoprotein lipase. Some vitamin E is transferred to the peripheral tissues, while chylomicron remnants are taken up by the liver. Here α-tocopherol is preferentially reincorporated into VLDLs by α-TTP. VLDLs facilitate distribution of α-tocopherol throughout the body. γ-Tocopherol appears to be degraded to the hydrophilic metabolite γ-CEHC by a cytochrome P450-dependent process in the liver and is excreted in the urine. The catabolism of α-tocopherol via hydrophilic metabolite in humans occurs only when the daily intake of α-tocopherol exceeds 150 mg. An excess of α-tocopherol and other isoforms that are not metabolized by cytochrome P450-dependent hydroxylases are excreted into the bile (67). The results of human studies show that α-tocopherol supplementation results in lowering plasma and tissue γ-tocopherol (68). Conversely, when γ-tocopherol is supplemented, both tissue γ- and α-tocopherol isomers markedly increase (69).

Conclusions

Based on the negative effects of hydroperoxides on oxidative status and their potential impact on recovery in small animal patients, it seems reasonable to protect PA from lipid oxidation prior to and during administration. This study was designed to investigate the value of vitamin E in decreasing the formation of lipid hydroperoxides in the HL:LD PA infusion bag and evaluate the potential benefits of lowering the hydroperoxide load delivered to the oxidatively-stressed patients.

CHAPTER III

PEROXIDATIVE PROTECTION OF PARENTERAL ADMIXTURE BY
d-α-TOCOPHEROL

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ABSTRACT

A practical alternative to traditional central line delivery of PA for small animal patients is utilization of a peripheral route. Admixtures delivered via this route should be a lower osmolarity to prevent complications; to attain this goal a HL:LD PA is formulated. Lipid peroxidation is a common sequella of PA containing high lipid content. This *in vitro* peroxidation can lead to oxidative injury of biological membranes *in vivo*. Despite this concern, peripheral PA has many benefits and continues to be utilized. Therefore, the objectives of this study were to measure lipid hydroperoxides in HL:LD PA; and to determine the optimal dose of d-α-tocopherol to minimize peroxidation in the PA during a 24 hour hang time.

This *in vitro* experiment included 3 identical blocks consisting of 14 bags (n = 7 control; n = 7 treatment) filled aseptically with 109 ml of HL:LD PA. Total lipid content per bag was 8 g as soybean oil (20% Intralipid). Natural d-α-tocopherol (Vital E-300) was added to the treatment bags as 8, 12, 16, 24, 32, 48, or 64 IU/g of lipid. Control bags contained d-α-tocopherol equivalent amounts of ethanol and benzyl alcohol. Bags were hung for 24 hours at room temperature under fluorescent light exposure. Hydroperoxides were measured by FOX assay and tocopherols by HPLC at times 0 and 24 hours. The

level of hydroperoxides was expressed as μM equivalents of tert-butyl hydroperoxide (μM equ TBH). A repeated measures analysis of variance was used for data analysis, with $P < 0.05$ considered significant. Hydroperoxides were detected in all PA at time 0 and 24. Mean TBH concentrations in control bags were 380 and 383 μM equ TBH at time 0 and 24, respectively. A 3-way interaction (time x treatment x α-tocopherol) was observed, $P = 0.0018$. D-α-tocopherol supplementation at 24-64 IU/g lipid decreased ($P < 0.0001$) TBH production from controls at time 0. By time 24 hours, significant reduction in TBH was observed with vitamin E concentrations of 48 and 64 IU/g lipid of d-α-tocopherol.

Lipid peroxidation of HL:LD PA occurs immediately following lipid administration, and d-α-tocopherol, as Vital E-300, appears to significantly minimize this peroxidation process *in vitro* based on concentration and time of exposure to lipid. This may have clinical implications for parenteral feeding in critically ill patients.

MATERIALS AND METHODS

Experimental design

This experiment was performed in a randomized complete block design with three blocks. Each block consisted of 14 bags (n = 7 treatments, n = 7 controls). Vitamin E (Vital E-300, Schering-Plough Animal Health Corp., Union, NJ) was added to the treated bags at the rate of 8, 12, 16, 24, 32, 48, or 64 IU d-alpha-tocopherol/g of lipid. Amounts of ethanol and benzyl alcohol equivalent to the given volume of Vital E-300 were added to each respective control bag. Administration bags were hung for 24 hours at room temperature (25ºC) and exposed to fluorescent room light.

Parenteral admixture preparation

Parenteral admixtures were compounded aseptically, using a syringe method, in a laminar flow hood into 150 mL polyvinylchloride (PVC) Containers (IntraVia, Baxter Healthcare Corporation, Deerfield, IL). Ingredients were added in the following order: amino acid solution without electrolytes (Aminosyn II 10%, Abbott Laboratories, North Chicago, IL; Lot No. 07023DM01), lipid emulsion (Intralipid 20%, Fresenius Kabi Clayton, L.P., Clayton, NC; glass bottle Lot No. NR67315B, Excel Container Lot No.1022688), Vitamin B-complex (Vedco, Inc., St. Joseph, MO; Lot No. 040564), Lactated Ringer's Solution (Baxter Healthcare Corporation, Deerfield, IL), d-α-tocopherol as Vital E-300, and glucose solution (Dextrose 50% Solution, Vedco, Inc., St. Joseph, MO; Lot No. 405181F). 20% Intralipid emulsions in glass bottles or PVC bags were stored according to the manufacturer's guidelines until use to maximize emulsion stability. Each bag contained a total of 109 mL of HL:LD PA, which provided 80% of energy from lipid emulsion, 20% of energy from glucose and 4 g of amino acids/100 kcals. Total lipid content per bag was 8 g in the form of soybean oil. The composition of the PA is reported in Table 1.

Sampling

Each bag was sampled immediately after compounding and thorough mixing (0 hours) and after 24 hours of hang time into aluminium foil-covered polystyrene tubes. Tubes were immediately saturated with nitrogen to remove oxygen, capped and frozen at -60 ºC until analysis. Analyses were conducted at one time for all samples. *In vitro* lipid peroxidation was assessed by measuring hydroperoxide production via ferrous oxidation / xylenol orange (FOX 2) assay, and the vitamin E concentration was assessed by measuring α- and γ-tocopherol concentrations using high-performance liquid chromatography (HPLC). Samples were analyzed in duplicate.

Assays

Ferrous Oxidation / Xylenol Orange Assay (FOX 2)

Hydroperoxides in the PA and in all newly opened 20% Intralipid bottles or bags were quantified with the FOX 2 assay (70). All reagents were purchased from Sigma Aldrich Chemical Company, St. Louis, MO. The FOX 2 assay method is based on the oxidation of ferrous to ferric ions by hydroperoxides under acidic conditions. Ferric ions are detected by absorbance of ultraviolet light at 560 nm after reaction with the ferric ion indicator xylenol orange, which generates a colored complex.

Briefly, FOX Reagent (100 mL) was prepared by dissolving 6.84 mg of xylenol orange, 6.75 mg $FeCl_2$, 220 μL H_2SO_4, 70 μL butylated hydroxytoluene (BHT) in 100 mL methanol while stirring. Each sample was diluted 1:10 by adding 100μL of PA to 900 μL of methanol. Standards were prepared by adding 0, 100, 200, 400, 600 μL of tert-butyl hydroperoxide (TBH) to 1000, 900, 800, 600, 400 μL of methanol, respectively. For each PA sample and standard, 1800 μL of FOX reagent was added into 200-μL aliquots of sample diluted with methanol or standard. Aliquots of PA were prepared in duplicates. Samples were incubated at room temperature (25ºC) for 30 minutes and absorbance was read at 560 nm on a spectrophotometer (Beckman DU 640 B, Beckman, Palo Alto, CA). Hydroperoxides were expressed in μM equivalents of TBH (μM equ TBH).

High-Performance Liquid Chromatography

Tocopherols were analyzed by HPLC as previously described by Hewavitharana and colleagues (71), with modification of column and solvent conditions. The solvent was a mixture of n-hexane (99.7%) and 2-propanol (0.3%) and an Agilent Technologies Zorbax Rx-SIL 150 mm x 4.6 mm, 5μ column was used. Briefly, all standards, internal standards and residues were dissolved in 0.01% butylated hydroxytoluene in cyclohexane (C_6H_6-BHT 0.01%). As an internal standard, 100 μL of retinol acetate 25 ppm was added to 200 μL of PA. Reagents were combined with a vortex mixer for 10 seconds and extracted with 3 x 500 μL of C_6H_6-BHT 0.01%. The residue was reconstituted in 200 μL of C_6H_6-BHT 0.01% and filtered through a Pall Gellman Acrodisc LC13 0.2μ filter (Pall Life Sciences, Ann Arbor, MI) before analysis.

Analysis of tocopherols was performed by HPLC with normal phase fluorescence detection. Tocopherols were separated using a column with hexanes containing 0.3% isopropanol at a flow rate of 1 mL/min. The fluorescence detector was set with an excitation wavelength of 295 nm and an emission wavelength of 330 nm. The injection volume was 10 μL. Data were expressed as mg/L.

Statistical analysis

Data are presented as the mean ± SEM. For each response variable, mixed effects, repeated measures analysis of variance was used to test for main effects of treatment, d-α-tocopherol concentration, and time, as well as their interactions. Significant interactions were further investigated with tests of simple main effects. All calculations were performed using the SAS System (ver. 9.12, SAS Institute Inc., Cary NC 27513). Statistical significance was defined as $P < 0.05$.

RESULTS

Hydroperoxide formation in the HL:LD parenteral admixture at 0 hours

The control and treated HL:LD PA contained detectable amounts of peroxides. Immediately after addition of the d-α-tocopherol, peroxide concentration markedly decreased. The concentration of peroxides decreased with increasing concentration of d-α-tocopherol. Significant reduction of peroxides was measured at concentrations 24 through 64 IU d-α-tocopherol/g lipid when compared with the control bags (Figure 6). The mean peroxide concentration in control bags at 0 hours was 380 μM equ TBH.

Hydroperoxide formation in the HL:LD parenteral admixture at 24 hour hang time (24 hours)

Detectable amounts of peroxides were measured in HL:LD PA 24 hours after addition of d-α-tocopherol and exposure of bags to fluorescent light (Figure 7). By 24 hours d-α-tocopherol concentrations of 48 and 64 IU/g lipid were required to significantly decrease the hydroperoxide level compared with control bags. The mean peroxide concentration in control bags at 24 hours was 383 μM equ TBH.

Concentrations of α- and γ-tocopherol in the HL:LD parenteral admixture treated with d-α-tocopherol

Alpha-tocopherol decreased in the bags containing 8, 12, 16, 32, or 48 IU d-α-tocopherol/g lipid (Table 2). Mean concentration of γ-tocopherol was similar at 0 hours in all PA bags irrespective of d-α-tocopherol supplementation level (range, 29.33 to 37.55 mg/L; mean 31.97 mg/L). Concentrations of γ-tocopherol did not change significantly (P = 0.452) during the 24-hour hang time (range, 29.65 to 34.73 mg/L; mean 32.96 mg/L) (Table 2).

28

Lipid emulsions in glass bottles and PVC bags

Detectable concentrations of peroxides were observed in all newly opened bottles and PVC bags of 20% Intralipid ranging from 262 to 449 μM equ TBH (mean, 314 μM equ TBH) (Table 3).

DISCUSSION

This study evaluated effects of d-α-tocopherol, as Vital E-300, on peroxidation of HL:LD PA commonly used in small animal practice. The results demonstrate that HL:LD PA formulated for intravenous administration contained measurable amounts of hydroperoxides. Additionally, hydroperoxides were present in all newly opened bottles and PVC bags of lipid emulsion. These findings are consistent with previous studies suggesting that soybean lipid emulsions undergo oxidative damage during storage (4-6), which results in the presence of hydroperoxides in the formulated PA solution. Further peroxidation of parenteral lipid emulsions can be influenced by time, temperature, light exposure, and the presence of oxygen (12). Although 20% Intralipid is manufactured under nitrogen to exclude oxygen, the PA compounding process introduces oxygen into this solution. In this study, all bags were compounded manually. This method likely introduced more oxygen into PA solutions than would use of an automated compounder.

Compounding the PA with lipid emulsion, amino acid solution, dextrose, vitamin B complex, and lactated Ringer's solution resulted in a numerically increased concentration of hydroperoxides compared with the concentration present in the lipid emulsion alone (Table 3). This study, however, was not designed to compare this aspect of compounding. Baseline concentration of hydroperoxides present in the HL:LD PA (mean, 380 μM equ TBH) was likely dependent on the concentration of hydroperoxides in the lipid emulsion (mean, 314 μM equ TBH), the introduction of oxygen during compounding, light exposure, and temperature. Recent research suggests that there are other possible contributors to hydroperoxide formation in the PA, such as amino acid peroxides, which are generated by photooxidation, especially in the presence of riboflavin, a known

photosensitizer (72). Both components, amino acids and riboflavin, were present in our PA and could contribute to the total hydroperoxide content of the final PA.

This study used an analytical procedure (FOX 2 assay) designed to measure lipid hydroperoxides in the presence of a high background of nonperoxidized lipid (70). This method has been used in previous studies to measure hydroperoxides not only in lipid emulsion (18) but also in edible oils (73), liposomes, and low-density lipoproteins (70, 74). Jiang et al showed that the FOX 2 assay also detected hydrogen peroxide (H_2O_2) in liposomes via catalase, which indicates the formation of H_2O_2 during lipid peroxidation (70). Conversely, Staprans et al observed no effect of catalase on total hydroperoxide level in edible oils (75). Therefore, depending on the type of sample analyzed, the FOX 2 assay measures total hydroperoxide content, including lipid hydroperoxides and H_2O_2. A known limitation of the FOX 2 assay is interference by ascorbate (76). We considered this analytical method appropriate because of our interest in total hydroperoxide concentrations and a type of sample that does not contain ascorbate. In all cases, this method was consistently reproducible and was simple to perform.

The concept of formation of hydroperoxides and protection of lipid emulsion in PA has been evaluated in humans over the past two decades. Initial studies focused on multivitamin sources to protect against lipid peroxidation. Results of these studies were contradictory. Lavoie et al suggested a prooxidative effect of multivitamins in PA (18), while Laborie et al reported peroxide formation induced by riboflavin and a possible protective effect of ascorbate on peroxide generation in PA (29). Conversely, Silvers et al showed a decrease in peroxide formation in Intralipid through multivitamin supplementation (30, 77). Neuzil et al also reported a protective effect of ascorbic acid in lipid emulsion, but final ascorbate concentration was high (1 mM/L) for use in human patients (11). Although vitamin E possesses high antioxidative activity, earlier studies indicated that addition of this vitamin to lipid emulsion was able to address a low vitamin E status in human patients during total parenteral nutrition (31).

Vitamin E is used to describe a family of eight molecules of related structure, four tocopherols and four tocotrienols. All of these molecules possess antioxidant activity, although α-tocopherol is chemically and biologically the most active because the

substitution pattern of methyl groups facilitates the transfer of the hydrogen from a hydroxyl group of the chromanol ring to a fatty acid peroxyl radical. This donation of a hydrogen atom can terminate the radical chain in the lipid autoxidation reaction. Several studies have demonstrated that the level of natural tocopherols differs between commercial lipid emulsions, as well as between lots of 20% Intralipid (5, 13). This study was able to measure hydroperoxides in newly opened bottles or PVC bags of 20% Intralipid and in freshly compounded PA. The results indicate that amounts of tocopherols were not sufficient to inhibit peroxidation in our *in vitro* model.

In the present study, addition of d-α-tocopherol in various concentrations influenced total concentration of peroxides measured immediately after compounding and over a 24-hour period. While addition of 24 IU or more d-α-tocopherol/g lipid decreased concentration of peroxides at 0 hours, only the two highest tested concentrations, 48 and 64 IU d-α-tocopherol/g lipid, remained significantly protective over 24 hours. Figures 1 and 2 indicate that the level of peroxide production was dependent on the d-α-tocopherol dose, but a time × dose interaction was also evident. These findings suggest that d-α-tocopherol served as an antioxidant in this *in vitro* model, having the ability to scavenge free radicals that perpetuate peroxidation of HL:LD PA over time.

Hydroperoxide concentrations in the control bags did not appear to change over the 24-hour hang time. We attribute this "steady state" of hydroperoxide levels to our closed system. Once the compounding was complete, there was no opportunity for oxygen, the major substrate for peroxidation, to enter the bag. Additionally, temperature was controlled over the 24-hour hang time, so there was no stimulus for additional hydroperoxide production. Conversely, in a clinical setting there are numerous opportunities for oxygen infusion into the delivery system. Examples of this would include connecting, detaching, and reattaching the intravenous line, using a filtered vent for PA infusion, and mechanical malfunctioning of the line, all of which would likely increase peroxide production in the PA delivery system.

Results of this study need to be interpreted in relation to the reported prooxidative effect of α-tocopherol. In this study, concentrations of supplemented d-α-tocopherol ranged from 1,600 to 12,800 IU/L (1,074 to 8,591 mg/L) of 20% Intralipid, and neither

concentration was found to be prooxidative. Steger et al reported a prooxidative effect of 160 mg/L (240 IU/L) α-tocopherol in 20% lipid emulsion during storage at 40 ± 0.5ºC (5). The prooxidative effect of α-tocopherol was not observed by Dupont in lipid emulsion at a concentration of 200 mg/L, in which oxidation was initiated at 37ºC by addition of 2.2'-azobis (amidinopropane) dihydrochloride (14). Similarly, the dose of α-tocopherol 6,460 mg/L of 20% lipid emulsion incubated with unstimulated neutrophils was not found to be prooxidative by Wu et al (78). These conflicting results were probably caused by very different experimental conditions. Optimal concentrations of supplemented d-α-tocopherol that markedly reduced hydroperoxides in our in vitro study were 3,221 and 6,443 mg/L of 20% Intralipid at 0 hours and 24 hours, respectively. These results are in agreement with those of Wu et al (78), who reported the optimal *in vitro* concentration of α-tocopherol to protect 20% lipid emulsion from phagocyte-induced lipid peroxidation to be between 3,455 and 6,460 mg/L.

The protective function of supplemental vitamin E in this study appears to be the direct action of increasing levels of the α-tocopherol isoform in the PA. Values from the product label indicate that 20% Intralipid contains a higher concentration of γ-tocopherol versus α-tocopherol (29 and 118 mg/L, respectively). The γ isoform did not appear to play a role in preventing or minimizing production of hydroperoxides from susceptible long-chain fatty acids in the 20% Intralipid emulsion. Gamma-tocopherol concentrations at 0 hours and 24 hours for the treated group averaged 31.97 mg/L and 32.96 mg/L, respectively. Conversely, d-α-tocopherol is converted into a fairly stable tocopheroxyl radical. This radical only reacts with another tocopheroxyl radical or a fatty acid peroxyl radical and forms stable, nonradical products. The reaction leads to the destruction of d-α-tocopherol as an antioxidant while simultaneously reacting with peroxyl radicals, an early intermediate product of PUFA lipid peroxidation. Indeed, this study showed marked decomposition of d-α-tocopherol over a 24-hour period. These results agree with those of Steger et al, who demonstrated decomposition of the tocopherol isomers in heat-stressed 20% Intralipid (5). Although we expected a significant decrease in α-tocopherol for all concentrations over 24 hours, the decrease measured in bags supplemented with 24 and

64 IU α-tocopherol/g lipid was only numerical. It is likely that large variance among bags at these concentrations was due to experimental error.

Formation of hydroperoxides in HL:LD PA *in vitro* should be considered potentially harmful and may represent a clinically significant risk in patients with insufficient antioxidant protective mechanisms in the face of increased oxidative stress. Increasing evidence suggests that hydroperoxides are toxic and can cause cell damage (28, 37). It has been shown that feeding oxidized fats increases concentrations of lipid peroxidation products in tissues where they are subsequently incorporated into membrane phospholipids (75). Peroxidized membranes become rigid and lose selective permeability and integrity (79). Peroxidation of membrane lipids yields cytotoxic metabolites such as hydroxynonenal, malondialdehyde, and other types of alkenals. The process of lipid peroxidation and its products can be detrimental to cell viability, resulting in cell death by apoptosis or necrosis (8). Peroxidative injury of biologic membranes is involved in a number of pathologic processes such as ischemia reperfusion injury, hemolytic anemia, inflammatory diseases, diabetes mellitus, obesity, cancer, aging, and cognitive disorders.

This study demonstrates that lipid peroxidation in HL:LD PA *in vitro* can be inhibited by adding d-α-tocopherol during compounding. Inhibition of lipid peroxidation was observed to be concentration dependent. The optimal concentration of d-α-tocopherol in HL:LD PA was between 24 and 48 IU/g lipid (3,221 - 6,443 mg/L of 20% Intralipid) at 0 hours and 24 hours, respectively. Numerous disease states in small animal patients are associated with increased oxidative stress and associated radical-induced cell damage. Although these patients are in need of nutritional support, delivery of a peroxide-rich nutrient source to these patients may perpetuate their compromised state. It is not clear if the concentrations of hydroperoxides produced in HL:LD PA reported in this study would have negative physiologic effects or if animal patients would benefit from receiving d-α-tocopherol enriched HL:LD PA. Therefore, further studies are required before vitamin E supplementation of PA is established in clinical practice.

CHAPTER IV

THE EFFECT OF VITAMIN E ENRICHED PARENTERAL ADMIXTURE ON
OXIDATIVE STATUS OF OBESE CATS

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ABSTRACT

Intralipid emulsion, a commonly utilized energy source in PA, is sensitive to peroxidation. HL:LD PA may be a significant source of lipid hydroperoxides in critically ill, oxidatively-stressed patients. Antioxidants, such as vitamin E, can minimize the damaging effects of oxidative stress *in vitro*, but the value it affords patients receiving HL:LD is not documented. Objectives of this study were to quantify oxidative damage and evaluate the benefits of d-α-tocopherol on oxidative status in obese cats receiving HL:LD PA.

Sixteen obese cats (n = 8 treatment; n = 8 control) received intravenous HL:LD PA over a total 48 hours. For each 24 hour period, HL:LD PA was delivered in cyclic fashion for 16 hours. Natural d-α-tocopherol (Vital E-300) was added (40 IU/g lipid, PA Toc$^+$) or not (PA Toc$^-$) to the daily PA. Plasma malondialdehyde (MDA), serum α- and γ-tocopherol, red blood cell (RBC) and white blood cell (WBC) total GSH (tGSH), and GPx were measured at baseline, 24, 48, and 96 hours. A repeated measures analysis of variance was used for data analysis, with P < 0.05 considered significant.

Cats exhibited a significant change in plasma MDA concentration during the experiment (time effect; P < 0.0001). Plasma MDA increased in both groups from

baseline to 24 and 48 hours (P < 0.0001). Plasma MDA appeared to increase in PA Toc⁻ cats from 24 to 48 hours. This increase in MDA was not apparent in PA Toc⁺ cats. Treatment did not have an effect on plasma MDA concentration. WBC-tGSH in PA Toc⁺ cats tended to increase from baseline to 24 and 48 hours (P = 0.07) while WBC-GPx activity did not change. RBC-tGSH and GPx changed over time (time effects; P = 0.0005; P = 0.0016, respectively). The PA Toc⁺ cats had higher RBC-tGSH concentration (treatment x time interaction; P = 0.012). Administration of Vital-E 300 increased serum α- and γ-tocopherol concentrations (treatment effect; P < 0.0001).

Although administration of HL:LD PA resulted in an immediate increase in lipid peroxidation, *in vitro* supplementation of d-α-tocopherol, as Vital-E 300, appeared to reduce subsequent production of hydroperoxides in obese cats by 48 hours of PA administration. Moreover, administration of d-α-tocopherol restored RBC-tGSH and tended to increase WBC-tGSH. Supplementation of d-α-tocopherol to HL:LD PA may provide a more physiologically compatible formulation for critically ill patients.

MATERIALS AND METHODS

Study design

Sixteen, domestic shorthaired, female spayed, adult, obese cats were used for this study. Cats ranged in age from 3 to 8 years (mean ± SD, 5.5 ± 1.63 years) and in body weight from 4.18 to 7.36 kg (5.35 ± 0.85 kg). The degree of obesity was determined by dual energy x-ray absorptiometry (DEXA) that revealed body fat percentages that ranged from 37.3 to 68.9 % (48.96 ± 8.8 %). Prior to the experiment, all cats were housed separately in an environmentally controlled room equipped with individual steel cages. All cats belonged to a research colony at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM). The protocol for this study was approved by the Virginia Tech Animal Care Committee (ACC #03-048-CVM).

Cats were determined to be clinically healthy based on results of physical examination and urinalysis. Four weeks prior to the experiment, all cats were fed the same commercial diet (Science Diet Feline Adult - dry) and had continuous access to fresh water. The dry diet was fed twice daily and provided energy to maintain an obese state in all cats.

Parenteral admixtures were compounded aseptically in a laminar flow hood, using 20- and 60-mL syringes. Nutrient solutions were transferred into 500 ml PVC infusion bags (IntraVia, Baxter Healthcare Corporation, Deerfield, IL) via a back-check valve (B. Braun Medical, Inc., Bethlem, PA) under a laminar flow hood. Total volumes of PA provided 75% of daily fluid requirements (55 mL/kg/day) and 75% of the resting energy requirements (RER = 70 x $BW_{kg}^{0.75}$). The proportion of nutrients in each PA supplied 80% of energy from lipids (Intralipid 20%, Fresenius Kabi, Baxter Healthcare Corporation, Deerfield, IL), 20% energy from carbohydrates (Dextrose 50% solution, Vedco, Inc., St. Joseph, MO) and 4 g of protein/100 kcal (Aminosyn II 10%, Abbott Laboratories, North Chicago, IL). Vitamin B-complex (Vedco, Inc., St. Joseph, MO) and potassium chloride (Abbott Laboratories, North Chicago, IL) were added at a rate of 0.5 mL/100 kcal and 20 mEq/L, respectively. All nutrient solutions used for this study originated from an identical lot. Lactated Ringer's solution (Baxter Healthcare

Corporation, Deerfield, IL) was added to achieve the desired total volume (0.75 x 55 mL/kg/day). The calculated total osmolarity of parenteral admixtures ranged from 662 to 725 mOsm/L (696 ± 19 mOsm/L). Natural d-α-tocopherol (Vital E-300, Schering Plough Animal Health Corp., Union, NJ) was added (40 IU/g lipid) to the each treatment PA bag (PA Toc[+]) daily. This dose of d-α-tocopherol was established in a previous *in vitro* experiment. Control PA bags (PA Toc[-]) were not supplemented with d-α-tocopherol. All PA bags were compounded daily, 30 minutes prior to use and were protected from light by covering with aluminium foil until attachment to the infusion pump.

Cats were randomly assigned to either treatment (n = 8) or control (n = 8) group. Morning blood samples were drawn into 7 mL lithium heparin and 5 mL serum vacutainer tubes via jugular venipuncture (baseline), after withholding food for 12 hours. Cats were anesthesized for catheter placement with an intramuscular injection of acepromazine (0.1 mg/kg) and ketamine (10 mg/kg). Anesthesia was prolonged by additional ketamine (5 mg/kg) when required. One cat was anesthesized with acepromazine (0.1 mg/kg), hydromorphone (0.1 mg/kg) intramuscularly, and anesthesia was maintained with isoflurane gas due to a known adverse reaction to ketamine. The medial aspect of the rear limb was aseptically prepared for catheter placement. A single lumen catheter (Argon arterial catheter 18 ga x 15 cm, Argon Medical Devices, Inc., Athens, TX) was inserted into the medial saphenous vein and secured with a splint and bandage.

During the experiment, cats were housed in the individual stainless steel cages (42" x 42") in a controlled environment that provided ambient temperature 21 - 23 ºC and 16 hours of fluorescent room light per day. Parenteral admixture was administered in a cyclic fashion to the treatment group (PA Toc[+]) and to the control group (PA Toc[-]) by infusion pump via saphenous catheter over 48 hours. The daily cycle was 12 hours on, 8 hours off, and 4 hours on within a 24 hour period. During the 8 hour dark regime, PA bags were transferred to a room with similar conditions; 21 - 23 ºC, fluorescent room light to ensure continuity of the environmental conditions for 24 hours.

General attitude, respiration rate, heart rate, capillary refill time and alertness were monitored for each cat every 2 hours. Hydration status was maintained by offering the remaining 25% of daily fluids through the oral route. Deficits between daily fluid requirements and the sum of fluids administered via PA and taken voluntarily were corrected by subcutaneous administration of Lactated Ringer's solution each morning. Body weights were obtained at baseline, 48, and 96 hours with the same digital laboratory scale.

After 48 hours of PA administration, the parenteral feeding was discontinued and catheters removed. The cats remained individually housed for an additional 48 hours. They were fed a commercial diet (Science Diet Feline Adult - dry) twice daily in an amount to meet their daily energy requirements from 48 to 96 hours. Water was offered ad libitum.

Blood samples were obtained at baseline, and 24, 48, 96 hours. All blood samples were drawn into 7 mL lithium heparin vacutainer tubes for plasma collection and into 5 mL serum vacutainer tubes via jugular venipuncture. Blood samples taken during PA administration were drawn 20 minutes after discontinuing the PA infusion to avoid hyperlipidemia. Blood samples were placed immediately on crushed ice and transported to the laboratory within 30 minutes for processing into RBC and WBC, plasma and serum aliquots.

Plasma was analyzed for malondialdehyde concentration (MDA; Bioxytech MDA-586), serum for α- and γ-tocopherols concentration by high-performance liquid chromatography, RBC and WBC for tGSH (Bioxytech GSH-420) concentration, and GPx (Bioxytech GPx-340) activity.

Isolation of red blood cell (RBC) lysate, white blood cells (WBCs), plasma and serum

Whole blood was collected into lithium heparin vacutainer tubes and rotated for 10 minutes on a nutator, until well mixed. Using a pipette, 250 µL of whole blood was transferred into a 1.5 mL microcentrifuge tube. Both, vacutainer and microcentrifuge tubes, were centrifuged at 2500 x g for 10 minutes at 4ºC. Plasma in the microcentrifuge
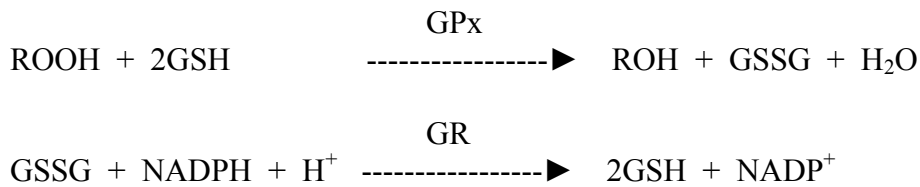
tube was removed with a Pasteur pipette, and the remaining RBC pellet was immediately snap-frozen by placing the tube in a dry ice/ethanol bath. Plasma in the vacutainer tube was removed using a Pasteur pipette into microcentrifuge tubes and snap-frozen immediately. The WBC layer was aspirated from the vacutainer tube using a Pasteur pipette and placed into 35 mL of lysis buffer (pH 7.2), incubated at room temperature for 10 minutes, and centrifuged at 1500 x g for 10 minutes at 4ºC. The supernatant was decanted and the WBC pellet was re-suspended in 5 mL of lysis buffer, to remove residual RBC, and 20 mL of Hanks Balanced Salt Solution (HBSS) (Invitrogen Corporation, Gibco, Grand Island, N.Y., USA). The re-suspended pellet was then incubated for 10 minutes at room temperature and centrifuged at 1500 x g for 10 minutes. The supernatant was removed and the WBC pellet re-suspended in 1 mL of HBSS. This pellet solution was transferred into a 1.5 mL microcentrifuge tube and snap-frozen in a dry ice/ethanol bath.

Serum aliquots were prepared by centrifuging serum vacutainer tubes at 2500 x g for 10 minutes at 4ºC and than transferring the supernatant to microcentrifuge tubes, which were snap-frozen in a dry ice/ethanol bath. All sample aliquots were stored at -80ºC until analysis.

<div align="center">Assays</div>

<div align="center">*Red blood cell and white blood cell glutathione peroxidase assay*</div>

Cellular GPx activity was determined using a commercial colorimeteric assay kit (Bioxytech GPx-340, Oxis International, Inc., Portland, OR) and an OxyScan Automated Oxidative Stress Analyzer. The Bioxytech GPx-340 assay measured the indirect activity of GPx enzyme. The enzyme activity in the cell homogenate was determined by adding reduced glutathione (GSH), glutathione reductase (GR), and nicotinamide adenine dinucleotide phosphate (NADPH). The enzyme reaction was initiated by adding the substrate, tert-butyl hydroperoxide (TBH).

$$\text{ROOH} + 2\text{GSH} \xrightarrow{\hspace{1em}\text{GPx}\hspace{1em}} \text{ROH} + \text{GSSG} + \text{H}_2\text{O}$$

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\hspace{1em}\text{GR}\hspace{1em}} 2\text{GSH} + \text{NADP}^+$$

The oxidation of NADPH to NADP$^+$ was accompanied by a decrease in absorbance at 340 nm. The rate of decrease in absorbance at 340 nm was directly proportional to the GPx activity in the sample.

Briefly, WBC pellet and RBC lysate were thawed at room temperature. One mL of distilled and deionized water was added to the microcentrifuge tube and RBC lysate and vortexed 10 seconds to dissolve the RBC pellet. Four µL of RBC lysate was then transferred into a microcentrifuge tube with 400 µL of sample buffer from the assay kit and vortexed. Fifty µL of RBC lysate in sample buffer was transferred to the curvette/carousel of the OxyScan and automatically analyzed. The WBC sample was vortexed for 10 seconds and then centrifuged at 10,000 x g for 5 minutes at 4ºC to precipitate cell membranes. Fifty µL of WBC supernatant was pipetted and transferred to the curvette/carousel of OxyScan for analysis. The GPx activity was expressed in mU/mL.

Red blood cell and white blood cell total glutathione assay

Total GSH was determined by a commercial colorimeteric assay kit (Bioxytech GSH-420, Oxis International, Inc., Portland, OR) using an OxyScan Automated Oxidative Stress Analyzer. The Bioxytech GSH-420 assay is based on the formation of a chromophoric thione. There were three steps to the reaction. The sample was first buffered and a reducing agent, tris(2-carboxyethyl)phosphine, was added to reduce GSSG to GSH. A chromogen, 4-chloro-1-methyl-7-trifluoromethylquinolinium methylsulfate, was then added forming thioethers with all thiols present in the sample. Upon addition of a base to raise the pH greater than 13, a β-elimination specific to the GS-thioether resulted in the chromophoric thione. Absorbance, measured at 420 nm, is directly proportional to the tGSH concentration.

Briefly, the WBC sample and RBC lysate were thawed at room temperature. One mL of distilled and deionized water was added to the RBC lysate tube, and vortexed 10 seconds to dissolve the RBC pellet. One hundred µL of the RBC lysate was transferred into a microcentrifuge tube with 300 µL of precipitation reagent. The tube was vortexed for 15 seconds and centrifuged at 10,000 x g for 5 minutes at room temperature. Fifty µL of sample supernatant was transferred to the curvette/carousel of OxyScan to be automatically analyzed. The WBC sample was vortexed for 10 seconds and centrifuged at 10,000 x g for 5 minutes to precipitate cell membranes. Fifty µL of the WBC supernatant was aspirated using a pipette and transferred to the curvette/carousel of OxyScan for analysis. Each sample was read in duplicate and the tGSH concentration was expressed in µMol/L.

Plasma malondialdehyde assay

Plasma MDA concentration was determined by use of a commercial colorimetric assay kit (Bioxytech MDA-586, Oxis International, Inc., Portland, OR). This assay is specific for free MDA and minimizes interference from other lipid peroxidation products, such as 4-hydroxyalkenals. This method is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (NMPI) with MDA at 45ºC. One molecule of MDA reacts with 2 molecules of NMPI to yield a stable carbocyanine dye with a maximum absorption at 586 nm.

Briefly, 10 µL of probucol was added to the each assay tube using a pipette. Plasma or each of eight standards (200 µL) was added, followed by 640 µL of N-methyl-2-phenylindole in acetonitrile diluted in 100% methanol (3:1 ratio). Each tube was individually vortexed. Next, 150 µL of concentrated hydrochloric acid was added and each tube was mixed by vortexing again. Tubes were incubated at 45ºC for 60 minutes using a water bath. After incubation, all tubes were centrifuged at 10,000 x g for 10 minutes at room temperature to obtain supernatant. Supernatants were removed using a pipette and each sample was divided into three wells of a flat bottom microplate. Absorbance was measured at 586 nm using a spectrophotometer (Beckman DU 640 B,

Beckman, Palo Alto, CA). Absorbance value of each sample was plotted to the standard curve and MDA concentration expressed in µMol/L. Each sample was read in triplicate.

Serum α- and γ-tocopherol high-performance liquid chromatography

All solvents were degassed with nitrogen prior to use and the analyses were run in subdued light. Two-hundred µL of plasma was added into a 16 dram screw-capped glass vial filled with nitrogen. Two-hundred µL of potassium hydroxide (600 g/L), 200 µL of 95% ethanol, 200 µL of sodium chloride (10 g/L) in water, and 625 µL of pyrogallol (60 g/L) in ethanol were added to the plasma. The mixture was vortexed, and the vials heated at 70 °C for 45 minutes. Tubes were then cooled in ice and the samples were extracted 3 times with 1 ml of cyclohexane containing 0.01% of butylated hydroxytoluene. Extracts were combined together and the solvent was evaporated under a stream of nitrogen. The residue was filtered through a Pall Gelman Acrodisc LC 13mm 0.2µ filter (Pall Life Sciences, Ann Arbor, MI). When necessary, a 1:10 or a 1:100 dilution of samples was prepared.

For each group of samples, two sets of external standards were prepared by adding 10 µL of α- and γ-tocopherol mix (10 ppm) in cyclohexane containing 0.01% butylated hydroxytoluene to 200 µL of 20% Intralipid or 200 µL of water. Recoveries were calculated as an average of the two sets.

Analyses of tocopherols were performed by HPLC with a normal-phase column. Tocopherols were separated with a column (Agilent Technologies Zorbax Rx-Si 150 x 4.6 mm, 5µ) with a mobile phase and consisted of a mixture of n-hexane and isopropanol (99.7:0.3) at a flow rate of 1 mL/min. Tocopherols were detected by their native fluorescence using a fluorescence detector set with an excitation wavelength of 295 nm and an emission wavelength of 330 nm. The injection volume was 10µL. Concentrations of α- and γ-tocopherols were expressed as µg/mL (ppm).

Data are presented as the mean ± SEM. For each response variable, mixed effects, repeated measures analysis of variance was used to test for main effects of treatment and time, as well as their interactions. Significant interactions were further investigated with tests of simple main effects. All calculations were performed using the SAS System (ver. 9.12, SAS Institute Inc., Cary NC 27513). Statistical significance was defined as $P < 0.05$.

RESULTS

Animals

Based on physical examination, cats in both groups remained healthy throughout the experiment. Body weights of all cats averaged 5.35 ± 0.85 kg, 5.32 ± 0.87 kg and 5.25 ± 0.85 kg (mean ± SD) at times 0, 48 and 96, respectively.

Plasma malondialdehyde concentration

All cats exhibited changes in plasma MDA concentration throughout the experiment (time effect; $P < 0.0001$) (Figure 9). Plasma MDA concentrations increased in both PA Toc$^+$ and PA Toc$^-$ groups at time 24 and 48 ($P < 0.0001$) compared to baseline. Plasma MDA increase was significantly higher in the PA Toc$^+$ group from baseline to time 24 ($P = 0.0418$). During the 24 to 48 hour period, MDA concentrations increased in PA Toc$^-$ cats (12.3 µMol/L vs. 13.5 µMol/L), but appeared to decrease in PA Toc$^+$ cats (12.6 vs. 12.5 µMol/L). Plasma MDA concentration approached baseline values by time 96 hours in both PA Toc$^-$ (baseline, 8.2 µMol/L; 96 hours, 5.7 µMol/L) and PA Toc$^+$ (baseline, 5.8 µMol/L; 96 hours, 6.8 µMol/L) treated groups (Figure 9).

White blood cell glutathione peroxidase activity and total glutathione concentration

The WBC-GPx activity did not change and there was no statistical difference observed between the groups over time (Figure 10).

The WBC-tGSH concentrations were not statistically different between groups during the experiment (Figure 11). However, the overall WBC-tGSH concentration in both groups of cats tended to increase from baseline to 24 and 48 hours (P = 0.07). There was a tendency for WBC-tGSH concentration to be higher in the PA Toc[+] group from baseline to time 24 and 48 (P = 0.07) compared with PA Toc[-] group.

Red blood cell glutathione peroxidase activity and total glutathione concentration

The RBC-GPx activity changed significantly in both groups (time effect; P = 0.0016) (Figure 12) but enzyme activity was not affected by the d-α-tocopherol (time x treatment interaction; P = 0.342). Cats in both groups showed a trend for increasing RBC-GPx activity (P = 0.06) by 24 and 48 hours compared to baseline.

Cats receiving PA Toc[+] showed a higher RBC-tGSH concentration (time x treatment interaction; P = 0.01) compared with the PA Toc[-] group (Figure 13). Overall RBC-tGSH concentration significantly decreased in both groups from baseline to 24 and 48 hours (P = 0.03). However, from 48 to 96 hours, cats in PA Toc[+] group maintained a higher RBC-tGSH concentration (P = 0.004) relative to cats receiving PA Toc[-].

Serum α- and γ-tocopherol concentrations

The PA Toc[+] cats had significantly higher serum levels of α-tocopherol relative to the PA Toc[-] cats (treatment x time interaction; P < 0.0001) (Figure 14). Cats in the PA Toc[+] group exhibited a significant increase in serum α-tocopherol concentration from baseline to 24 hours (P < 0.0001), and a significant decrease from 48 to 96 hours (P < 0.0001). Concentrations in the PA Toc[+] group did not change from 24 to 48 hours. Serum α-tocopherol level in PA Toc[+] cats appeared to decrease in the 48 hours after HL:LD PA infusion was discontinued.

The PA Toc[+] cats had significantly higher serum γ-tocopherol concentration when compared to the PA Toc[-] group (treatment effect; P < 0.0001). Gamma-tocopherol concentration changed over time for both groups (time effect; P < 0.0001) (Figure 15). Serum γ-tocopherol concentration increased significantly from baseline to time 24 and 48

hours in PA Toc$^+$ (P < 0.0001) and in PA Toc$^-$ groups (P < 0.0001). The serum γ-tocopherol level in PA Toc$^+$ treated cats appeared to approach baseline values at 48 hours after HL:LD PA infusion was discontinued.

<div align="center">DISCUSSION</div>

Our study was designed to test the effect of α-tocopherol (Vital E-300) supplemented HL:LD PA in oxidatively-stressed cats. The presence of oxidative stress in study subjects was based on the premise that a state of extreme obesity results in imbalance of antioxidant defences. To enhance obesity-induced oxidative damage, cats were maintained in an obese state (BCS $\geq$ 8/9) for three months prior to the start of the experiment. The degree of obesity was quantified by DEXA which allows the body to be viewed as fat tissue, lean tissue and bone mineral compartments, and to calculate the percentage of body fat.

Although, the patho-mechanisms of oxidative stress in many disease states have been identified over the past two decades, there is accumulating evidence that obesity is a predictor of systemic oxidative stress (80). Multiple studies have demonstrated obesity-induced oxidative stress in humans with only a few comparable studies in animals. Chang et al (81) observed lower liver GSH concentration, Mn-SOD, and GPx activities in untrained obese rats compared to trained obese and non-obese controls. Tanner et al (82) also studied changes in plasma MDA during weight loss in obese cats. In that study, plasma MDA concentration decreased with decreasing body fat mass of study subjects. Based on results of recent human and animal research, obesity may provide a reasonable model of oxidative stress.

Serum α- and γ-tocopherol concentrations increased in cats given PA Toc$^+$ in the present study. Vital E-300, is marketed as a sole source of d-α-tocopherol, yet our post-experiment HPLC analysis of the product revealed the presence of γ-tocopherol with a α-tocopherol : γ-tocopherol ratio of 200:1. This finding may explain elevations of serum γ-tocopherol in the PA Toc$^+$ treatment group. Although, serum vitamin E levels reached supra-physiological levels, concentrations approached baseline two days after PA Toc$^+$

administration was discontinued. Additionally, even though the HL:LD PA was discontinued for 20 minutes before drawing the blood sample, some cats (8 blood samples) exhibited a degree of hyperlipidemia. Hyperlipidemia, caused by the presence of HL:LD PA with d-α-tocopherol in the blood stream, could result in high serum vitamin E concentrations. Treated cats in our study received 74.3 ± 3.85 mg of d-α-tocopherol/kg body weight/day intravenously which appears to be within a safe range. A review of the literature by Bendich et al (83) revealed that cats can tolerate up to 200 mg/kg of vitamin E without apparent toxicosis, and that deleterious effects were not observed until daily doses exceeded 1g/kg. However, several points need to be carefully assessed and further investigated. First, the kinetics of vitamin E transport are different when absorbed orally, or when given intravenously (84). Second, possible adverse effects of higher doses of vitamin E in the presence of clotting disorders should be considered (85).

One principal finding of this study was that short term administration of HL:LD PA resulted in increased lipid peroxidation *in vivo*. Additionally, high serum concentrations of α- and γ-tocopherol did not affect the degree of lipid peroxidation in the d-α-tocopherol treated vs. non-treated group. We also observed that lipid peroxidation in PA Toc[+] cats ceased after the first 24 hours of parenteral feeding, while it continued to increase in PA Toc[-] cats. This observation may indicate that d-α-tocopherol, at given concentrations, may provide a protective effect after 24 hours of HL:LD PA administration. Since we discontinued the HL:LD PA administration after 48 hours, we could not observe development of changes for a longer period. By two days post PA administration, plasma MDA concentrations returned to baseline values in both groups. Studies in rats demonstrated that the liver eliminates MDA from the circulation by the action of aldehyde dehydrogenase and thiokinase, resulting in MDA half-life of approximately 2 hours. Ten to 30% of MDA bind to proteins which prolongs elimination to more than 12 hours (86). The decrease in MDA concentration measured in our study at 96 hours could be explained as a cessation of MDA production and rapid excretion of MDA after lipid delivery was discontinued. The MDA colorimetric assay used in our study measured free MDA, therefore metabolism of protein-bound MDA could not be

evaluated. These results demonstrate that HL:LD PA administration promotes lipid peroxidation in obese cats and that d-α-tocopherol appears to initiate protection between 24 and 48 hours of HL:LD PA administration.

Our results agree with previous reports of increased *in vivo* lipid peroxidation in subjects receiving intravenous lipid emulsion and in oxidatively-stressed obese human subjects (4, 45). Pironi et al (10) measured lipid peroxidation as serum MDA in 12 adult human patients receiving home parenteral nutrition (HPN) and in 40 healthy control subjects. The controls were not receiving parenteral nutrition. The HPN patients had a higher concentration of serum MDA than control subjects (2.19 ± 0.82 μMol/L vs. 1.43 ± 0.35 μMol/L; $P < 0.003$) and lower plasma α-tocopherol concentration ($P < 0.002$). These results suggest that diseased human patients given PA containing lipid emulsion showed increased *in vivo* lipid peroxidation. Since excessive fat stores can serve as a target for lipid peroxidation, administration of lipid emulsion may represent an additional substrate that perpetuates oxidative damage. Our results should therefore be assessed in the context of obesity-induced lipid peroxidation. This relationship was tested by Sojiljkovic et al (87) in humans. A 20% Intralipid emulsion with heparin was infused into obese (n = 10) and non-obese (n = 12) human subjects. After a two hour infusion period, F_2-isoprostanes formed nearly 2.5 times faster in the blood of the obese (14.9 ± 2.8 ng/mL) compared with non-obese (4.6 ± 2.8 ng/mL) subjects ($P < 0.05$). Obesity-enhanced lipid peroxidation was also observed by Riserus et al (88). In a randomized, placebo-controlled, double blind study, obese adult men (n = 24) received oral linoleic acid supplement or placebo. Men receiving CLA exhibited a 50% increase in urinary F_2-isoprostane excretion ($P < 0.01$) when compared with men receiving placebos. Finally, an obesity study by Olusi et al (80) revealed higher ($P < 0.001$) plasma MDA concentrations (4.75 ± 0.15 μMol/L) in 250 obese, but otherwise healthy, adult humans relative to 50 age- and sex-matched non-obese control subjects (2.53 ± 0.04 μMol/L). These researchers concluded that obesity alone, without contributions of hypertension, diabetes and hyperlipidemia, causes significant lipid peroxidation. Both groups of obese cats in our study had a 79% increase in plasma MDA concentration from baseline (mean 7.0

47

μMol/L) at the first 24 hours of HL:LD PA administration (mean 12.5 μMol/L). The HPN patients from Pironi's study had a 65% increase of serum MDA concentration and obese patients in Olusi's study had a plasma MDA concentration that was 88% higher compared to healthy controls. While our study showed similar trends, the higher baseline and post-infusion total concentration of MDA in cats, relative to humans, can most likely be contributed to differences between species, study designs and disease models used. Additionally, the total amount of administered lipids in this study was higher (2.85 g lipid/kg body weight/day) compared to amounts administered to human HPN patients (0.97 g lipid/kg body weight/day). Our data and results of recent human research indicate that despite proven benefits of parenteral feeding in properly selected patients, lipid emulsions rich in linoleic acid have the potential to increase *in vivo* lipid peroxidation. These results obtained from obese subjects imply that obesity may markedly contribute to the severity of lipid peroxidation. Although we did not include a lean cat group in our experiment to confirm this relationship, data from other studies suggest that lipid peroxidation in cats in our study was potentially magnified by severe obesity. Nevertheless, consequences of lipid peroxidation-induced oxidative damage on the health status of small animal patients are still unknown.

To assess oxidative status of cats in our study, we identified several biomarkers of oxidative stress such as tGSH, MDA concentrations, and GPx activity. Many researchers have concluded there is no single biomarker of lipid peroxidation or oxidative damage. A reason for the poor correlation between concentrations of oxidative stress biomarkers could be explained by their different metabolic rates. Although, biomarkers of damage to various molecules can increase in cells undergoing severe oxidative stress, the time course of such damage can be very different (89). Therefore, the approach to assess oxidative status of an individual is to monitor multiple biomarkers in various types of samples (90). Reduced glutathione and GSSG (tGSH) are biologically important intracellular thiols that protect cells against several toxic ROS and are used to assess exposure of cells to oxidative stress (91). Malondialdehyde is one of many aldehydes formed during lipid peroxidation of PUFA and has been reported to respond to alterations

in antioxidant nutrient status (92). Malondialdehyde belongs to the group of biomarkers of oxidative stress that were consistently elevated in human obese subjects (56). Other observed changes in obese humans include elevated plasma F_2-isoprostanes, plasma TBARS and lowered erythrocyte SOD and GPx activities (56). In many studies, F_2-isoprostanes, specific end products of the peroxidation of arachidonic acid, are considered to be the most reliable biomarkers of lipid peroxidation (89). 20% Intralipid does not contain arachidonic acid and cats are unable to elongate and desaturate linoleic acid from PA to form arachidonic acid. Therefore, MDA was considered a more reliable measure of lipid peroxidation than F_2-isoprostanes in our study.

The importance of vitamin E as an antioxidant within biological membranes has been well documented (33). *In vitro*, vitamin E protects RBC against hemolysis induced by oxidative agents (93). Moreover, vitamin E deficiency has been associated with higher RBC sensitivity to oxidative damage (94). As vitamin E enters the circulation, it is incorporated into cell membranes. Its presence may enhance the ability of cells to resist an oxidative attack when challenged with a source of reactive oxygen species such as in the HL:LD PA. The role of vitamin E is to interrupt the propagation of oxygen radical mechanisms within the cell membrane and decrease formation of hydroperoxides from highly reactive peroxyl radicals. Once formed, hydroperoxides are reduced by GPx which catalyzes the GSH/GSSG redox-system of the cell. Results of our study demonstrated a significant change of RBC-GPx activity over 96 hours with a trend of increased RBC-GPx activity during HL:LD PA administration in both groups. RBC-GPx activity was not, however, affected by d-α-tocopherol supplementation. Data regarding the activity of RBC-GPx in patients receiving HL:LD PA or obese individuals are limited and show variable responses to oxidative stress insults. Pironi et al (10) showed significantly lower RBC-GPx in 12 HPN adult patients compared to 40 healthy controls (P < 0.0003). Conversely, an animal study with Wistar rats (n = 24) undergoing gastrectomy and receiving either 20% medium-chain triglyceride (MCT)/long-chain triglyceride (LCT) (1:1) or 20% LCT intravenous emulsion revealed significantly higher RBC-GPx activity in the LCT group (P < 0.05). Data from obesity studies show contradictory findings.

Beltowski et al (95) studied effects of diet-induced obesity of eight week duration on oxidative status of Wistar rats (n = 30). Obese rats tended to have increased blood-GPx activity compared to controls. Conversely, Ozata et al (57) measured significantly lower RBC-GPx activity in 76 obese men (P = 0.001) compared to the 24 non-obese controls. The discrepancy between our results and those of Pironi et al and Ozata et al, could be due to the duration of PA administration and/or length of the obese state of the study subjects. Antioxidant enzyme activity may be stimulated in the early phases of obesity or with short-term oxidative stress, as described in our study. On the other hand, antioxidant enzyme activity may be decreased in persistent obesity or prolonged PA administration, as seen in HPN patients. It is possible that once oxidative stress persists for a sufficient time, antioxidative enzymes become depleted, leading to a low level of activity.

Maintenance of GSH levels is essential to sustain the thiol groups of cellular enzymes, and the intramembranous and cytoskeletal proteins in the reduced state (Figure 8). The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed by two cytosolic enzymes, γ-glutamylcysteine synthetase (GSC) and GSH synthetase. This pathway occurs in all cell types, including erythrocytes (96). The rate of GSH synthesis in erythrocytes is controlled by a GSH-induced feedback inhibition which acts on GSC (97). Maintenance of an adequate concentration of GSH within erythrocytes is a very dynamic process. The human erythrocyte has a relatively high turnover rate for GSH, such that all GSH may be replaced in 1.5 days (98). Red blood cell GSH concentrations may be influenced by a variety of factors. It is known that cellular GSH concentrations are reduced in response to protein malnutrition, oxidative stress, and many pathological conditions (99). More specifically, oxidative stress can induce an efflux of GSSG from erythrocytes (100). The concentration of RBC-tGSH decreased, while RBC-GPx tended to increase in both groups of cats in our study during PA administration. The antioxidative reaction to reduce hydroperoxides and other ROS is the oxidation of GSH to GSSG, and is driven by the enzyme GPx. Thus the RBC-GPx should be active as long as the GSH is available for the reaction. The GSSG should consequently be re-converted to a reduced state (GSH) by NADPH-dependent GR. Unfortunately, the total GSH assay

that we used in our study could not distinguish GSH from GSSG. Therefore, decreases in the RBC-tGSH with a trend of increased RBC-GPx activity can possibly be explained as an efflux of GSSG from RBC in response to oxidative stress while GSH synthesis remained unchanged. While RBC-tGSH decreased in all cats during PA administration, PA Toc^+ animals maintained higher RBC-tGSH concentration compared with PA Toc^- animals. This observation is in agreement with other studies. Significantly higher RBC-tGSH concentrations were shown in 10 healthy human volunteers, receiving orally 1000 IU vitamin E/day for 10 days, relative to 10 untreated control subjects (374.8 ± 17.3 µg/mL vs. 267.5 ± 15.7 µg/mL; $P < 0.001$) (101). Based on our results we conclude that PA Toc^+ administration does not prevent the decrease of RBC-tGSH, but it appears to minimize the degree of depletion.

Leukocytes represent a powerful defense system against invading microorganisms. During particle ingestion, granulocytes and macrophages produce large amounts of highly reactive molecules, leading to an increase in energy and oxygen consumption. This requires strictly aerobic conditions, when O_2 is reduced to $O_2^{\bullet-}$, and H_2O_2. Various reaction pathways then lead to formation of more destructive species such as $OH^\bullet$, hypochlorous acid (HOCl) and nitric oxide ($NO^\bullet$) (102). Although, the amount of ROS produced by stimulated phagocytes is higher than in all other cell types, the generated ROS and antioxidative mechanisms are basically identical. This means that the activated phagocyte is directly exposed to its own toxic metabolites which may represent a threat to its viability and function. One of the most potent intracellular antioxidant systems within the phagocyte is glutathione redox-system (103), that we assessed in our study as tGSH. For analysis, we harvested WBC samples that included all leukocyte types. Our results showed an unchanged activity of WBC-GPx and a trend towards increased concentration of WBC-tGSH throughout the PA administration in both groups of cats. These findings can possibly be explained as an increased resistance of WBC to oxidative stress. The primary function of phagocytes is their microbicidal property and simultaneous self-antioxidative protection. Therefore, it is critical to maintain the glutathione redox-system active for cell protection against autoxidation. To guarantee sufficient protection of

antioxidants within the phagocyte, antioxidative systems are either membrane-bound or located in specialized organelles (peroxisomes). Other antioxidants are freely diffusible within the cytosol or in the mitochondrial matrix. If oxidative stress was prolonged, the cell damage would possibly lead to destruction of antioxidative systems and WBCs would loose the ability to maintain the tGSH pool.

Leukocytes, in theory, could serve as a reasonable biological sample to assess oxidative stress *in vivo* due to their fast turnover (hours) and activity of antioxidative systems. To our knowledge, there are no published data about alterations of WBC-GPx and WBC-tGSH in subjects receiving PA or in obese individuals, but there have been several *in vitro* studies reporting the responsiveness of the glutathione redox-system in leukocytes to oxidative stress. Tsuchiya et al demonstrated decreased oxygen generation in phagocytes that underwent oxidative stress by exposure to H_2O_2 and $O_2^{\bullet-}$ (104). Additionally, Roos et al reported that deficiencies in the glutahione redox-system contributed to severe dysfunctions in the phagocytic and bactericidal properties of granulocytes (105). Finally, Spielberg et al studied phagocytic cells unable to properly regenerate GSH. These cells exhibited normal generation of ROS after stimulation, but were rapidly inactivated due to the autoxidation (106). Based on our *in vivo* observations, the WBC glutathione redox-system of obese cats receiving HL:LD PA did not respond to increased lipid peroxidation, measured as plasma MDA. Therefore, more *in vivo* studies are required to evaluate responsiveness of WBC to obesity and HL:LD PA associated oxidative stress.

In conclusion, our study demonstrated that PA Toc$^+$ administration plays a role in several oxidative stress responses in obese cats given this type of nutritional support. In the previous *in vitro* study, we demonstrated the peroxidative protection of HL:LD PA using 32 to 48 IU d-α-tocopherol/g lipid, immediately after compounding. This protective effect lasted for 48 hours of HL:LD PA hang-time. While the dose of 40 IU d-α-tocopherol/g lipid was administered to the cats in our study, it did not show protection against *in vivo* lipid peroxidation. The lack of protective effect *in vivo* could be explained by reliance of α-tocopherol on redox-active reagents to ensure its function. An example

of a redox-active reagent is ascorbate which is necessary to reduce the tocopheroxyl radical back to its active form, in order to prevent α-tocopherol from being destroyed as an antioxidant. However, ascorbate concentrations were not measured in our study and prevented us from exploring this relationship. Additionally, recent findings in human research do not clearly support the expected benefits of high dose of vitamin E (400 IU or more/day) taken orally long term (1 year or more). Even though the antioxidative activity of vitamin E has led to the expectation that long term dietary supplementation could provide a prevention against oxidative stress-related chronic pathologies, data from large meta-analysis have not provided supporting evidence (107). Conversely, several animal studies reported significantly reduced mortality rates in endotoxic rats and septic guinea pigs receiving vitamin E supplementation as a sole antioxidant (108, 109). Furthermore, a review of literature by Bulger et al. revealed that vitamin E appears to be a potent immunomodulator *in vitro* and in animal models of inflammatory syndromes (110). These results imply that vitamin E may have therapeutic potential for critically ill small animal patients.

In summary, obesity and HL:LD PA induced oxidative stress *in vivo* supports use of antioxidants in the prevention of oxidative cell damage. Our study shows that vitamin E has potential to improve antioxidative status of the animal. The relative safety of vitamin E makes it an attractive agent for additional human and animal studies. Further experiments are needed to elucidate whether d-α-tocopherol supplemented HL:LD PA can reach beyond the scope of antioxidative benefits, improve health status and hasten recovery from critical illness.

LITERATURE CITED

1. Lippert AC, Fulton RB, Jr., Parr AM. A retrospective study of the use of total parenteral nutrition in dogs and cats. J Vet Intern Med 1993;7:52-64.

2. Pyle SC, Marks SL, Kass PH. Evaluation of complications and prognostic factors associated with administration of total parenteral nutrition in cats: 75 cases (1994-2001). J Am Vet Med Assoc 2004;225:242-50.

3. Reuter JD, Marks SL, Rogers QR, Farver TB. Use of total parenteral nutrition in dogs: 209 cases (1988-1995). Vet Emerg Crit Care 1998;8:201-213.

4. Pitkanen O, Hallman M, Andersson S. Generation of free radicals in lipid emulsion used in parenteral nutrition. Pediatr Res 1991;29:56-9.

5. Steger PJ, Muhlebach SF. Lipid peroxidation of IV lipid emulsions in TPN bags: the influence of tocopherols. Nutrition 1998;14:179-85.

6. Laborie S, Lavoie JC, Pineault M, Chessex P. Protecting solutions of parenteral nutrition from peroxidation. JPEN J Parenter Enteral Nutr 1999;23:104-8.

7. Meydani M. Vitamin E. The Lancet 1995;345:170-175.

8. Davies KJ. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. IUBMB Life 2000;50:279-89.

9. Helbock HJ, Motchnik PA, Ames BN. Toxic hydroperoxides in intravenous lipid emulsions used in preterm infants. Pediatrics 1993;91:83-7.

10. Pironi L, Ruggeri E, Zolezzi C, et al. Lipid peroxidation and antioxidant status in adults receiving lipid-based home parenteral nutrition. Am J Clin Nutr 1998;68:888-93.

11. Neuzil J, Darlow BA, Inder TE, Sluis KB, Winterbourn CC, Stocker R. Oxidation of parenteral lipid emulsion by ambient and phototherapy lights: potential toxicity of routine parenteral feeding. J Pediatr 1995;126:785-90.

12. Steger PJ, Muhlebach SF. In vitro oxidation of IV lipid emulsions in different all-in-one admixture bags assessed by an iodometric assay and gas-liquid chromatography. Nutrition 1997;13:133-40.

13. Pironi L, Guidetti M, Zolezzi C, et al. Peroxidation potential of lipid emulsions after compounding in all-in-one solutions. Nutrition 2003;19:784-8.

14. Dupont IE. Peroxidation of lipid emulsions: effects of changes in fatty acid pattern and alpha-tocopherol content on the sensitivity to peroxidative damage. Clin Nutr 1999;18:113-6.

15. Simic M, Jovanovic S, E N. Mechanisms of lipid oxidative processes and their inhibition. In: Angelo A, ed. Lipid oxidation in food. New York, N.Y.: American Chemical Society, 1992:14-32.

16. Halliwell B, Chirico S. Lipid peroxidation: its mechanism, measurement, and significance. Am J Clin Nutr 1993;57:715S-724S; discussion 724S-725S.

17. Gutteridge JM. Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clin Chem 1995;41:1819-28.

18. Lavoie JC, Belanger S, Spalinger M, Chessex P. Admixture of a multivitamin preparation to parenteral nutrition: the major contributor to in vitro generation of peroxides. Pediatrics 1997;99:E6.

19. Balet A, Cardona D, Jane S, et al. Effects of multilayered bags vs. ethylvinyl-acetate bags on oxidation of parenteral nutrition. JPEN J Parenter Enteral Nutr 2004;28:85-91.

20. Remillard R, Thatcher,CD. Parenteral nutritional support in the small animal patient. Vet Clin North Am Small Anim Pract 1989;19:1287-1306.

21. Weissman C. Nutrition in the intensive care unit. Crit Care 1999;3:R67-75.

22. Remillard RL, Armstrong PJ, Davenport DJ. Assisted feeding in hospitalized patients: Enteral and parenteral nutrition. In: Hand MS, Thatcher CD, Remillard RL, Roudebush P, eds. Small Animal Clinical Nutrition. 4th ed: Walsworth Publishing Co., Marceline, MS, 2000:351-399.

23. Payne-James J, Khawaja HT. First choice for total parenteral nutrition: the peripheral route. JPEN J Parenter Enteral Nutr 1993;17:468-78.

24. Chandler ML, Guilford WG, Payne-James J. Use of peripheral parenteral nutritional support in dogs and cats. J Am Vet Med Assoc 2000;216:669-673.

25. Chan DL, Freeman LM, Labato MA, Rush JE. Retrospective evaluation of partial parenteral nutrition in dogs and cats. J Vet Intern Med 2002;16:440-445.

26. Directors ABo. Guidelines for the use of parenteral and enteral nutrition in adult and pediatric patients. JPEN Journal of Parenteral and Enteral Nutrition 1993;17 (Suppl.):16SA.

27.   Remillard RL. Angell Memorial Animal Hospital, Boston, MA, USA. Unpublished data. January 1998.

28.   Pitkanen OM. Peroxidation of lipid emulsions: a hazard for the premature infant receiving parenteral nutrition? Free Radic Biol Med 1992;13:239-45.

29.   Laborie S, Lavoie JC, Chessex P. Paradoxical role of ascorbic acid and riboflavin in solutions of total parenteral nutrition: implication in photoinduced peroxide generation. Pediatr Res 1998;43:601-6.

30.   Silvers KM, Sluis KB, Darlow BA, McGill F, Stocker R, Winterbourn CC. Limiting light-induced lipid peroxidation and vitamin loss in infant parenteral nutrition by adding multivitamin preparations to Intralipid. Acta Paediatr 2001;90:242-9.

31.   Schwalbe P, Buttner P, Elmadfa I. Development of vitamin-E-status of premature infants after intravenous application of all-rac-alpha-tocopheryl acetate. Int J Vitam Nutr Res 1992;62:9-14.

32.   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.

33.   Schneider C. Chemistry and biology of Vitamin E. Mol Nutr Food Res 2005;49:7-30.

34.   Burton GW. Autoxidation of biological molecules. 1. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants in vivo. J Am Chem Soc 1981;103:6472-6477.

35.   Liebler DC, Kling DS, Reed DJ. Antioxidant protection of phospholipid bilayers by a-tocopherol. Control of a-tocopherol status and lipid peroxidation by ascorbic acid and glutathione. J Biol Chem 1986;261:12114-12119.

36.   Kamal-Eldin A, Appelqvist LA. The chemistry and antioxidant properties of tocopherols and tocotrienols. Lipids 1996;31.

37.   Kaneko T, Kaji K, Matsuo M. Cytotoxicities of a linoleic acid hydroperoxide and its related aliphatic aldehydes toward cultured human umbilical vein endothelial cells. Chem Biol Interact 1988;67:295-304.

38.   Cheeseman KH. Initiation and pathobiological consequences of lipid peroxidation. In: Nohl H, Esterbauer, H., Rice-Evans, C., ed. Free radicals in the

environment, medicine and toxicology. London: The Richelieu Press Limited, 1994:81-96.

39. Aust SD. Sources of iron for lipid peroxidation in biological systems. In: Halliwell B, ed. Oxygen radicals and tissue injury. Bethesda,MD: Federation of American Societies for Experimental Biology, 1987:27-33.

40. Gutteridge JM. Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides. FEBS Lett 1986;201:291-295.

41. Kamal-Eldin A, Marjukka M, Lampi A-M. The challenging contribution of hydroperoxides to the lipid oxidation mechanism. In: Kamal-Eldin A, ed. Lipid oxidation pathways. Uppsala: AOCS Press, 2003:1-36.

42. Bird RP, Draper HH, Basrur PK. Effect of malonaldehyde and acetaldehyde on cultured mammalian cells: production of micronuclei and chromosomal aberrations. Mutat Res 1982;101:237-246.

43. Cutler MG, Schneider R. Tumours and hormonal changes produced in rats by subcutaneous injections of linoleic acid hydroperoxide. Food Cosmet Toxicol 1974;12:451-459.

44. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 1991;11:81-128.

45. Van Gossum A, Shariff R, Lemoyne M, Kurian R, Jeejeebhoy K. Increased lipid peroxidation after lipid infusion as measured by breath pentane output. Am J Clin Nutr 1988;48:1394-9.

46. Linseisen J, Hoffmann J, Lienhard S, Jauch KW, Wolfram G. Antioxidant status of surgical patients receiving TPN with an omega-3-fatty acid-containing lipid emulsion supplemented with alpha-tocopherol. Clin Nutr 2000;19:177-84.

47. Thurlow PM, Grant JP. Vitamin E and total parenteral nutrition. Ann N Y Acad Sci 1982;393:121-32.

48. Lespine A, Fernandez Y, Periquet B, et al. Total parenteral nutrition decreases liver oxidative metabolism and antioxidant defenses in healthy rats: comparative effect of dietary olive and soybean oil. JPEN J Parenter Enteral Nutr 2001;25:52-9.

49. Vandewoude MG, Vandewoude MFJ, De Leeuw IH. Vitamin E status in patients on parenteral nutrition receiving Intralipid. JPEN J Parenter Enteral Nutr 1986;10:303-305.

50. Fenster CP, Weinsier RL, Darley-Usmar VM, Pate RP. Obestiy, aerobic exercise, and vascular disease: the role of oxidant stress. Obes Res 2002;10:964-968.

51. Smith MA, Sayre LM, Perry G. Primary involvement of oxidative damage and redox imbalance in Alzheimer and other neurodegenerative diseases. Oxid Stress Dis 1999;3:115-126.

52. Lin BF, Lai CC, Lin KW, Chiang BL. Dietary oxidized oil influences the levels of type 2 T-helper cell-related antibody and inflammatory mediators in mice. Br J Nutr 2000;84:911-7.

53. Goode HF, Cowley HC, Walker BE, Howdle PD, Webster NR. Decreased antioxidant status and increased lipid peroxidation in patients with septic shock and secondary organ dysfunction. Crit Care Med 1995;23:646-651.

54. Matsuoka T. Glycation-dependent reactive oxygen species-mediated suppression of the insulin gene promoter activity in HIT cells. J Clin Invest 1997;99:144-150.

55. Spiteller G. Lipid peroxidation in aging and age-dependent diseases. Exp Gerontol 2001;36:1425-57.

56. Vincent HK, Taylor AG. Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans. Int J Obes (Lond) 2005.

57. Ozata M, Mergen M, Oktenli C, et al. Increased oxidative stress and hypozincemia in male obesity. Clin Biochem 2002;35:627-31.

58. Ohrvall M, Tengblad S, Vessby B. Lower tocopherol serum levels in subjects with abdominal adiposity. J Intern Med 1993;234:53-60.

59. Bakker SJ, RG IJ, Teerlink T, Westerhoff HV, Gans RO, Heine RJ. Cytosolic triglycerides and oxidative stress in central obesity: the missing link between excessive atherosclerosis, endothelial dysfunction, and beta-cell failure? Atherosclerosis 2000;148:17-21.

60. Maachi M, Pieroni L, Bruckert E, et al. Systemic low-grade inflammation is related to both circulating and adipose tissue TNF-alpha, leptin and IL-6 levels in obese women. Int J Obes Relat Metab Disord 2004;28:993-7.

61. Guzik TJ, West NE, Black E, et al. Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. Circ Res 2000;86:E85-90.

62. Podrez EA, Abu-Soud HM, Hazen SL. Myeloperoxidase-generated oxidants and atherosclerosis. Free Radic Biol Med 2000;28:1717-25.

63. Skrha J, Sindelka G, Kvasnicka J, Hilgertova J. Insulin action and fibrinolysis influenced by vitamin E in obese Type 2 diabetes mellitus. Diabetes Res Clin Pract 1999;44:27-33.

64. Keaney JF, Jr., Larson MG, Vasan RS, et al. Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham Study. Arterioscler Thromb Vasc Biol 2003;23:434-9.

65. Furukawa S, Fujita T, Shimabukuro M, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest 2004;114:1752-61.

66. Chaudiere J, Ferrari-Iliou R. Intracellular antioxidants: from chemical to biochemical mechanisms. Food Chem Toxicol 1999;37:949-962.

67. Hacquebard M, Carpentier YA. Vitamin E:absorption, plasma transport and cell uptake. Curr Opin Clin Nutr Metab Care 2005;8:133-138.

68. 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;59:1025-1032.

69. Clement M, Bourre JM. Graded dietary levels of RRR-gamma-tocopherol induce a marked increase in the concentrations of alpha- and gamma-tocopherol in nervous tissues, heart, liver and muscle of vitamin-E-deficient rats. Biochim Biophys Acta 1997;1334:173-181.

70. Jiang ZY, Woollard AC, Wolff SP. Lipid hydroperoxide measurement by oxidation of Fe2+ in the presence of xylenol orange. Comparison with the TBA assay and an iodometric method. Lipids 1991;26:853-6.

71. Hewavitharana AK, Lanari MC, Becu C. Simultaneous determination of vitamin E homologs in chicken meat by liquid chromatography with fluorescence detection. J Chromatogr A 2004;1025:313-7.

72. Brawley V, Bhatia J, Karp WB. Hydrogen peroxide generation in a model paediatric parenteral amino acid solution. Clin Sci (Lond) 1993;85:709-12.

73. Nourooz-Zadeh J, Tajaddini-Sarmadi J, Birlouez-Aragon I, Wolff SP. Measurement of hydroperoxides in edible oils using the ferrous oxidation in xylenol orange assay. J Agric Food Chem 1995;43:17-21.

74. Jiang ZY, Hunt JV, Wolff SP. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. Anal Biochem 1992;202:384-9.

75. Staprans I, Rapp JH, Pan XM, Kim KY, Feingold KR. Oxidized lipids in the diet are a source of oxidized lipid in chylomicrons of human serum. Arterioscler Thromb 1994;14:1900-5.

76. Wolff SP. Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. Methods Enzymol 1994;233:182-189.

77. Silvers KM, Darlow BA, Winterbourn CC. Lipid peroxide and hydrogen peroxide formation in parenteral nutrition solutions containing multivitamins. JPEN J Parenter Enteral Nutr 2001;25:14-7.

78. Wu GH, Jarstrand C, Nordenstrom J. Phagocyte-induced lipid peroxidation of different intravenous fat emulsions and counteractive effect of vitamin E. Nutrition 1999;15:359-64.

79. Udilova N, Jurek D, Marian B, Gille L, Schulte-Hermann R, Nohl H. Induction of lipid peroxidation in biomembranes by dietary oil components. Food Chem Toxicol 2003;41:1481-9.

80. Olusi S. Obesity is an independent risk factor for plasma lipid peroxidation and depletion of erythrocyte cytoprotectic enzymes in humans. Int J Obes (Lond) 2002;26:1159-1164.

81. Chang SP, Chen YH, Liu IM, Cheng JT. Increase of anti-oxidation by exercise in the liver of obese Zucker rats. Clin Exp Pharmacol Physiol 2004;31:506-511.

82. Tanner AE, Martin J, Thatcher CD, Saker KE. Nutritional amelioration of oxidative stress induced by obesity and acute weight loss. 2005 Nestle Purina Nutrition Forum. St. Louis, MO, USA, 2005.

83. Bendich A, Machlin LJ. Safety of oral intake of vitamin E. Am J Clin Nutr 1988;48:612-9.

84. Traber MG, Carpentier YA, Kayden HJ, Richelle M, Galeano NF, Deckelbaum RJ. Alterations in plasma alpha- and gamma-tocopherol concentrations in

response to intravenous infusion of lipid emulsions in humans. Metabolism 1993;42:701-9.

85. Takahashi O. Haemorrhagic toxicity of a large dose of alpha-, beta-, gamma- and delta-tocopherols, ubiquinone, beta-carotene, retinol acetate and L-ascorbic acid in the rat. Fd Chem Foxic 1995;33:121-128.

86. Sui SM, Draper HH. Metabolism of malondialdehyde in vivo and in vitro. Lipids 1982;17:349-355.

87. Stojiljkovic MP, Lopes HF, Zhang D, Morrow JD, Goodfriend TL, Egan BM. Increasing plasma fatty acids elevates F2-isoprostanes in humans: implications for the cardiovascular risk factor cluster. J Hypertens 2002;20:1215-21.

88. Riserus U, Vessby B, Arnlov J, Basu S. Effects of cis-9,trans-11 conjugated linoleic acid supplementation on insulin sensitivity, lipid peroxidation, and proinflammatory markers in obese men. Am J Clin Nutr 2004;80:279-83.

89. Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol 2004;142:231-55.

90. Dotan Y, Lichtenberg D, Pinchuk I. Lipid peroxidation cannot be used as a universal criterion of oxidative stress. Prog Lipid Res 2004;43:200-27.

91. Afzal M, Afzal A, Jones A, Armstrong D. A rapid method for the quantification of GSH and GSSG in biological samples. Methods Mol Biol 2002;186:117-22.

92. Mayne ST. Antioxidant nutrients and chronic disease: use of biomarkers of exposure and oxidative stress status in epidemiologic research. J Nutr 2003;133 Suppl 3:933S-940S.

93. Rose CS, Gyorgy P. Hemolysis with alloxan and alloxan-like compounds and the protective action of tocopherol. Blood 1950;5:1062-1074.

94. Brownlee NR, Huttner JJ, Panaganamala RV, Cornwell DG. Role of vitamin E in glutathione induced oxidant stress: methemoglobin, lipid peroxidation and hemolysis. J Lipid Res 1977;18:635-644.

95. Beltowski J, Wojcicka G, Gorny D, Marciniak A. The effect of dietary-induced obesity on lipid peroxidation, antioxidant enzymes and total plasma antioxidant capacity. J Physiol Pharmacol 2000;51:883-96.

96.   Wu G, Fang Y, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. J Nutr 2004;134:489-492.

97.   Jackson RC. Studies in the enzymology of glutathione metabolism in human erythrocytes. Biochem J 1969;111:309-15.

98.   Lyons J, Rauh-Pfeiffer A, Yu YM, et al. Blood glutathione synthesis rates in healthy adults receiving a sulfur amino acid-free diet. Proc Natl Acad Sci U S A 2000;97:5071-5076.

99.   Lu SC. Regulation of glutathione synthesis. Curr Top Cell Regul 2000;36:95-116.

100.   Smith JE. Relationship of in vivo erythrocyte glutathione flux to the oxidized glutathione transport system. J Lab Clin Med 1974;83:444-50.

101.   Costagliola C, Libondi T, Menzione M, Rinaldi E, Auricchio G. Vitamin E and red blood cell glutathione. Metabolism 1985;34:712-714.

102.   Splettstoesser WD, Schuff-Werner P. Oxidative stress in phagocytes - "The enemy within". Microsc Res Tech 2002;57:441-455.

103.   Droge W, Schulze-Osthoff K, Mihm S, et al. Functions of glutathione and glutathione disulfide in immunology and immunopathology. Faseb J 1994;8:1131-8.

104.   Tsuchiya M, Thompson DF, Suzuki YJ, Cross CE, Packer L. Superoxide formed from cigarette smoke impairs polymorphonuclear leukocyte active oxygen generation activity. Arch Biochem Biophys 1992;299:30-7.

105.   Roos D, Weening RS, Voetman AA. Protection of human neutrophils against oxidative damage. Agents Actions 1980;10:528-35.

106.   Spielberg SP, Boxer LA, Oliver JM, Allen JM, Schulman JD. Oxidative damage to neutrophils in glutathione synthetase deficiency. Br J Haematol 1979;42:215-23.

107.   Miller ER, 3rd, Pastor-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.

108.   Ashorobi RB, Williams PA. Indomethacin and alpha-tocopheral enhanced survival in endotoxic rats. Cent Afr J Med 1995;41:216-9.

109. Peck MD, Alexander JW. Survival in septic guinea pigs is influenced by vitamin E, but not by vitamin C in enteral diets. JPEN J Parenter Enteral Nutr 1991;15:433-6.

110. Bulger EM, Maier RV. An argument for vitamin E supplementation in the management of systemic inflammatory response syndrome. Shock 2003;19:99-103.

111. Packer L, Obermuller-Jevic UC. Vitamin E: and introduction. In: Packer L, Traber MG, Kraemer K, Frei B, eds. The antioxidant vitamins C and E. Champaign, IL: AOCS Press, 2002:133-151.

112. Rice-Evans C. Erythrocytes, oxygen radicals and cellular pathology. In: Dipak K, Walter,BE, ed. Oxygen radicals: systemic events and disease processes. Pratteln: Thur AG Offsetdruck, 1990:1-30.

Figure 1. Mechanism of lipid peroxidation (adapted from (17)).
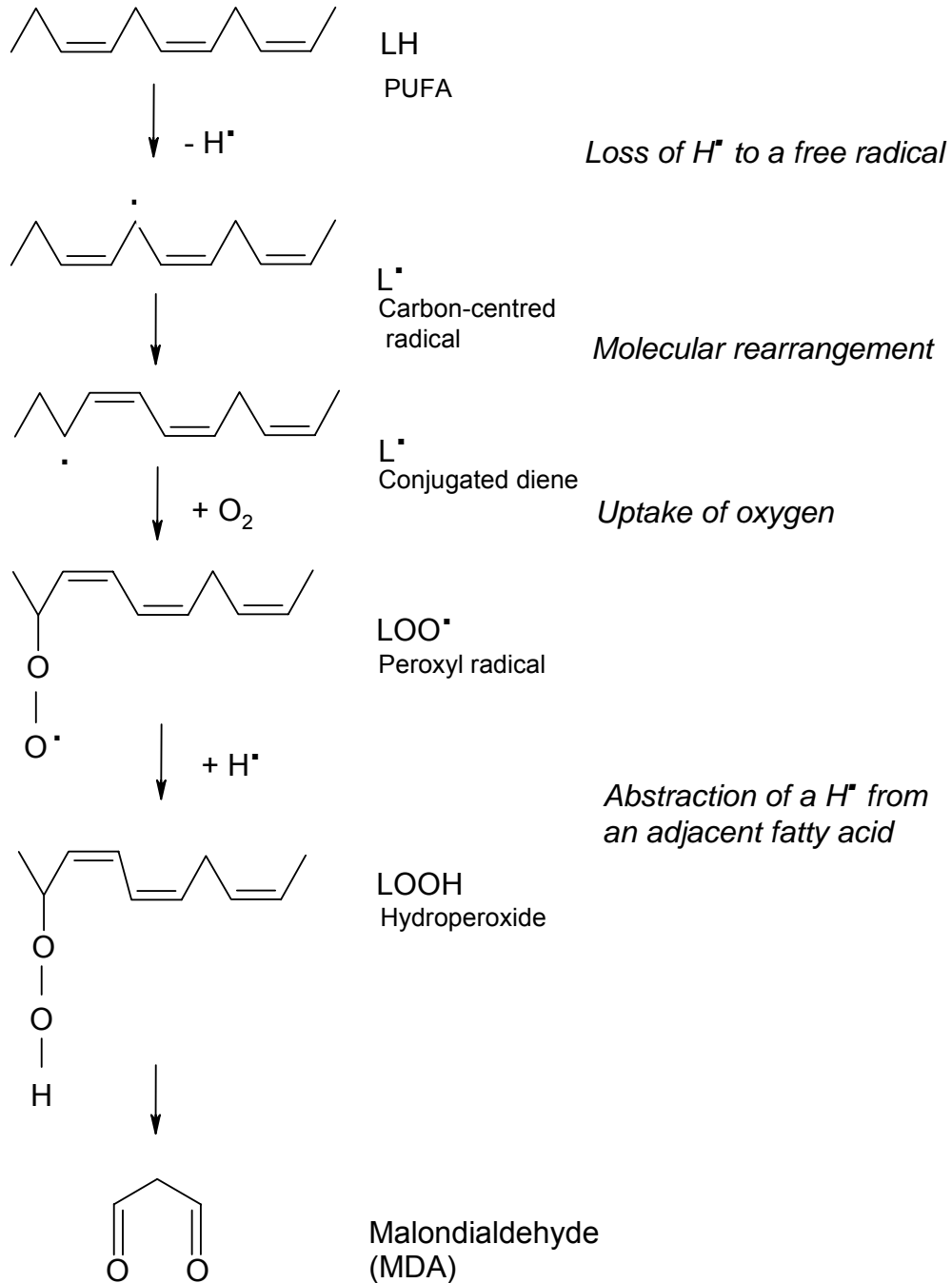
Figure 2. The structures of tocopherols and tocotrienols.
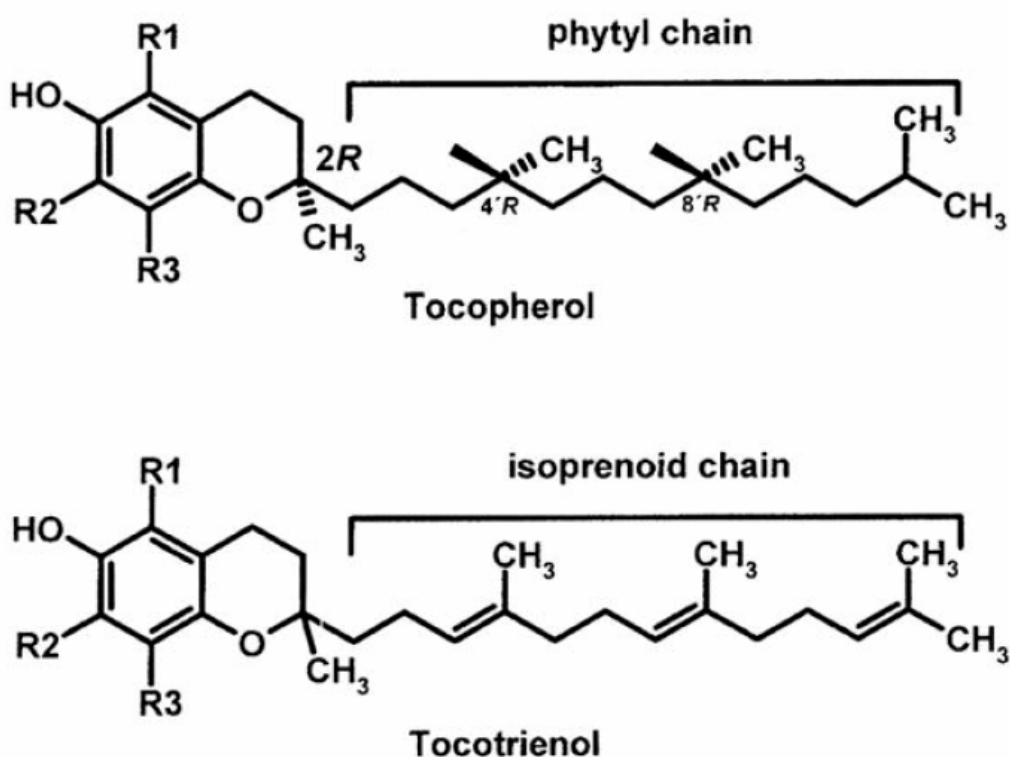
The four isoforms of tocopherols and tocotrienols:

α-:	$R_1 = CH_3$	$R_2 = CH_3$	$R_3 = CH_3$
β-:	$R_1 = CH_3$	$R_2 = H$	$R_3 = CH_3$
γ-:	$R_1 = H$	$R_2 = CH_3$	$R_3 = CH_3$
δ-:	$R_1 = H$	$R_2 = H$	$R_3 = CH_3$

The natural configuration of α-tocopherol is 2R, 4'R, 8'T. Important for the antioxidant activity is the hydroxyl group at C-6 of the chromanol ring which can donate its hydrogen atom to terminate the radical chain in the autoxidation reaction. The *in vitro* antioxidant activity is dependent on the substitution pattern of methyl groups at the aromatic ring. The side chain has no influence on the antioxidant activity. The biological activity of each isomer is mainly determined by its binding specificity to the α-tocopherol transfer protein.

Figure 3. Contribution of adiposity to oxidative stress (adapted from (50)).
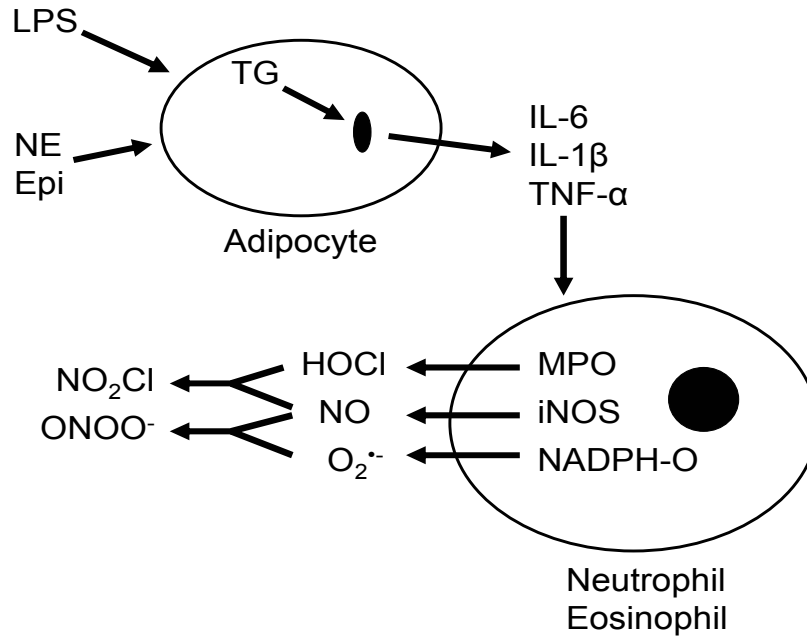
Adipocytes produce cytokines [interleukin (IL) and tumor necrosis factor-alpha (TNF-α) in response to lipopolysaccharides (LPS), catecholamines [norepinephrine (NE) and epinephrine (Epi)], or intracellular triglycerides (TG). Cytokines stimulate neutrophils and eosinophils to produce ROS/RNS. Enzymes that generate ROS/RNS in response to cytokines include myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate [NAD(P)H]-oxidase (NADPH-O). Peroxynitrite (ONOO$^-$) and nitryl chloride (NO$_2$Cl) may damage host tissues.

Figure 4. Co-operative interactions of vitamin E, reduced glutathione (GSH) and glutathione peroxidase (GPx), and their metabolic coupling to NADPH-generating pathways (adapted from(66)).
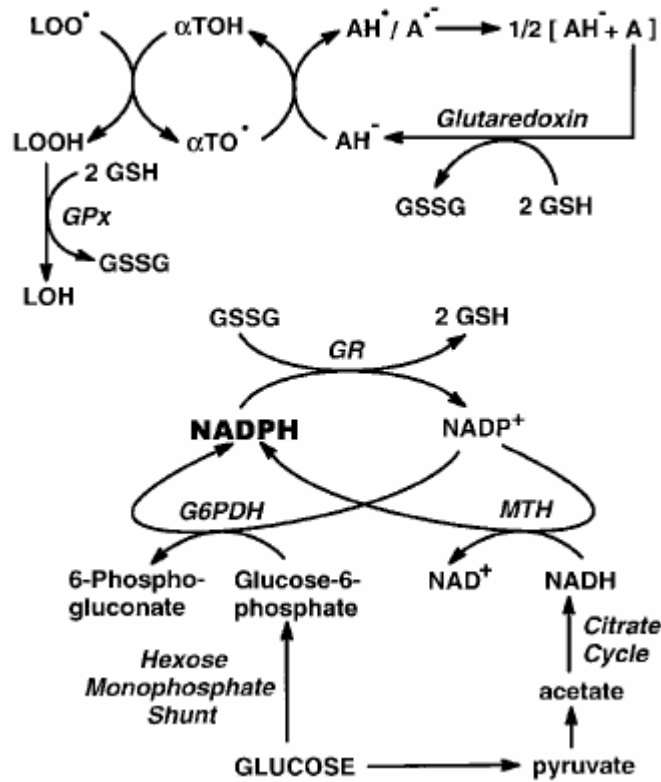
The peroxyl radical (α-TO$^\bullet$) produced upon scavenging of lipid peroxyl radicals (LOO$^\bullet$) is reduced back to α-tocopherol (α-TOH) by ascorbate (AH$^-$) at the membrane/water interface. This mechanism of free radical translocation takes advantage of the spontaneous dismutation of the ascorbyl radical (AH$^\bullet$ / A$^{\bullet-}$) into non-radical species. Hydroperoxide degradation by phospholipids hydroperoxide GPx or another glutathione peroxidase, and ascorbate regeneration by glutaredoxin both produce GSSG, which is reduced back to GSH by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent glutathione reductase. The antioxidant protection generally relies on cytosolic and mitochondrial pathways of NADPH production.

Figure 5. Absorption, transport, and metabolism of α-tocopherol (α-T) and γ-tocopherol (γ-T) in peripheral tissues of humans (adapted from (32)).
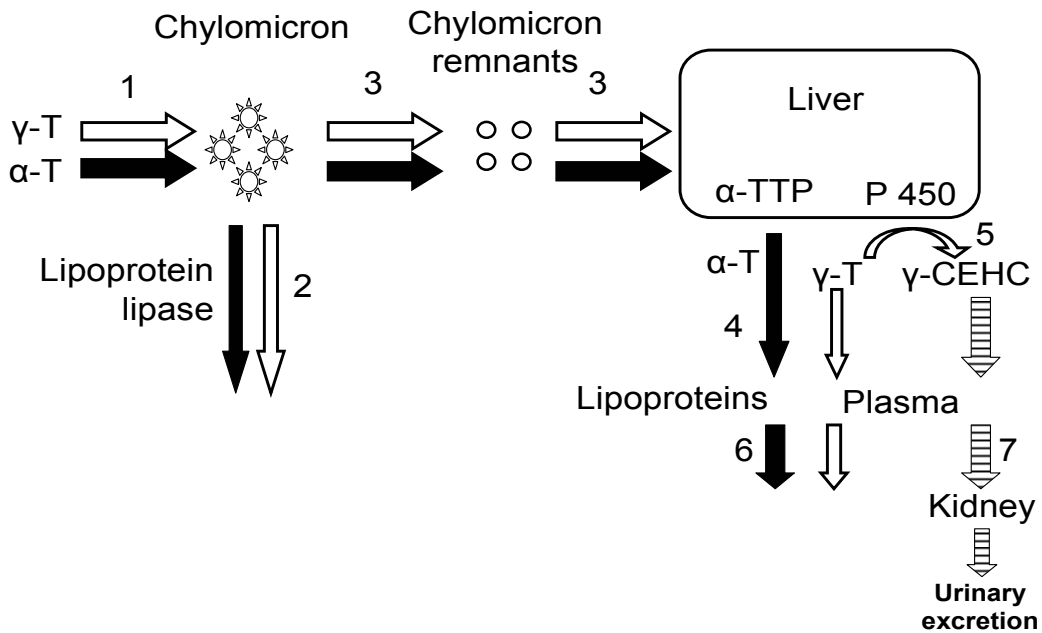
1) Both α-T and γ-T are similarly absorbed by the intestine along with dietary fat and are secreted into chylomicron particles. 2) Some of the chylomicron-bound vitamin E is transported to peripheral tissues with the aid of lipoprotein lipase. 3) the resulting chylomicron remnants are subsequently taken up by the liver. 4) In the liver, most of the remaining α-T but only a small fraction of γ-T is reincorporated into nascent VLDLs by α-tocopherol transfer protein (α-TTP). 5) Substantial amounts of γ-T are probably degraded by a cytochrome P450-mediated reaction to 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (γ-CEHC). 6) Plasma vitamin E is further delivered to tissues and organs by LDL and HDL. 7) γ-CEHC is excreted into urine.

Figure 6.   Effect of d-α-tocopherol on hydroperoxide concentrations in a HL:LD PA at 0 hours.
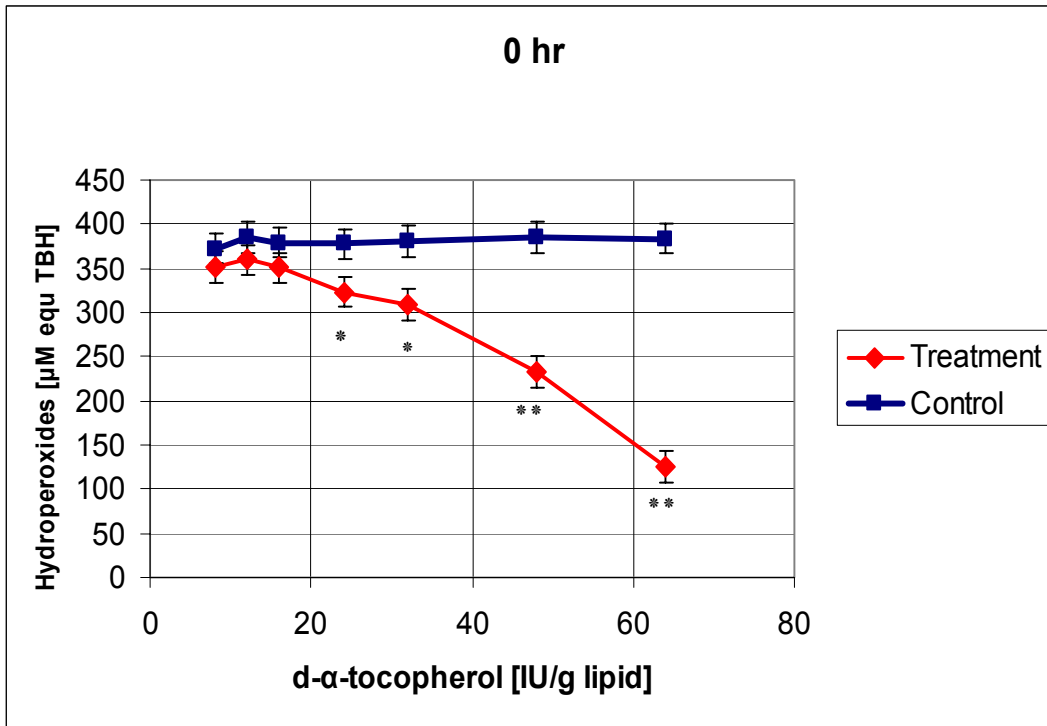
Hydroperoxides decreased immediately (0 hr) in treated bags (♦) as d-α-tocopherol increased.

Values are means ± SEM of three bags (n = 3).

Asterisks: significant differences between control (■) and treatment (♦) group (* P < 0.01; ** P < 0.0001).

**Figure 7.** Effect of d-α-tocopherol on hydroperoxide concentrations in HL:LD PA at 24 hours.
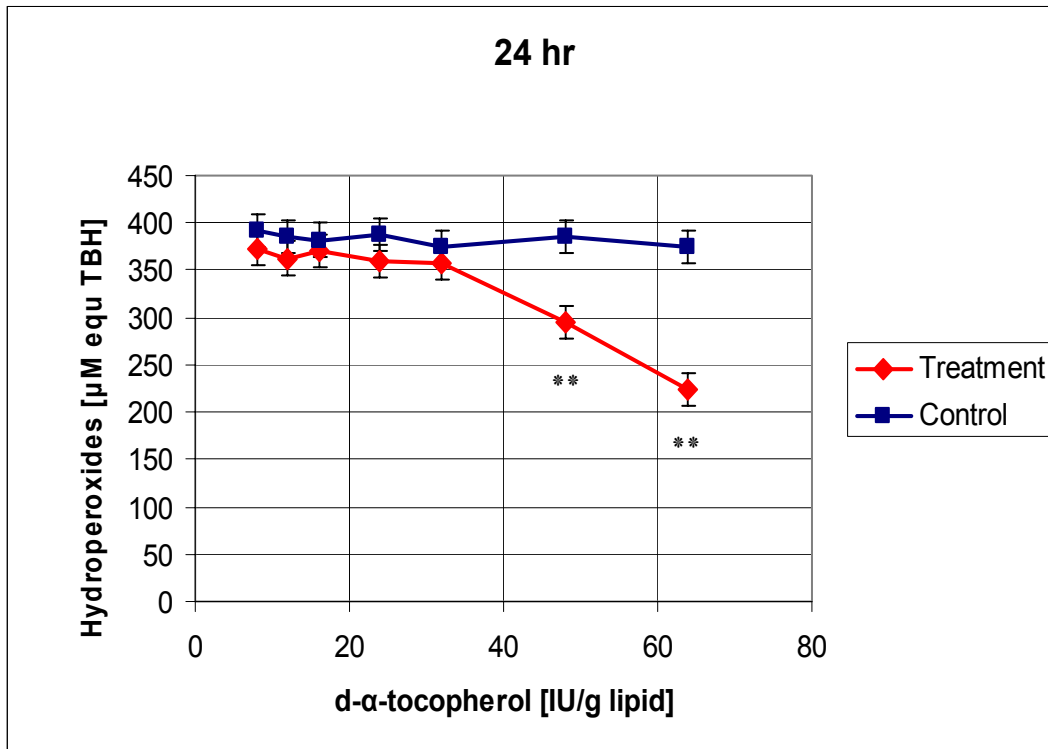
Higher concentrations of d-α-tocopherol were needed to lower hydroperoxide production in treated bags (♦) after 24 hr hang time.

Values are means ± SEM of three bags (n = 3).

Asterisks: significant differences between control (■) and treatment (♦) group (** P < 0.0001).

**Figure 8.** The intracellular antioxidant defenses of the erythrocyte (adapted from(112)).
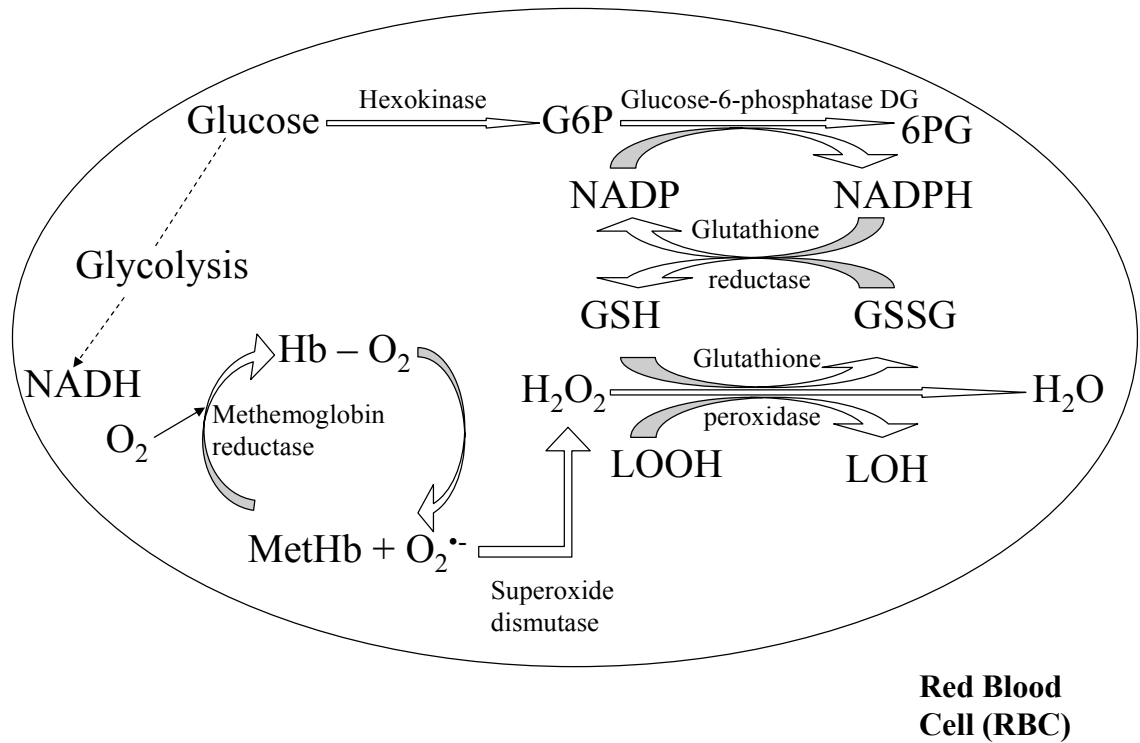
On autoxidation of hemoglobin, nicotinamide adenine dinucleotide (NADH)-dependent methemoglobin reductase converts the methemoglobin back to deoxyhemoglobin. Cu, Zn-superoxide dismutase catalyzes the reaction of the superoxide ($O_2^{\bullet-}$) formed from hemoglobin oxidation and dismutases it to hydrogen peroxide (H_2O_2). Reduced glutathione (GSH) and glutathione peroxidase (GPx) detoxify the H_2O_2. The cell is equipped with nicotinamide adenine dinucleotide phosphate (NADPH)-dependent glutathione reductase to re-convert the oxidize glutathione (GSSG) to GSH.

Figure 9.   Plasma malondialdehyde (MDA) concentration in obese cats given HL:LD
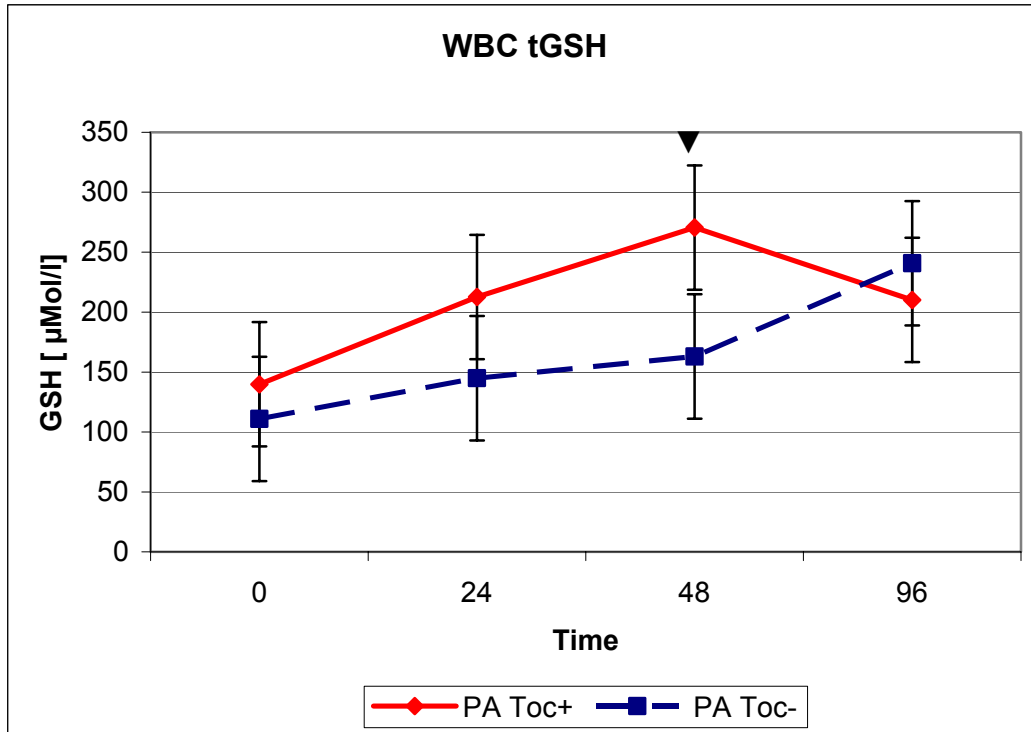PA supplemented or not with d-α-tocopherol.
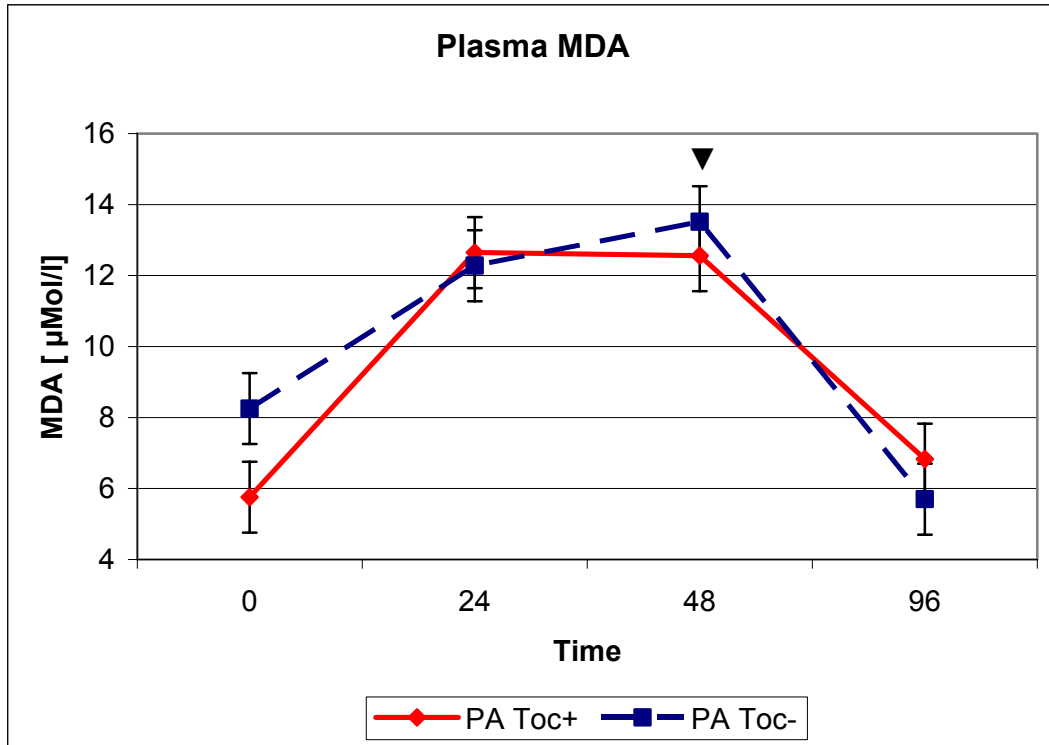
Effect of d-α-tocopherol on plasma malondialdehyde (MDA) concentrations in cats (n =
8 per group) receiving parenteral admixture not-supplemented with d-α-tocopherol (PA
Toc[-]: control group; interrupted line) and supplemented with 40 IU/g lipid d-α-tocopherol
(PA Toc[+]: treatment group; solid line). Plasma MDA increased from baseline to time 24
and 48 (P < 0.0001) in both groups. MDA concentrations were not different between
groups (treatment x time interaction; P = 0.08). The arrow (▼) indicates the time when
PA administration was discontinued. Values depict the means ± SEM of each sample
analyzed in triplicate.

Figure 10. White blood cell (WBC) glutathione peroxidase (GPx) activity in obese cats given HL:LD PA supplemented or not with d-α-tocopherol.
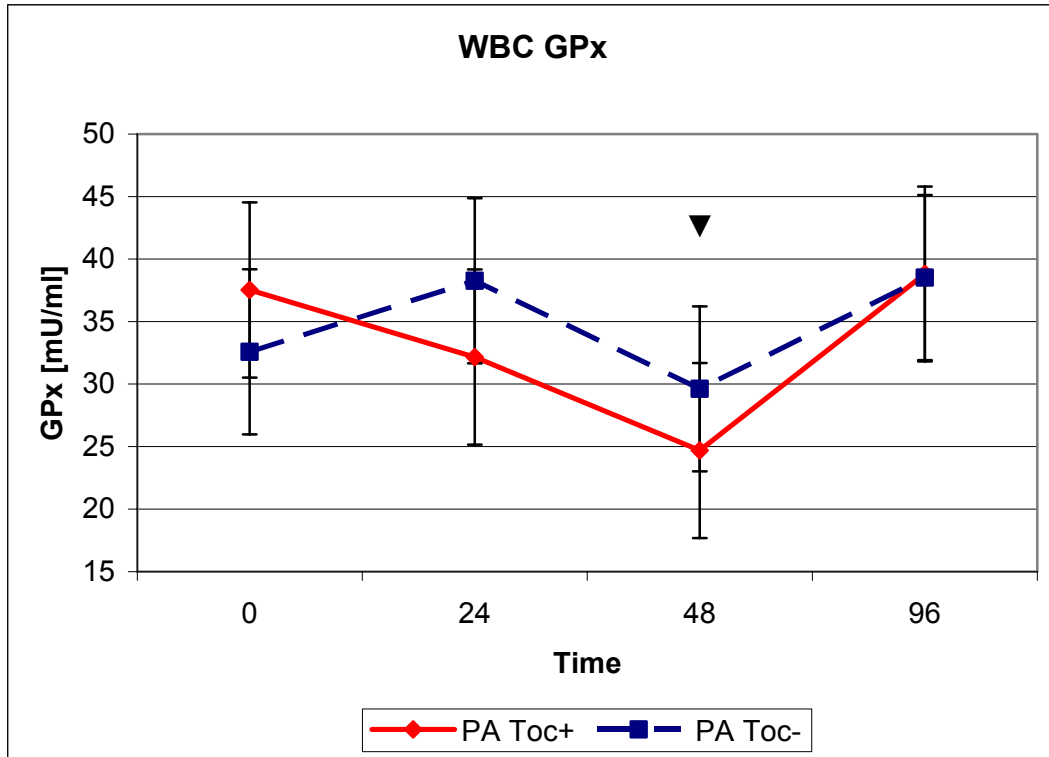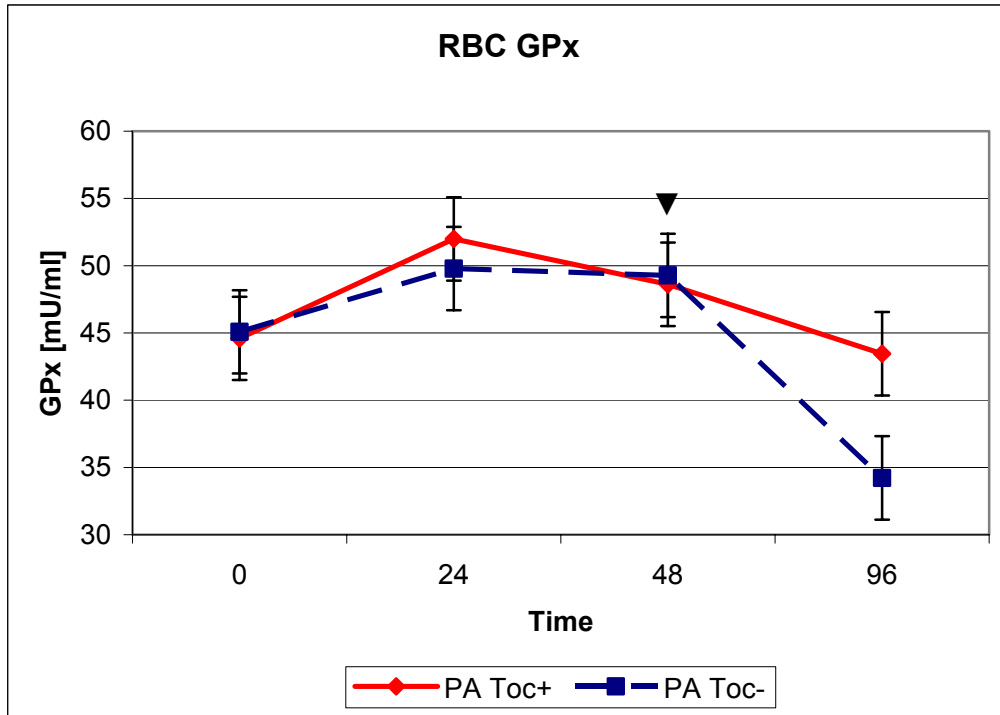

Effect of d-α-tocopherol on white blood cell (WBC) glutathione peroxidase (GPx) activity in cats (n = 8 per group) receiving parenteral admixture not-supplemented with d-α-tocopherol (PA Toc[-]: control group; interrupted line) and supplemented with 40 IU/g lipid d-α-tocopherol (PA Toc[+]: treatment group; solid line). There was no statistically significant difference between the groups and no change in enzyme activity over time. The arrow (▼) indicates the time when PA administration was discontinued. Values depict the means ± SEM of each sample analyzed in duplicate.

Figure 11. White blood cell (WBC) total glutathione (tGSH) concentration in obese cats given HL:LD PA supplemented or not with d-α-tocopherol.
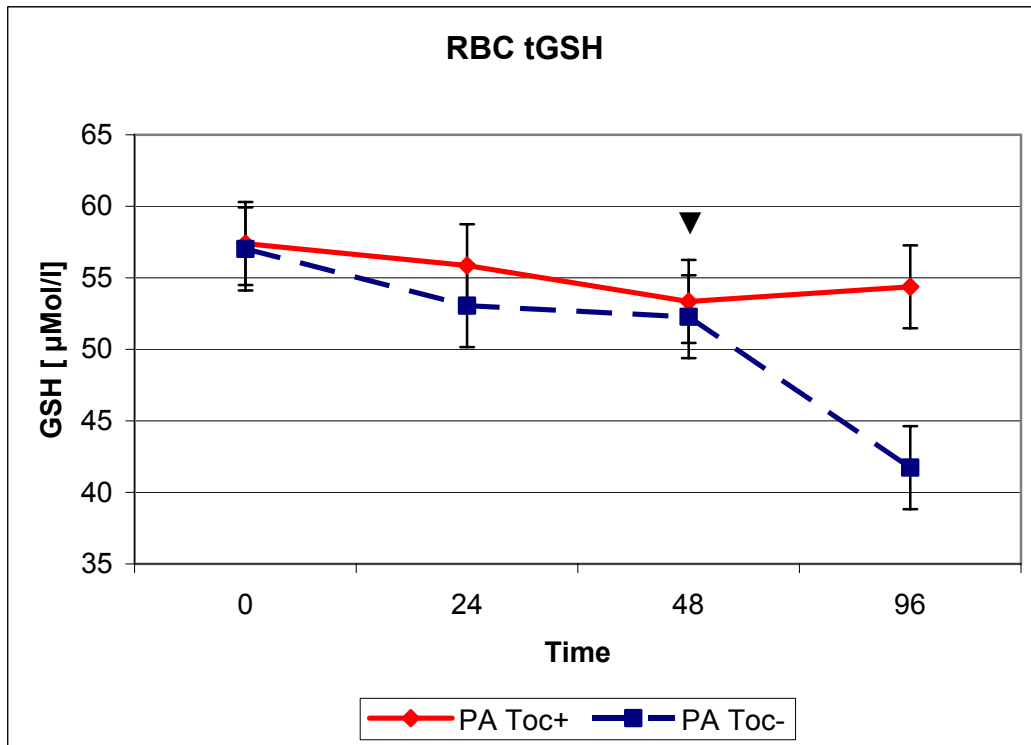

Effect of d-α-tocopherol on white blood cell (WBC) total glutathione (tGSH) concentration in cats (n = 8 per group) receiving parenteral admixture not-supplemented with d-α-tocopherol (PA Toc⁻: control group; interrupted line) and supplemented with 40 IU/g lipid d-α-tocopherol (PA Toc⁺: treatment group; solid line). The WBC-tGSH concentration tended to increase in the PA Toc⁺ group from baseline to time 24 and 48 (P = 0.07). There was no statistically significant difference in WBC-tGSH between the groups. The arrow (▼) indicates the time when PA administration was discontinued. Values depict the means ± SEM of each sample analyzed in duplicate.

Figure 12. Red blood cell (RBC) glutathione peroxidase (GPx) activity in obese cats given HL:LD PA supplemented or not with d-α-tocopherol.
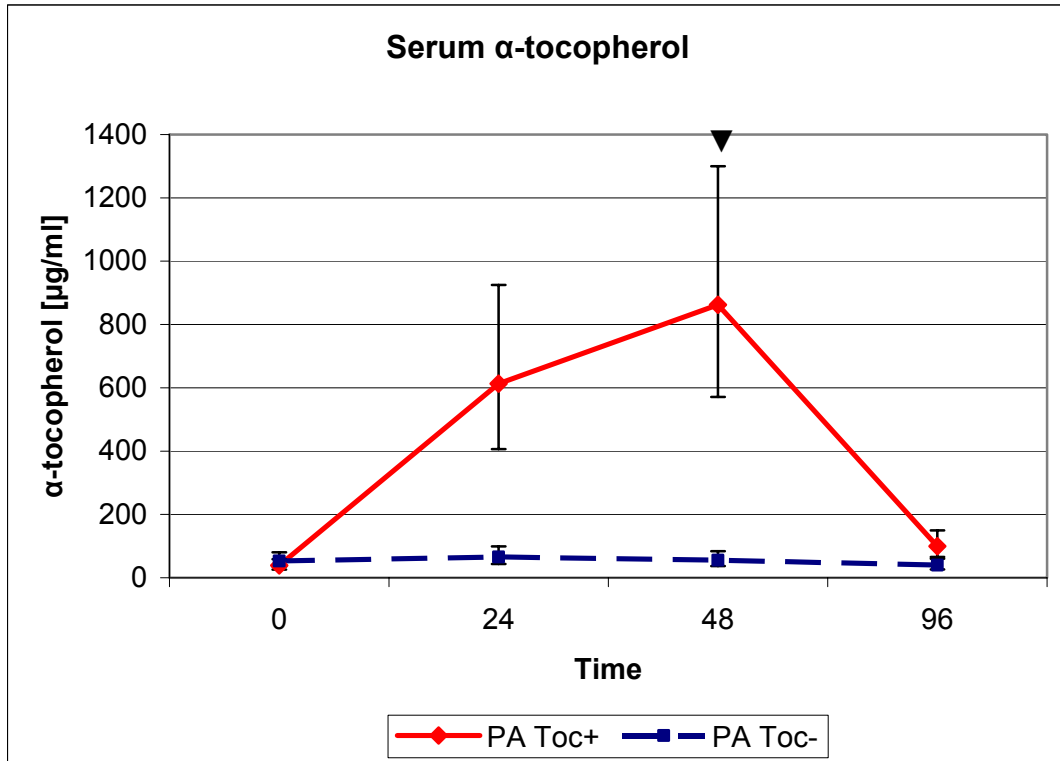

Effect of d-α-tocopherol on red blood cell (RBC) glutathione peroxidase (GPx) activity in cats (n = 8 per group) receiving parenteral admixture not-supplemented with d-α-tocopherol (PA Toc[-]: control group; interrupted line) and supplemented with 40 IU/g lipid d-α-tocopherol (PA Toc[+]: treatment group; solid line). The cats in both groups exhibited RBC-GPx activity changes during experiment (time effect; P = 0.0016). Overall RBC-GPx activity tended to increase in both groups from baseline to 24 and 48 hr (P = 0.06). The arrow (▼) indicates the time when PA administration was discontinued. Values depict the means ± SEM of each sample analyzed in duplicate.

Figure 13. Red blood cell (RBC) total glutathione (tGSH) concentration in obese cats given HL:LD PA supplemented or not with d-α-tocopherol.


Effect of d-α-tocopherol on red blood cell (RBC) total glutathione (tGSH) in cats (n = 8 per group) receiving parenteral admixture not-supplemented with d-α-tocopherol (PA Toc[-]: control group; interrupted line) and supplemented with 40 IU/g lipid d-α-tocopherol (PA Toc[+]: treatment group; solid line). The cats in PA Toc[+] group had higher RBC-tGSH concentration (treatment x time interaction; P = 0.012). The overall RBC-tGSH concentration decreased in both groups from baseline to 24 and 48 hr (P = 0.031). The RBC-tGSH was higher from 48 to 96 hr in the PA Toc[+] group (P = 0.004).The arrow (▼) indicates the time when PA administration was discontinued. Values depict the means ± SEM of each sample analyzed in duplicate.

Figure 14. Serum α-tocopherol concentration in obese cats given HL:LD PA
supplemented or not with d-α-tocopherol.

Effect of d-α-tocopherol on serum α-tocopherol concentrations in cats (n = 8 per group) receiving parenteral admixture not-supplemented with d-α-tocopherol (PA Toc[-]: control group; interrupted line) and supplemented with 40 IU/g lipid d-α-tocopherol (PA Toc[+]: treatment group; solid line). D-α-tocopherol supplementation significantly increased (P < 0.0001) serum α-tocopherol concentration in the PA Toc[+] group during parenteral admixture administration. The arrow (▼) indicates the time when PA administration was discontinued. Values depict the geometric means ± confidence intervals of each sample analyzed in duplicate.

Figure 15. Serum γ-tocopherol concentration in obese cats given HL:LD PA supplemented or not with d-α-tocopherol.
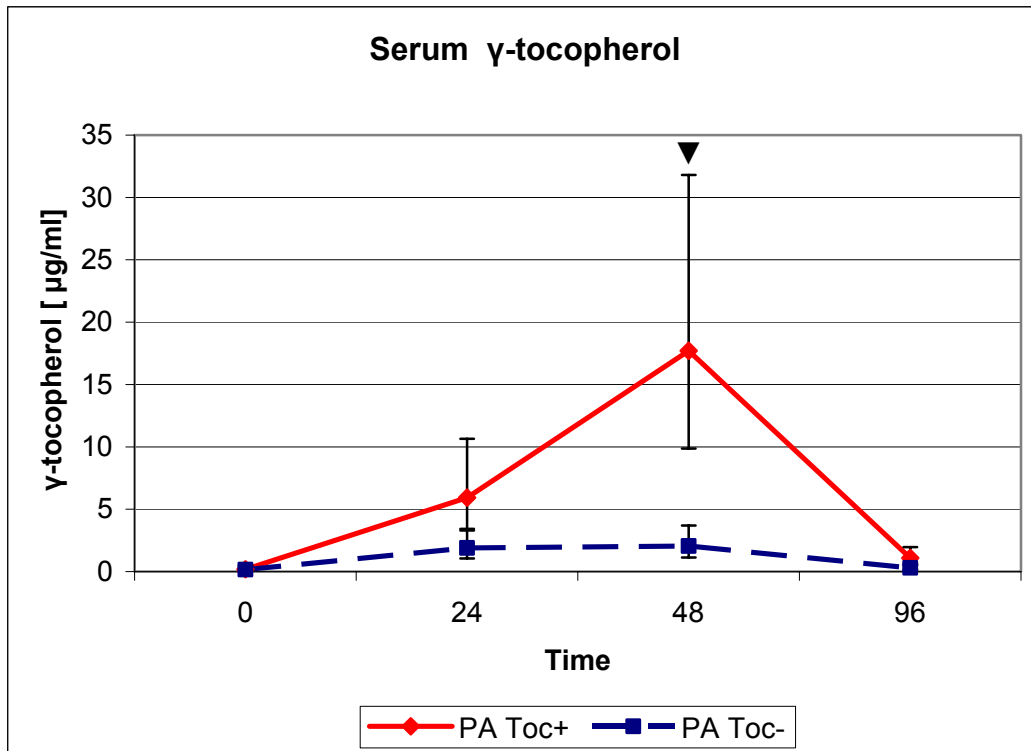
Effect of d-α-tocopherol on serum γ-tocopherol concentrations in cats receiving parenteral admixture not-supplemented with d-α-tocopherol (PA Toc[-] : control group; interrupted line) and supplemented with 40 IU/g lipid d-α-tocopherol (PA Toc[+]: treatment group; solid line). The cats in PA Toc[-] group exhibited significantly higher serum γ-tocopherol concentration relative to control group (treatment effect; $P < 0.0001$). The serum γ-tocopherol concentration increased from the baseline to 24 and 48 hr in both PA Toc[+] ($P < 0.0001$) and PA Toc[-] ($P < 0.0001$). The arrow (▼) indicates the time when PA administration was discontinued. Values depict the geometric means ± confidence intervals of each sample analyzed in duplicate.

Table 1. Composition of high lipid : low dextrose parenteral admixture (HL:LD PA) prior to d-α-tocopherol supplementation.

ITEM	CONCENTRATION IN THE PRODUCT[a]	TOTAL AMOUNT IN THE PA BAG
Amino acid solution without electrolytes	**10 g/100 mL**	**40 mL**
Sodium (Na^+)[b]	45.3 mEq/L	1.812 mEq
Lipid emulsion	**20 g/100 mL**	**40 mL**
Mg^{++}	0.008 mEq/L	0.0003 mEq
Ca^{++}	0.014 mEq/L	0.0006 mEq
Na^+	3.5 mEq/L	0.1400 mEq
K^+	0.87 mEq/L	0.0348 mEq
Cl^-	3.1 mEq/L	0.1240 mEq
Phosphorus (from phospholipids)	15 mM/L	0.6000 mM
α- tocopherol	approx 29 mg/L	1.16 mg
γ- tocopherol	approx 118 mg/L	4.72 mg
δ- tocopherol	approx 35 mg/L	1.40 mg
Glucose	**50 g/100 mL**	**11.7 mL**
Lactated Ringer's		**16.8 mL**
Na^+	130 mEq/L	2.184 mEq
Cl^-	109 mEq/L	1.831 mEq
K^+	4 mEq/L	0.067 mEq
Ca^{++}	2.7 mEq/L	0.045 mEq
Vitamin B Complex		**0.5 mL**
Thiamine Hydrochloride (B1)	12.5 mg/mL	6.25 mg
Niacinamide	12.5 mg/mL	6.25 mg
Pyridoxine Hydrochloride (B6)	5 mg/mL	2.5 mg
d-Panthenol	5 mg/mL	2.5 mg
Riboflavin (B2)	2 mg/mL	1 mg
Cyanocobalamin (B12)	5 μg/mL	2.5 μg
Total volume		**109 mL**
Calories Provided		**116 kcal**

[a] These values were obtained from product labels; see text for manufacturer information.
[b] Includes Na^+ from the antioxidant, sodium hydrosulfite and pH adjustor.

Table 2. Concentration of α-tocopherol[a] (α-T) and γ-tocopherol[a] (γ-T) measured by HPLC in HL:LD PA treated with d-α-tocopherol[b].

d-α-tocopherol added [IU/g lipid][c]	α-T [μg/mL] 0 hr	α-T [μg/mL] 24 hr	P value	γ-T [μg/mL] 0 hr	γ-T [μg/mL] 24 hr	P value
8	326.7	142.5	< 0.0001	29.3	29.7	= 0.9234
12	511.3	302.9	= 0.011	31.6	33.3	= 0.2834
16	556.9	386.5	= 0.011	30.0	34.6	= 0.6278
24	781.6	737.6	= 0.671	29.3	33.8	= 0.1898
32	1307.0	923.9	= 0.032	34.9	34.7	= 0.9593
48	2068.1	1429.7	= 0.029	37.6	31.8	= 0.0994
64	2096.7	2022.1	= 0.840	31.1	32.9	= 0.5604

Statistical significance was defined as $P < 0.05$.
[a] Values depict the means of three bags ($n = 3$).
[b] d-α-tocopherol was supplied as a commercial product Vital E – 300.
[c] IU, international units; 1 mg d-α-tocopherol equivalent corresponds to 1.49 IU.

Table 3. Concentration of hydroperoxides in newly opened lipid emulsions (20% Intralipid).[a]

Lipid emulsion	1 Glass bottle	2 Glass bottle	3 PVC bag	4 PVC bag	5 PVC bag	6 PVC bag
Peroxides [μM equ TBH]	276.8	313.7	262.4	310.6	449.4	270.9

[a] Values depict the means of one sample per bottle or PVC bag analyzed in duplicate.

VITA

Iveta Becvarova was born on March 20, 1975 in Velke Mezirici, Czech Republic. In June 1999, she graduated from University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic with doctor of veterinary medicine degree. She began to work in a small animal clinical practice from August 1999 till May 2003. In June 2003, Iveta began work on her combined Residency/Master of Science degree in the Department of Large Animal Clinical Science at Virginia-Maryland Regional College of Veterinary Medicine. Her research interests focus on clinical nutrition with an emphasis on oxidative stress in critically ill small animal patients.