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Lindsay Fitz Hendey
University of Dayton

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THE EFFECT OF AGE AND DIET ON IMMUNE FUNCTION IN FELINES

Thesis

Submitted to

The College of Arts and Sciences of the

UNIVERSITY OF DAYTON

In Partial Fulfillment of the Requirements for

The Degree

Master of Science in Biology

By

Lindsay Fitz Hendey

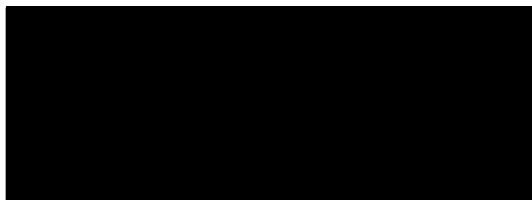
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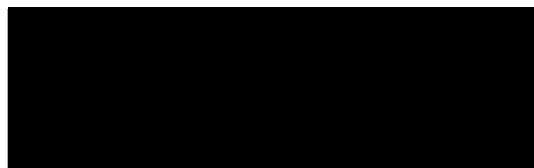
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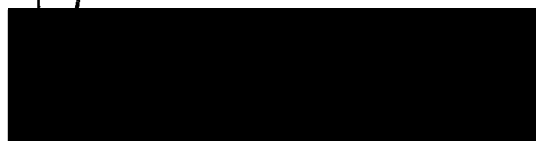
APPROVED BY:



Robert J. Kearns, Ph.D., Thesis Advisor



John J. Rowe, Ph.D., Department Chair, Faculty Reader



Marie-Claude Hofmann, Ph.D., Faculty Reader

ABSTRACT

THE EFFECT OF THIOMAXTM ON IMMUNE FUNCTION IN YOUNG AND AGED FELINES

Hendey, Lindsay Fitz
University of Dayton

Advisor: Dr. Robert J. Kearns

Aging is associated with several physiological changes. An important physiological change seen in aging is a shift in the balance between oxidants and antioxidants, in which increased oxidants, or free radicals, seen with aging lead to increased cellular damage, also called oxidative stress. Immune function of elderly individuals seems to be significantly impacted through a decline of immunologic function as well as dysregulation of inflammatory and specific immune responses. Oxidative stress and the accumulation of free radicals have been implicated in the decline of immune function seen in the aged. Antioxidants, both intracellular and exogenous, counteract free radical damage to cells through the inactivation of free radicals. Through their free radical inactivation and scavenging capabilities, dietary antioxidants have been shown to ameliorate several age-related disease states associated with the accumulation of free radicals and improve immune function in the elderly.

The objective of this study was to determine whether the antioxidant cocktail ThiomaxTM had any effect on immune function, through measurement of mitogen-induced lymphocyte proliferation, lymphocyte subsets, antibody production, and prostaglandin E₂ (PGE₂) production in young and old felines. Similarly, the effects of

ThiomasTM on glutathione status, and oxidative stress, as measured by serum lipid peroxidation, were measured in these animals. Twenty young (2.68-2.75 years) and aged (9.12-16.22 years) felines were maintained on a control or ThiomasTM diet throughout the treatment period. Aged felines exhibited a significant decline in mitogen-induced lymphocyte proliferation, CD3+ and CD8+ T cell subsets, and levels of the intracellular glutathione, whereas IgM production was significantly greater compared to young felines. Conversely, PGE₂ and lipid peroxidation levels were significantly lower in aged compared to young felines. However, ThiomasTM supplementation did not significantly impact any of the parameters measured in either young or aged felines. Further studies need to be conducted with optimal doses of ThiomasTM to further test its effect on immune function in young and aged felines.

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LITERATURE REVIEW

While several theories of aging have been proposed, a great amount of attention has been focused on the free radical theory of aging, supported by increasing amounts of evidence in recent years. This theory is based on the age related shift in the balance of oxidants and antioxidants, both intracellular such as glutathione or dietary such as vitamin E and carotenoids, in which increased oxidants in the aged individual leads to increased cellular damage (1, 2). This increase in cellular damage, or oxidative stress, is observed later in life through deterioration of several cellular processes and oxidative stress has also been implicated in several disease states. Immune function of elderly individuals seems to be significantly impacted through a decline of immunologic function as well as dysregulation of inflammatory and specific immune responses (3, 4). Several aspects of elderly immune function are impacted including cell-mediated and humoral immunity, as well as the production and secretion of cytokines. This impact on the elderly immune system seems to be due, but not limited, to oxidative stress on cells of the immune system by oxidants, or free radicals (5). While much research has been centered on the cause of the general decline of the elderly immune system and the accumulation of free radicals in aged individuals, several researchers are focusing on the effects of antioxidants and the possibility of using these to reverse or restore some of the damaging effects of oxidative stress.

The production of free radicals occurs as a normal function of aerobic metabolism occurring in the mitochondria (2). There are several biological sources of free radicals including the lipooxygenase and cyclooxygenase pathways in the plasma membrane,

which produce leukotrienes and prostaglandins, respectively, electron transport and ubiquinone in the mitochondria, as well as the microsomal membranes of the endoplasmic reticulum (6). Phagocytic cells, such as macrophages and neutrophils, also produce free radicals in what is called the “respiratory burst”. This respiratory burst in phagocytes is defined by an increased intake of oxygen, which is converted to toxic byproducts with potent antimicrobial activity (7, 8). These free radicals include several molecules such as reactive oxygen and nitrogen intermediates. These molecules include superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (OH^{\bullet}), hydrogen peroxide (H_2O_2), hypochlorous acid ($HOCl$), nitric oxide (NO), and peroxynitrite ($ONOO^-$) (7, 8). Free radicals are deleterious to several cellular processes; one being through lipid peroxidation, or autoxidation. Lipid peroxidation is accomplished through the production of a free radical, which reacts with a lipid, thereby oxidizing it (9). This process is described as a chain reaction since oxidation of the lipid results in the production of a subsequent radical (9). The first lipid oxidation event can produce an alkyl radical from the lipid molecule itself, through removal of hydrogen, and the alkyl radical can react with molecular oxygen to form lipid peroxyl radicals (LOO^{\bullet}) (6). Therefore, subsequent radicals produced by the first oxidation of lipids can propagate the oxidation of other lipids in the membrane, possibly resulting in detrimental effects to the cell membrane (9). Lipid peroxidation is detrimental to cells since the autoxidation of the lipids in the cell membrane disrupts the fluidity and compromises the integrity of the membrane. Cells of the immune system are particularly susceptible to membrane damage through free radical attack since their membranes have a high percentage of polyunsaturated fatty acids (10). Cells such as macrophages and neutrophils can incur increased oxidative damage during

the respiratory burst, potentially causing harm to them as well as invading microorganisms (8). Signal transduction can also be affected by lipid peroxidation of the cell membrane through increased rigidity of the membrane (11). Additionally, aging is associated with an increase in membrane rigidity (6). There is also an age associated increase in membrane damage due to oxidative stress. A study by Hayashi and Miyazawa (12) demonstrated a significant increase in lipid hydroperoxides, a by-product of lipid peroxidation and an indicator of oxidative stress, in aged rat hepatocyte plasma membranes as compared to those in young rats.

Oxidatively damaged proteins also accumulate in the cells of aged individuals, possibly leading to loss of cellular and biochemical function (11, 13). Alterations in the proteolytic capabilities of aged cells has also been observed; compromising their ability to degrade altered and inappropriately folded proteins and contributing to the accumulation of oxidatively damaged proteins (14). As a consequence of free radical attack on the cell membrane, normal metabolic production of free radicals, and exposure to environmental factors, DNA experiences a heavy dose of oxidative lesions. This can lead to mutations and DNA damage, and although there are several DNA repair mechanisms in the cell, as a person or animal ages these lesions accumulate faster than can be repaired (2). Cells which can remain in a quiescent state for years, such as lymphocytes, are more susceptible to DNA damage by free radicals, and studies suggest an accumulation of DNA damage in lymphocytes with aging (15). Since mitochondria are the site of oxidative phosphorylation in eukaryotes and a major source of free radicals, mitochondrial DNA is subject to a much higher level of oxidative damage than experienced by nuclear DNA (11). In addition to the DNA damage caused by oxidative

stress in the mitochondria, organelles also experience significant oxidative damage to membrane lipids and cellular proteins, all of which seem to contribute to the decline in mitochondrial function seen in the aged (11). This decline in mitochondrial function, commensurate with a decline in energy production, resulting from oxidative stress has been hypothesized to contribute the process of aging (11).

The accumulation of free radicals has been associated with several disease states including cardiovascular disease, cancer, and viral infections. Cardiovascular disease (CVD) is a major cause of morbidity among the aging (16). Atherosclerosis is a component of CVD and is associated with chronic inflammation of the arterial walls as well as an accumulation of plaque in these arteries (17). Oxidative stress, or an accumulation of free radicals, has been implicated in the pathogenesis of CVD. Following injury to arteries, either bacterial, viral, or physical, endothelial cells of the artery release chemotactic molecules, which recruit monocytes to the area of injury and initiate an inflammatory response (17). In addition to the recruitment of monocytes, arterial endothelial cells increase the uptake and modification of lipids from blood; specifically, there is an increased uptake of low-density lipoproteins (LDLs), which deliver cholesterol to cells (16). Free radicals produced by activated monocytes/macrophages and arterial endothelial cells oxidize the LDLs leading to the formation of plaque in the arterial wall, or atherosclerosis (18).

Cancer is seen with increased frequency in the aged (17). Oxidative stress has been implicated in the pathogenesis of cancer since free radicals attack DNA leading to strand breaks and base and nucleotide damage (19). DNA damage caused by free radical attack can initiate mutagenesis of genes, leading to carcinogenesis and possibly loss of

tumor suppressor genes (17). Higher levels of free radicals have also been associated with certain types of cancer. In a study performed by Ray et al. (19), women with breast cancer had significantly higher levels of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet -}$) than control women. It could be hypothesized that the increased production of free radicals in cancer could compound the damage incurred during this disease.

Viral infections such as pneumonia, influenza, and the human immunodeficiency virus (HIV) pose a major health threat to the elderly and the population as a whole. The aged-associated decline in immune function increases the susceptibility of elderly individuals to these infections, which are a chief component of morbidity in the elderly (20, 21). An increased production of free radicals is associated with viral infections, in part due to the respiratory burst of phagocytes in response to infection (22, 23). In mice infected with the influenza virus, increased H_2O_2 concentration was detected in the lungs (21). Oxidative stress is also documented in HIV infections. Allard et al. (23) found increased oxidative stress in HIV infected humans, as measured by increased lipid peroxidation, compared to non-infected individuals. The production of free radicals has also been associated with several aspects of a viral infection. Lung damage seen in influenza infections may be attributable to the effects of free radicals on these tissues (21). Free radicals may also contribute to successful viral replication. Hydrogen peroxide (H_2O_2) has been shown *in vitro* to enhance HIV replication (22, 23). A cascade of events following the expression of influenza hemagglutinin (HA) is suggested to increase the production of free radicals (24). The increased production of free radicals and subsequent oxidative stress in viral infections can be particularly detrimental to

elderly individuals already experiencing elevated oxidative stress, increased susceptibility to infections, and decreased immune function.

Additionally, the accumulation of free radicals may also contribute to alterations in immune function. The process of aging is associated with a decline in immune function leading to increased susceptibility to infections (4). Dysregulation of the immune and inflammatory responses, also seen in aged individuals, can lead to autoimmune and chronic inflammatory diseases such as multiple sclerosis and systemic lupus (4). Free radicals can impact several aspects of the immune system including cell-mediated immunity, humoral immunity, as well as secretion of cytokines and inflammatory mediators.

Cell-mediated immunity is the branch of the immune system dealing primarily with the elimination of intracellular pathogens and altered self-cells. Helper T cells (T_H cells) and cytotoxic T lymphocytes (CTLs) characterized by the membrane marker $CD4^+$ and $CD8^+$, respectively, are the effector cells generated during a cell-mediated immune response (25, 26). T_H cells secrete various cytokines that activate phagocytic cells for pathogenic clearance and CTLs mediate killing of altered-self cells, such as tumors and virally infected cells (26).

Cell-mediated immunity is impacted in several ways in the aged. For example, in the aged there seems to be an aged related shift in lymphocyte subsets. Aged individuals have a higher proportion of memory (antigen experienced) T lymphocytes compared to naïve (antigen inexperienced) T lymphocytes (27). Nagel et al. (28) reported a significant decrease in cytotoxic T lymphocytes ($CD8^+$) in elderly humans as compared to young subjects, while there was no significant difference in helper T ($CD4^+$) cells.

Another study found a significant decrease in lymphocytes expressing the membrane marker CD3⁺, which represents the total mature T cell population, in the elderly, which may be reflected in the decrease of CD8⁺ T lymphocytes (28). There seems to be conflicting evidence as to whether there is a reduction in the total T cell population in the elderly, however, results confirm an aged-related shift of increased memory T cells and decreased naïve T cells. This trend may be partially explained by thymic atrophy. The thymus is the lymphoid organ in which T cell maturation occurs, and even starting early in life thymic function and size decrease (29). Therefore, less mature naïve T lymphocytes may leave the thymus in older individuals (30), supported by evidence of decreased naïve T cell values in the elderly. This age-related decline in production of naïve T cells may contribute to the loss of immunologic vigor in response to new or rarely encountered antigens (27).

The proliferative response of lymphocytes to mitogenic and antigenic stimulation is also depressed in the aged (31, 32). Proliferation of splenic murine lymphocytes stimulated with concanavalin A (ConA), a known T cell mitogen, was significantly decreased in aged animals when compared to young animals (31). Additionally, other studies have shown aged splenic murine T lymphocytes showed a reduced proliferative response to mitogenic stimulation (27, 33). Isolated peripheral blood mononuclear cells (PBMCs) from elderly human subjects stimulated with the T cell mitogens Con A and phytohemagglutinin (PHA) showed decreased ³H-thymidine incorporation (34). Elderly humans also had a decreased response to and production of the cytokine interleukin 2 (IL-2), which has been shown to control mitogen-induced proliferation in lymphocytes, when compared with young subjects (35). While elderly human and animal T

lymphocytes exhibit decreased proliferative response to non-specific stimulation (mitogens), these cells also show decreased proliferation in response to stimulation with specific antigens (32). In a study examining the immune response to the influenza vaccine, Gardner et al. (36) found elderly human subjects had a significantly reduced proliferative response to stimulation with a trivalent influenza vaccine compared to young subjects, both pre- and post-immunization with the same trivalent influenza vaccine. Delayed-type hypersensitivity (DTH), an *in vivo* correlate of cell-mediated immunity, has also been shown to decrease in the aged (37).

Free radicals have been implicated in a decrease in cell-mediated immunity in the elderly. In an experiment conducted by Pahlavani and Harris, young and aged exposed to H_2O_2 and xanthine-xanthine oxidase (X/XO) showed reduced proliferation in response to Con A stimulation compared to controls (31). The increased susceptibility of lymphocytes, because of their high membrane lipid content, to free radical attack can lead to damage of cellular receptors important to proliferation (3). The experimental evidence demonstrating the diminished cell-mediated immune function in elderly humans and animals and the damaging role of free radicals elucidates one factor in the decline of immune function in the elderly.

Humoral immunity is characterized by the interaction of B cells with antigen resulting in the activation, proliferation, and differentiation into effector plasma cells, which secrete antibodies (38). The development of an effective humoral immune response involves the synergism of helper T cells (T_H cells, $CD4^+$), cytokines secreted from these activated T_H cells, and the subsequent activation of a population of B cells to secrete antibodies and produce memory B cells (25). Humoral immunity does not seem

to be affected as significantly as cell-mediated immunity in the aged, but there are several age related changes (39). The percentage of memory B cells in the elderly is significantly lower than those in young individuals (32). Memory B cells are critical to mount a rapid and effective humoral immune response, and this decline of memory B cells seen in the aged could be implicated in the increased susceptibility to bacterial and viral infections in the aged. Additionally, the response to vaccines is depressed in the elderly (36). This decreased humoral immune response to vaccines is distinguished by a lower antibody response and an increased lag in activation of the response compared to younger individuals (39). An antibody response is considered protective if antibody titers are ≥ 40 after vaccination, and there is a distinct decrease in protective and intact antibody responses to vaccines, specifically to the trivalent influenza vaccine, in the elderly (36).

In the aged immune system, a major component of dysregulation is seen in the inappropriate secretion of cytokines and inflammatory mediators (40). There also seems to be a shift in the cytokine profile in aged individuals, leading to dysregulation of T_H1 and T_H2 lymphocyte subset function (20). Cytokines produced by T_H1 cells are involved in cell-mediated immunity and include IL-2 and interferon γ (IFN- γ), while cytokines produced by T_H2 cells are involved primarily in B cell activation and include interleukin 4 (IL-4) and interleukin 6 (IL-6) (41). IL-6, tumor necrosis factor α (TNF- α), and IFN- γ , important inflammatory cytokines, have also been reported to increase with age. IL-6 is involved in T cell activation, B cell differentiation, acute phase responses and TNF- α mediates inflammation, fever responses, and direct cytotoxicity to altered self cells (24, 42). IFN- γ inhibits viral replication, activates macrophages, and is involved in immunoglobulin class switching (24, 42). IL-6 and TNF- α levels detected in the serum

of unstimulated control mice were significantly higher in old mice compared to young mice (43). Moreover, stimulated elderly human T cells ($CD3^+$) expressed significantly elevated levels of $TNF-\alpha$ and $IFN-\gamma$ than young subjects (44). Macrophages are a major source of IL-6 and in normal, healthy individuals IL-6 is not detectable in the serum, however IL-6 is detectable in older individuals (40). A study by Beharka et al. found that detectable levels of IL-6 were produced by unstimulated aged murine macrophages in culture (4). Additionally, significantly higher levels of IL-6 were detected in supernatants of cultured elderly human peripheral blood mononuclear cells (PBMCs) than in young subjects (45). Elevated levels of IL-6 and $TNF-\alpha$ have been associated with several disease states including human and canine rheumatoid arthritis (46). However, there does seem to be some discrepancy as to whether there is a spontaneous, i.e. unstimulated and therefore dysregulated, increase in these cytokines in the aged compared to young individuals. Beharka et al. (40) reported no age-related increase in IL-6 production in human PBMCs, either unstimulated or stimulated with the T cell mitogens Con A and PHA (40). Others have suggested that the increase in inflammatory cytokines such as IL-6 and $TNF-\alpha$ may be due to age-related diseases and health status in the elderly, which were not monitored as closely in previous studies using aged individuals (24, 40, 45).

Interleukin 2 (IL-2) is an important cytokine involved in the clonal expansion of T cell populations (27). One unequivocal finding in the investigation of the aging immune system is the decreased production of IL-2 in the aged compared to young subjects. Several studies have found a significant decrease in IL-2 production in stimulated culture supernatants obtained from aged mouse and rat splenic lymphocytes (3, 27, 31), elderly human PBMCs (35) and serum (45) compared to young subjects. Because of the

importance of IL-2 in the proliferation of activated helper T cells, the age-related decline in IL-2 production is implicated in decreased cell-mediated immunity in aged animals and humans.

Macrophages have an intimate association with cell-mediated immunity through their role as antigen presenting cells and secretion of cytokines (TNF- α , IL-1, IL-6) and inflammatory mediators such as prostaglandins. Prostaglandins are important lipid inflammatory mediators with diverse biological functions. Prostaglandins are produced by the degradation of phospholipids and the subsequent oxidation of arachidonic acid in the membranes of various cell types and metabolized via the cyclooxygenase pathway(47). Macrophages are the major source of prostaglandin E₂ (PGE₂), with activated macrophages producing higher levels of PGE₂ than resting macrophages. While normal levels of PGE₂ exert a positive effect on cell-mediated immunity, high levels exert a suppressive effect (3, 48). Inappropriate secretion, or dysregulation of secretion, of PGE₂ has been implicated in several disease states such as chronic inflammatory disorders (4), atherosclerosis and cancer (47). Dysregulated secretion of PGE₂ in the murine model has been reported, with significantly higher production seen in aged compared to young animals (47-49). This increase of PGE₂ found in aged subjects may be due to several factors including the fact that aged individuals experience a heavy oxidative stress load and increased lipid peroxidation, and in the case of macrophages the free radicals produced by the respiratory burst may contribute to increased oxidative stress. Prostaglandin production is initiated by peroxidation of membrane polyunsaturated fatty acids, such as arachidonic acid, and the increased lipid peroxidation

in aged individuals due to oxidative stress may lead to dysregulated and increased production of PGE₂ (5).

An important consequence of the elevated secretion of PGE₂ in aged individuals is the immunosuppressive effect on cell-mediated immunity, specifically reduced proliferative capacity of T cells, demonstrated by co-culture experiments. Beharka et al. performed co-culture experiments in order to elucidate the age-related changes in T cells and macrophages and reported that co-cultures of aged murine macrophages and T cells produced significantly elevated levels of PGE₂ compared to young macrophage and T cell co-cultures (3). Additionally, when young co-cultures were exposed to similar levels of PGE₂ produced by aged macrophages, reduced lymphocyte proliferation and IL-2 production were observed (3). This and other studies support the suppressive effect on cell-mediated immunity of elevated levels of PGE₂ in the aged.

Antioxidants counteract the effects of free radicals by scavenging and inactivating these molecules, through enzymatic and non-enzymatic mechanisms. The cell has several natural, or endogenous, antioxidants including the enzymes superoxide dismutase, catalase, and glutathione peroxidase, glutathione reductase, and the ubiquitous tripeptide thiol, glutathione (5). Superoxide dismutase catalyzes the degradation of superoxide anion (O₂^{•-}) into hydrogen peroxide (H₂O₂) and oxygen (O₂), while catalase reacts with H₂O₂ to form water (H₂O) and O₂ (5). Glutathione peroxidases are selenium-dependent enzymes that reduce hydroperoxides (H₂O₂) to water at the expense of reduced glutathione (GSH) (50). Superoxides and hydroperoxides are continuously produced during aerobic respiration and these enzymes are important in inactivating these molecules before they can be converted into more potent oxidizing agents, such as

peroxynitrite (ONOO⁻) and hydroxyl radicals (OH[•]), which can act as initiators of the chain reaction in lipid peroxidation (50). Glutathione (GSH), a hydrophilic scavenger, has potent free radical scavenging capacity in itself, due to its sulfhydryl group, as well as participating in the reaction of glutathione peroxidases (50). GSH is involved in several cellular functions such as protein and DNA synthesis and is an important source of reducing power for the cell (10, 51). GSH also has been shown to modulate mitogen stimulated lymphocyte activation and proliferation (52). GSH depletion negatively impacts proliferation, whereas GSH enhancement positively influences lymphocyte proliferation (53, 54). GSH levels have been shown to decrease with age, and this age-related decrease in GSH might contribute to the decline in cell-mediated immunity in the aged (10, 51). Hu et al. showed that GSH depletion significantly increased the mitogen-stimulated production of PGE₂, an indicator of oxidative stress and inflammation, by human skin fibroblasts and human umbilical vein endothelial cells (55). These results demonstrate that GSH depletion may cause stimulated cells to produce increased amounts of inflammatory mediators (55).

While the cell has several natural antioxidants, there are a variety of exogenous compounds with antioxidant properties available to the cell through diet. Compounds such as vitamin E (α -tocopherol) and carotenoids, such as β -carotene and lutein, function as free radical scavengers (9, 10, 37, 56-58). These compounds are located in lipoproteins and membranes where they act to inactivate peroxy radicals, thereby interrupting the propagation step of lipid peroxidation, and blocking the formation of hydroperoxides (50). Selenium and zinc are essential trace elements, their importance in an antioxidant system partially stems from the fact that zinc is a cofactor for superoxide

dismutase and selenium is a cofactor for glutathione peroxidase (5). Several other compounds have antioxidant properties such as melatonin (59), strawberry extract (58), α -lipoic acid (60), and the synthetic antioxidant n-propyl gallate (61).

Through their free radical inactivation and scavenging capabilities, dietary antioxidants have been shown to ameliorate several age-related disease states associated with the accumulation of free radicals and improve immune function in the elderly.

Antioxidant status declines with age as demonstrated by decreased amounts of plasma vitamins, carotenoids, and enzymatic antioxidants in the elderly (62), therefore antioxidant supplementation may increase antioxidant status in the elderly.

Antioxidants such as vitamin E and carotenoids are transported in the blood in conjunction with lipoproteins, specifically LDLs (56). Vitamin E and carotenoids protect the protein portion of LDLs from oxidative modification, and the subsequent build-up of arterial plaque, which may lead to progressive cardiovascular disease (2, 18).

Antioxidant supplementation, particularly with vitamin E, has been found to reduce production of chemokines, which recruit immune cells to the endothelium of vessels and promote inflammation associated with cardiovascular disease (16). Antioxidants have also been shown to reduce the risk of cancer, and epidemiological evidence has shown that increased intake of carotenoid rich foods, such as green leafy vegetables and red and orange colored fruits, has been associated with a decreased risk of certain types of cancers (56). This association of antioxidants with reduced cancer risk may be due in part to the free radical scavenging and inactivation and protection of DNA from oxidative damage and possible mutagenesis, since carotenoids and vitamin E exert a protective effect and contribute to genome stability (63, 64). Natural killer (NK) cells are a subset

of lymphocytes that have cytotoxic effects on tumor cells, through non-specific or antibody-dependent mechanisms, and are an important cell in immunosurveillance against cancer (65). It has previously been demonstrated that β -carotene enhanced NK cell activity in elderly males(65). Enhanced NK cell activity through antioxidant supplementation may help to reduce the incidence of cancer in the elderly. Antioxidants also help ameliorate the effects of viral infections, in part by reducing the increased oxidative stress accompanied with viral infections, and have been shown to enhance elderly individuals protective response to infection. Hayek et al. demonstrated that vitamin E supplementation reduced viral lung titer in aged mice to comparable levels of young mice (66). Antioxidant supplementation with vitamin E and vitamin E plus glutathione significantly reduced lung viral titers and levels of lipid peroxidation markers, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), in the liver of old mice compared to control old mice (21). Antioxidant supplementation during a viral infection has also been shown to increase levels of IFN- γ , a critical cytokine expressed during a viral infection in which production is depressed in the elderly, as well as reduced levels of TNF- α , an inflammatory cytokine whose secretion has been shown to be dysregulated in the elderly (20, 24). Antioxidant supplementation may also reduce the severity of HIV infections by contributing to the reduction of oxidative stress and lipid peroxidation (22, 23).

Antioxidant supplementation also works to reduce or restore several of the age-related alterations in immune function. Antioxidants enhance and modulate several parameters of elderly immune function including cell-mediated and non-specific immune function, as well as secretion of cytokines and inflammatory mediators. Chandra et al.

found elderly subjects given a daily micronutrient supplementation, including several antioxidants, had significantly higher percentages of total T cells (CD3⁺) and T_H cells (CD4⁺), significantly higher lymphocyte proliferation in response to mitogenic stimulation by PHA, and significantly increased production of IL-2 and IL-2 receptor than control subjects (67). Daily micronutrient supplementation in older humans found that supplementation significantly enhanced the delayed-type hypersensitivity (DTH) response, a direct *in vivo* correlate of cell-mediated immunity, compared to control subjects (68). Vitamin E supplementation in old mice significantly increased splenic T cell proliferation compared to control old mice, significantly increased IL-2 production compared to control old mice, and vitamin E supplemented old mice had IL-2 levels comparable to young mice, both control and vitamin E supplemented (27). In a study performed by Meydani et al., vitamin E supplementation of elderly human subjects significantly increased Con A induced lymphocyte proliferation and IL-2 production of isolated PBMCs, and significantly reduced serum lipid peroxide concentration compared to baseline values before supplementation (37). The increase in the proportion of memory T cells and decrease of naïve T cells is well documented in the aged. Adolfsson et al. found that vitamin E supplementation in mice increased IL-2 production in elderly naïve T cells and enhanced the ability of elderly naïve T cells to progress through 2 cell cycle divisions, demonstrating that vitamin E may play a role in increasing the immune response to newly encountered antigens and decreasing the susceptibility to new infections in the elderly (27). The carotenoid β -carotene has been shown to modulate cell-mediated immune function in animals and humans. Enhanced T and B cell lymphocyte proliferation was seen in rats supplemented with β -carotene compared to

controls (69). β -carotene supplementation of young human males showed an increase, though not significant, in T_H cells ($CD4^+$), but did show a significant increase in the CD4:CD8 ratio compared to the controls (70). Additionally, supplementation of elderly humans with β -carotene showed an increase of CD4+ helper T cells compared to controls(71). However, other studies found little or no effect of β -carotene supplementation on cell-mediated immunity in women (72, 73) or in elderly individuals (74), but this may possibly be due to differences in research methodology or experimental design. Dietary supplementation with glutathione has also been shown to enhance cell-mediated immunity. A study conducted by Furukawa et al. in mice confirmed the age related decrease in glutathione concentration as well as decreased cell-mediated immune responses (mitogenic lymphocyte proliferation and delayed-type hypersensitivity response) compared to young mice (75). Glutathione supplementation in this study increased glutathione concentration and cell-mediated immune responsiveness in the aged mice (75).

Antioxidants have been shown to modulate cytokine expression in the aged and ameliorate some of the age-related dysregulation in secretion of selected cytokines and inflammatory mediators. Vitamin E has been shown to decrease production of IL-6 in human PBMCs (76) and decrease production of TNF- α in rat whole blood cultures (77). In a study by Beharka et al., vitamin E and melatonin significantly decreased IL-6 production in murine peritoneal macrophages (4). Glutathione supplementation of human alveolar macrophages *in vitro* decreased TNF- α and IL-6 production (78).

Increased PGE₂ production in aged individuals has been implicated in several disease states and contributes to the suppression of T cell function. Antioxidants, in

particular vitamin E, have been shown to modulate the age-related increase in PGE₂ production by macrophages. Vitamin E supplementation significantly reduces PGE₂ production in murine macrophages compared to controls (47-49). Vitamin E and melatonin significantly reduced unstimulated PGE₂ production by murine macrophages compared to controls (4). Macrophage production of PGE₂ contributes to the reduced proliferative capacity and production of IL-2 by T cells in old mice, and vitamin E supplementation enhanced IL-2 production and T cell proliferation and this effect was mediated by the reduction of PGE₂ production by macrophages (3).

Several studies have shown that antioxidant supplementation reduces oxidative stress in the cell through free radical scavenging, ameliorates several age-related diseases and conditions, and enhances immune function. However, most of these studies have been performed in rodents or humans. In recent years, there has been an increased interest in the effects of proper nutrition and antioxidant supplementation in companion animals (felines and canines) in order to maintain health with age. This interest is two-fold, in that companion animals can serve as another animal model of antioxidant supplementation and the benefits of companion animals to humans as pets and in a functional capacity such as seeing eye dogs, search and rescue dogs, and animals which help disabled owners function normally. Evidence also suggests a positive influence of companion animals to human health (79).

An important aspect in canine and feline health is proper immune function, and like humans and murines, immune function decreases with age in companion animals (79). Changes in lymphocyte subsets have been observed in aged companion animals. Kearns et al. demonstrated that the percentage of total T cells, CD4⁺ helper T cells, and B

cells decreased in aged canines, with no apparent change in CD8⁺ cytotoxic T cells in these animals (80). Greeley et al. also demonstrated an age related decrease in CD4⁺ T cells and B cells in the canine (81). A study by Greeley et al. performed in the canine found a significantly reduced proliferative response of lymphocytes to mitogens and a decrease, though not significant, of antibody titers in response to vaccination in old dogs compared to young (81). Other studies have also confirmed the age-related decrease in mitogen-induced lymphocyte proliferation as well as other age-related changes in immune parameters, such as decreased DTH response and antibody production in response to antigenic challenge and an increase in PGE₂ production, in canine and feline (80, 82, 83).

Several studies have examined the effect of antioxidant supplementation on immune function in companion animals, both in young and aged canines and felines. Chew et al. showed that dietary supplementation with β -carotene in young dogs significantly increased DTH skin response, plasma IgG concentration, and percentages of CD4⁺ helper T cells compared to unsupplemented dogs (84). Similarly, β -carotene supplementation in old dogs increased mitogen-induced lymphocyte proliferation, DTH skin response to PHA (a nonspecific antigen), and percentages of CD4⁺ helper T cells compared to control animals (80). However, β -carotene does not seem to modulate immune function in felines (85). Dietary supplementation with the carotenoid lutein in cats significantly increased lymphocyte proliferation in response to Con A and pokeweed mitogen (PWM), increased skin DTH responsiveness to vaccine (a specific antigen), increased percentages of CD4⁺ helper T cells and B cells, as well as significantly increasing plasma IgG compared to unsupplemented cats (86). Similarly,

supplementation with dietary lutein in dogs significantly increased mitogen-induced lymphocyte proliferation to Con A, PHA, and PWM, skin DTH responsiveness to PHA, increased percentages of CD4⁺ helper T cells and CD8⁺ cytotoxic T cells as well as cells expressing the MHC class II cell surface marker, which are found on antigen presenting cells, compared to dogs on a control diet (87). This study also found that dietary lutein significantly increased plasma IgG concentration in response to vaccine compared to unsupplemented dogs (87). A study by Hayek et al. found that vitamin E supplementation significantly increased lymphocyte proliferation in old and young cats in response to the mitogens Con A and PWM, respectively (83). This same study also found that vitamin E supplementation in cats significantly reduced the production of PGE₂ compared to control cats, and vitamin E also produced a trend towards significance in reducing PGE₂ production in aged cats compared to aged controls (83). However, vitamin E supplementation did not significantly increase mitogen-induced lymphocyte proliferation in dogs (82).

The numerous studies just reviewed have shown age-related alterations in immune function in companion animals and that antioxidant supplementation can enhance immune function in both young and aged companion animals. ThiomaxTM, manufactured by Health Span Sciences, is a proprietary antioxidant cocktail composed of α -lipoic acid and n-propyl gallate. It has been demonstrated that α -lipoic acid and n-propyl gallate have effective antioxidant and free radical scavenging capabilities (60, 61, 88-90). ThiomaxTM is a supplement used and tested in humans, however, no studies have been undertaken to determine the efficacy of ThiomaxTM in companion animals. Therefore, the objective of this study was to test the effects of the antioxidant cocktail

ThiomaxTM on immune function, through measurement of mitogen-induced lymphocyte proliferation, lymphocyte subsets, antibody production, and PGE₂ production, as well as testing the effects of ThiomaxTM on glutathione status, and oxidative stress, as measured by serum lipid peroxidation, in young and aged felines. This study confirmed the age-related decrease in selected immune parameters and glutathione concentration, however no consistent effect of ThiomaxTM supplementation was found in young or old cats.

MATERIALS AND METHODS

Animals and Diet:

Twenty young (2.68-2.75 years) and aged (9.12-16.22 years) felines were used to assess the effects of the antioxidant cocktail ThiomaxTM on immune function. Animals were housed and maintained at the Iams Research and Development Facility in Lewisburg, OH with 12-hour light and dark cycles and adequate exercise. Animals were maintained on a nutritionally complete baseline diet, developed by Iams, for 4 weeks prior to commencement of the study. After that time, felines from the young and aged groups were randomized and assigned either to the control or ThiomaxTM diet (Table 1) formulated by Iams. The animals were maintained on this diet for 60 days. Blood was collected by veinipuncture into heparinized tubes on days 0, 30, and 60 of the treatment period. Veterinarians from this facility drew blood from the animals at the desired time points throughout the study.

Isolation of Peripheral Blood Mononuclear Cells:

A 10 ml sample of heparinized whole blood was collected from each animal on days 0, 30, and 60 of the treatment period and transported from the Iams facility in Lewisburg, OH to the University of Dayton. Five hundred microliters was removed from each sample and centrifuged at 956 x g at 4°C for 10 minutes. The serum was drawn off and resulting pellet of blood cells was frozen at -70°C for use in measuring

Table 1: Composition of Control and ThiomaxTM diet fed to young and aged felines

Diet Composition	Diet Type	
	Control	ThiomaxTM
Acid Fat Hydrolysis	18.9%	19.2%
Ash (dry sample)	6.5%	6.7%
Calcium	1.1%	1.1%
Moisture	7.0%	6.6%
Phosphorus	0.9%	1.0%
Protein	36.6%	32.2%
Gross Energy	5090.4 Cal/gm	5124 Cal/gm
Thiomax TM	0.00%	0.08%

intracellular glutathione levels. The remaining whole blood was centrifuged in 50 ml centrifuge tubes (Fisher) at 827 x g at 22°C for 10 minutes and serum was collected and stored at -70°C for use in measuring levels of lipid peroxidation. Peripheral blood mononuclear cells (PBMCs) were obtained by diluting the pelleted whole blood up to 30 ml with 1X PBS (Gibco), underlaying with 15 ml of Histopaque (Sigma), and centrifuging at 670 x g at 22°C for 40 minutes. The resulting layer of PBMCs was drawn off and placed in a sterile 50 ml centrifuge tube (Fisher). Cells were resuspended in 1X PBS (Gibco) and centrifuged at 206 x g at 22°C for 10 minutes. This step was then repeated for a total of 2 washes. The resulting pellet of PBMCs was resuspended in 2 ml of media consisting of RPMI-1640 (Sigma) containing penicillin (100 U/ml) and streptomycin (100 µg/ml)(Sigma) and gentamicin (50 µg/ml) (Gibco) and cell counts were performed. Isolated PBMCs were used in lymphocyte proliferation assays and for the measurement of PGE₂ production.

Lymphocyte Proliferation Assay (LPA):

Isolated peripheral blood mononuclear cells (PBMCs) were then used to measure proliferation in response to mitogenic stimulation with concanavalin A (ConA), phytohemagglutinin (PHA) and pokeweed mitogen (PWM). Con A and PHA are T cell mitogens, whereas PWM is a T and B cell mitogen. Isolated PBMCs were adjusted to a cell concentration of 2×10^6 cells/ml in complete media consisting of RPMI-1640 (Sigma) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/ml) and streptomycin (100 µg/ml)(Sigma) and gentamicin (50 µg/ml) (Gibco). One hundred microliters of cells were dispensed into individual wells of a 96-well flat-bottom microtiter plate (Costar). PBMCs were then stimulated in triplicate with 100 µl ConA

(Sigma), PHA (Sigma), and PWM (Sigma) at final concentrations of 5.0µg/ml, 2.5µg/ml, and 1.0µg/ml. Complete media alone added in triplicate wells served as controls. The cells were incubated for 72 hours at 37°C in a humidified incubator with 5% CO₂. Eighteen hours prior to the termination of the experiment, each well was pulsed with 1µCi ³H-thymidine (50µCi ³H-thymidine/ml) (ICN Biomedical). Tritiated thymidine incorporation was measured by liquid scintillation (Beckman LS3801) and the proliferative response of PBMCs to mitogenic stimulation was expressed as corrected counts per minute (cCPM). Corrected counts per minute were calculated by subtracting the counts per minute (cpm) of mitogen-stimulated cultures from the cpm of unstimulated cultures (media alone).

Prostaglandin E₂ (PGE₂) Production:

Isolated PBMCs were adjusted to a cell concentration of 5x10⁵ cells/ml with complete media, consisting of 5% fetal bovine serum (Gibco) and antibiotics as previously described. One milliliter of each cell suspension was dispensed into individual wells of a 24-well cluster plate (Costar). In order to stimulate PGE₂ production, 100 µl of Con A (25µg/ml) was added to individual wells for a final concentration of 2.5µg/well Con A. Cells were incubated for 18-20 hrs at 37°C in a humidified incubator with 5% CO₂. Cells were collected and centrifuged at 4,768 x g at 4°C for 10 minutes. Culture supernatants were collected and stored at -70°C until time of assay.

PGE₂ production was measured using the Correlate-CLIA High Sensitivity PGE₂ Chemiluminescence Enzyme Immunoassay Kit (Assay Designs, Inc. cat # 910-001). Briefly, supernatants were diluted 1:10 in complete media and PGE₂ standards were

prepared according to the protocol provided. All reagents were allowed to warm to room temperature and all wells were performed in duplicate.

Into predetermined wells of a 96-well microtiter plate coated with goat anti-mouse antibodies, 100 µl of complete RMPI-1640 was pipetted into the non-specific binding wells (NSB) and Bo (maximum binding) wells. One hundred microliters of PGE₂ standards and samples were pipetted into the appropriate wells. Fifty microliters of assay buffer was pipetted into the NSB wells. Fifty microliters of blue Conjugate (alkaline phosphatase conjugated with PGE₂) was pipetted into each well, except the Total Activity (TA) and Blank wells. Fifty microliters of the yellow Antibody (monoclonal antibody to PGE₂) was pipetted into each well, except the Blank, TA, and NSB wells. The plate was then incubated at room temperature with shaking for 2 hours at 500 rpm. The plate was washed three times with wash solution and firmly tapped dry after final wash. Five microliters of blue Conjugate was pipetted into the TA wells followed by 200ul of CLIA Substrate (alkaline phosphatase substrate) pipetted into every well with a 10 second delay between wells. The plate was shaken for 1 hour at room temperature. The plate was read on a Victor² 1420 luminometer (Wallac, Perkin Elmer Life Sciences), reading each well for 2 seconds with a 10 second delay between each well. Data was analyzed with AssayZap software (Biosoft) using a 4- parameter logistic curve program.

Measurement of Intracellular Glutathione:

Intracellular glutathione, a natural intracellular antioxidant, was measured from erythrocyte lysates using the Colorimetric Assay for Glutathione (Oxford Biomedical

Research, #GT 10). In order to obtain erythrocyte cell lysates, erythrocyte pellets, collected previously from 0.5 ml whole blood, were resuspended in 1 ml of 5% metaphosphoric acid (MPA)(Sigma) at 4°C. Samples were mixed thoroughly by vortexing and centrifuged at 3000 x g at 4°C for 10 minutes. The upper clear aqueous layer was collected for use in the glutathione assay. A standard curve was generated as per protocol using reduced glutathione (Calbiochem) dissolved in 5% MPA (Sigma) and diluted appropriately with assay buffer. Samples were diluted by adding 200 µl of sample to 700 µl of assay buffer (1:5 dilution). Nine hundred microliters of assay buffer alone served as the blank. To standards, samples, and blank, 50 µl of Reagent 1, the chromogenic reagent, was added with thorough mixing. Fifty microliters of Reagent 2, 30% NaOH, was added to standards, samples, and blank with thorough mixing. Samples and standards were incubated in a 25°C water bath for 10 minutes in the dark. Final absorbance was measured at 400nm on a Genesys 2 spectrophotometer (Spectronic). Results were then extrapolated from the standard curve using the following equation:

$$[\text{GSH}] = \{A / (\epsilon_{400} \times l)\} \times D$$

where:

[GSH] is the initial glutathione concentration in the sample, µM

A is the measured absorbance

ϵ_{400} is the apparent molar extinction coefficient of product measured at 400 nm (slope of the corresponding straight line of standard curve)

l is the optical path length

D is the dilution factor of sample

Measurement of Lipid Peroxidation:

Lipid peroxidation, which correlates to oxidative stress and cellular damage, was measured in previously collected serum samples. Lipid peroxidation was quantitated using the colorimetric assay BIOXYTECH® LPO-560 (OxisResearch, #LPO-560). Briefly, each sample was performed in duplicate, with one aliquot serving as the blank and the other aliquot serving as the test sample used to measure lipid peroxidation. Ninety microliters of each serum sample was incubated with 10 µl of catalase, in order to eliminate H₂O₂ interference from the samples and obtain a more accurate lipid peroxide measurement. Samples were incubated at room temperature for 2 minutes. Ten microliters of reducing reagent, tris(2-carboxyethyl)phosphine (TECP), was added to each sample blank in order to reduce the lipid peroxides in the sample to the corresponding alcohols, and 10 µl of deionized water was added to each test sample. Samples were mixed by vortexing and incubated at room temperature for 30 minutes. Nine hundred microliters of working reagent, prepared previously as per protocol, was added to each sample. Each sample was mixed by vortexing for 30 seconds and incubated at room temperature for 60 minutes.

Samples were centrifuged at 12,000 x g for 10 minutes and supernatants were collected. The samples were read at 560 nm on a Genesys 2 spectrophotometer (Spectronic), with deionized water serving as a blank to zero the instrument. The lipid peroxides were calculated by subtracting the blank sample, treated with reducing reagent, from the test samples. This yielded the net absorbance, which was due to lipid peroxide content. The concentration of lipid hydroperoxides was calculated using the following equation:

$$[\text{LOOH}] = (\text{Net } A_{560} / \epsilon) * \delta$$

Where:

$[\text{LOOH}]$ = Concentration of lipid peroxides in sample (μM)

Net A_{560} = Net absorbance at 560 nm

$\epsilon = 0.0431 \text{ mM}^{-1}\text{cm}^{-1}$, apparent molar extinction coefficient of the hydroperoxide

δ = Dilution factor, 11.2

Measurement of Total IgG and IgM Antibody Titer:

After day 60 of the treatment period, animals were maintained on their respective diets and vaccinated with Fel-O-Vax® (Fort Dodge Animal Health-Division of Wyeth, Madison, NJ) according to protocol. After a two week period, serum samples were obtained to designate a day 0 time point, after which all animals were boosted with the same vaccine. Serum samples were then obtained from all animals at day 7, 14, and 21. Collected serum samples were used to measure total IgG and IgM antibody titer using the Cat IgG and IgM ELISA Quantitation Kits (#E20-117 and E20-100), ELISA Starter Accessory Package (#E101), and a quantitative ELISA immunoassay protocol, all obtained from Bethyl Laboratories (Bethyl Laboratories, Inc., Montgomery, TX).

Reagents obtained from the Cat IgG and IgM ELISA Quantitation Kits and ELISA Starter Accessory Package were allowed to warm to room temperature and all steps were performed at room temperature. According to the provided protocol, the wells of the 96-well microtiter plates were coated with capture antibody, either goat anti-cat IgG-Fc affinity purified antibody for IgG detection or goat anti-cat IgM affinity purified antibody for IgM detection. The affinity purified antibodies were diluted 1:100 in

coating buffer and 100 μ l was added to the wells of the plate. The plate was incubated for 60 minutes then washed twice with wash solution. Two hundred microliters of postcoat solution was added to the wells and the plate was incubated for 30 minutes, then washed twice with wash solution. Cat reference serum was diluted as per protocol in sample diluent to serve as standards and previously collected serum samples from day 0, 7, 14, and 21 were diluted in sample diluent at a range of 1:4,000 to 1:10,000 (determined by preliminary testing). One hundred microliters of standards and samples were added to appropriate wells and incubated for 60 minutes, after which the plate was washed 4 times with wash solution. The detection antibody/horseradish peroxidase (HRP) conjugate, either goat anti-cat IgG-Fc HRP for IgG detection or goat anti-cat IgM HRP for IgM detection, were diluted appropriately in sample diluent. One hundred microliters were added to each well and incubated for 60 minutes, after which the plate was washed 4 times with wash solution. The enzyme substrate solution was prepared by mixing equal volumes of TMB peroxidase substrate and peroxidase solution B (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and 100 μ L of substrate was added to each well. Plates were incubated 5-15 minutes (depending on intensity of color change), after which 100 μ L of 2 M H₂SO₄ were added to each well. Plates were read at 450 nm on a Dynex MRX plate reader and data was generated using Revelation software (Dynex Laboratories).

Flow Cytometric Analysis

Analysis of lymphocyte subsets on blood collected from old and young felines on day 0, 30, and 60 of the treatment period was performed with single-color flow cytometry by Dr. Boon Chew's lab at Washington State University, Pullman, WA. Mouse monoclonal antibodies (mAbs) to feline antigens used to quantitate the lymphocyte

subsets were obtained from VMRD (Pullman, WA). Briefly, PBMCs were separated on Histopaque-1119 (Sigma, St. Louis, MO) and contaminating red blood cells were lysed in NH_4Cl (8.4 mg/ml) and cells were washed three times with phosphate buffered saline (PBS, pH 7.4). Isolated PBMCs were resuspended to 1×10^7 cells/ml in PBS supplemented with 2% gamma globulin-free horse serum, 5% goat serum and 0.2mg/ml sodium azide. A total of 5×10^5 cells were incubated for 30 minutes on ice with monoclonal antibodies that recognize feline Total T cells (CD3+ cell, CF54A), CD4+ cells (CAT30A, helper T cells), and CD8+ cells (25-2A, cytotoxic T cells). Cells were then washed three times and secondary staining was performed by incubating cells with fluorescein isothiocyanate (FITC)-conjugated goat F(ab')_2 anti-mouse immunoglobulin (Ig) G (H+L) antibody (Caltag, Burlingame, CA) to visualize the bound mAb. Stained cells were fixed in 4% paraformaldehyde in preparation for acquisition and determination by flow cytometry (FACScan, Becton Dickinson, San Jose, CA) after gating the lymphocyte population by forward and side scatter analysis. For each sample, 5,000 total events were acquired for analysis. Appropriate negative controls were included to correct for background fluorescence. Data were expressed as the percentage of positive-staining cells corrected for cells stained nonspecifically with the secondary antibody.

Statistics:

All statistical analyses were performed using the Statistical Analysis System (SAS) statistical package version 8.0 (SAS Institute, Cary, NC). The General Linear Model Procedure was used to analyze data with age, diet, and day as main effects. For analysis of the effects of ThiomaxTM on lymphocyte subsets, values for young and old felines from day 30 and 60 were grouped together. Significant differences were

identified by the Least Squared Means test and Tukey's honestly significant difference (HSD) post hoc test. Significance was set at $p \leq 0.05$.

RESULTS

Aging is associated with several physiological changes. An important physiological change seen in aging is a shift in the balance between oxidants and antioxidants, in which increased oxidants, or free radicals, lead to increased cellular damage, also called oxidative stress. Antioxidants, both intracellular and exogenous, counteract free radical damage to cells through the inactivation of these molecules. The overall goal of this research was to determine whether or not supplementation with the antioxidant cocktail ThiomaxTM could reduce the damage caused by the age related increase in oxidative stress and possibly restore function to levels comparable to those seen in the young. In order to test this hypothesis, we choose several parameters in which age related oxidative stress has a significant impact, using young and old felines. These parameters include immune function, secretion of inflammatory mediators, lipid peroxidation, and intracellular antioxidants. In order to assess the impact of age and ThiomaxTM supplementation on immune function we examined several immune parameters. Lymphocyte function, dealing mainly with T lymphocytes, was assessed using mitogen-induced lymphocyte proliferation as a measure of lymphocyte responsiveness and flow cytometric analysis in order to determine T cells subsets and any subsequent age related shifts. Humoral immunity was assessed by measuring IgM and IgG antibody levels in response to vaccine challenge. Secretion of PGE₂ was measured in these animals since PGE₂ is a molecule associated with inflammation and modulation of cell-mediated immune responses. The innate intracellular antioxidant status in these animals was measured through intracellular glutathione levels and the extent of cellular

damage by oxidative stress was measured through lipid peroxidation levels in these animals.

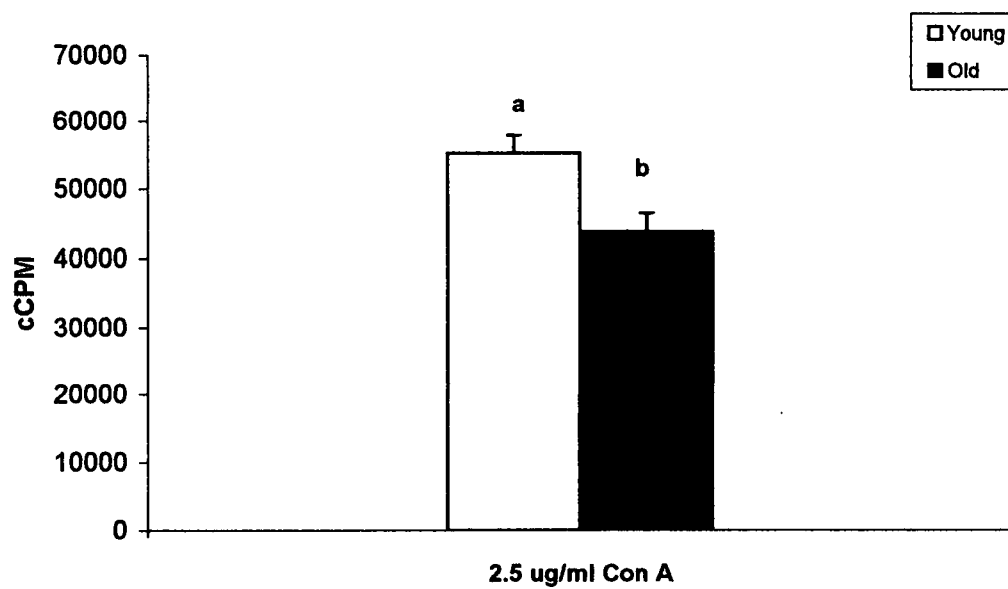
Effect of Age on the Immune Function

In order to assess lymphocyte function, lymphocyte proliferation assays were performed on isolated peripheral blood mononuclear cells (PBMCs) obtained from young and aged felines. The T cell mitogens Con A and PHA, and the T and B cell mitogen PWM were used to stimulate lymphocyte proliferation *in vitro* (data for PHA was not significant). The optimal concentration for Con A and PWM was determined to be 2.5 µg/ml. Young felines had significantly greater lymphocyte proliferation than old felines when stimulated with 2.5 µg/ml Con A (Figure 1A). Similarly, stimulation with 2.5 µg/ml PWM resulted in greater lymphocyte proliferation in young felines compared to old felines, however this difference was not significant ($p \leq 0.10$) (Figure 1B).

Based on differences in proliferation, experiments were designed to determine whether there were any changes in the distribution of T cell subsets. Flow cytometric analysis was performed in order to measure T lymphocyte subsets in young and old felines. When feline PBMCs from young and old felines were stained with CD3 antibody (total T cells), there was a significant decline ($p \leq 0.05$) in total T cell population in old felines compared to young (Figure 2A). Staining with CD4 antibody identified T helper cells, and there was a strong trend towards a reduced ($p = 0.06$) T helper cell population in old cats (Figure 2B). Similarly, there was a significant decline ($p \leq 0.01$) in

Figure 1: The effect of age on mitogen-induced lymphocyte proliferation. Peripheral blood mononuclear cells (PBMCs) from both young and old felines were stimulated with 2.5 µg/ml Con A (**1A**) or 2.5 µg/ml PWM (**1B**). After 72 hours, ^3H -thymidine incorporation was assessed as a correlate of cellular proliferation. Values were expressed as corrected counts per minute (cCPM). Different letters denote a statistically significant difference between groups ($p \leq 0.05$).

A



B

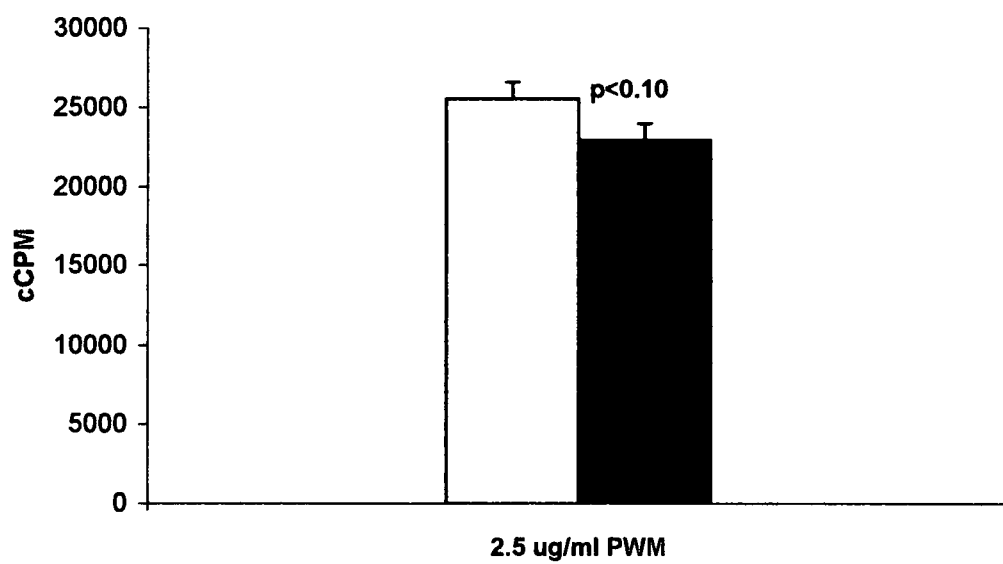
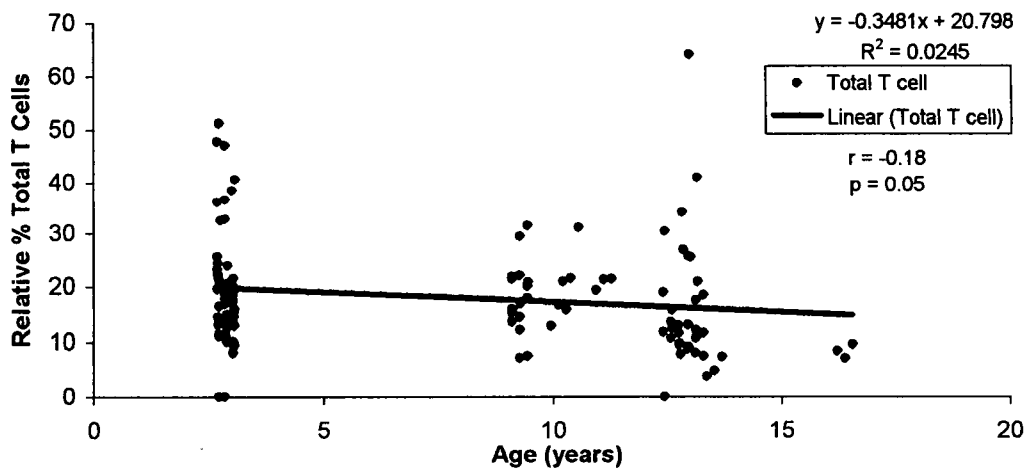
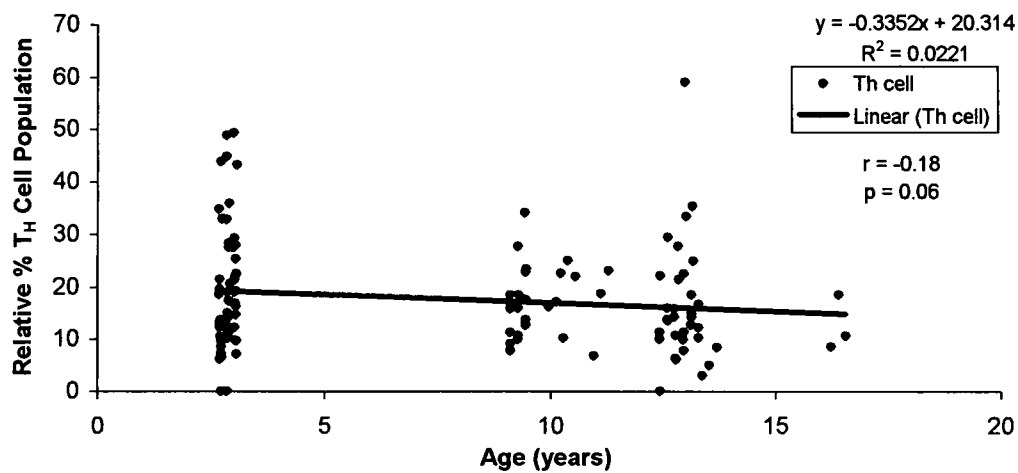


Figure 2: The effect of age in felines on T cell subsets using single color flow cytometry. PBMCs from young and aged felines were labeled with monoclonal antibodies (mAb) to CD3 (total T cells) (2A), CD4 (T-helper cells) (2B), and CD8 (cytotoxic T cells) (2C). Cells stained with the respective mAb were labeled with fluorescein isothiocyanate (FITC)-conjugated goat F(ab')² anti-mouse IgG+IgM (H+L)^d. Labeled cells were run on a flow cytometer to obtain relative numbers of the respective T cell populations. Significance was determined by an R² analysis and significance was set at $p \leq 0.05$.

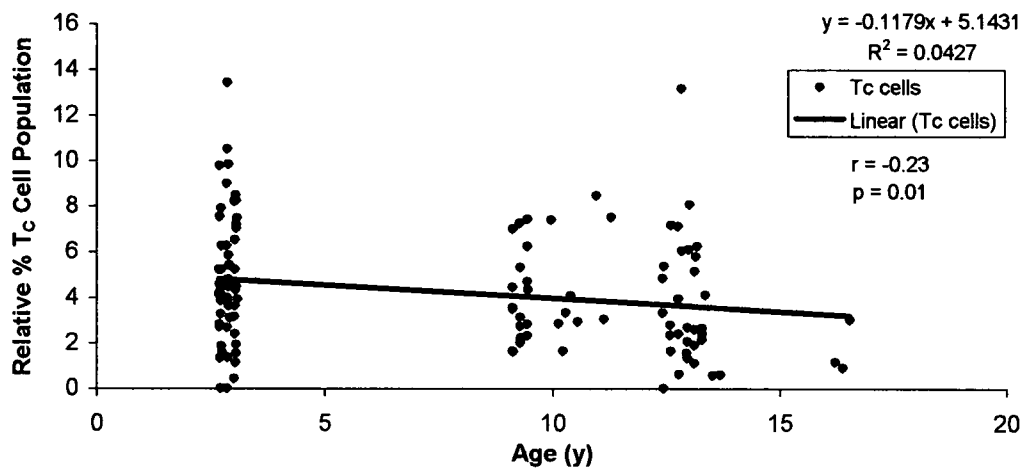
A - Total T cells (CD3⁺)



B - Helper T (T_H) Cells (CD4⁺)



C - Cytotoxic T (T_C) Cells (CD8⁺)



cytotoxic T cells stained with CD8 antibody in old felines compared to young felines (Figure 2C).

Humoral immunity was assessed in young and old felines by measuring IgM and IgG antibody levels in response to antigenic challenge with Fel-O-Vax®. Old felines had significantly higher IgM antibody levels at days 0 and 7 post-challenge than young felines, however there was no statistical difference at days 14 and 21 (Figure 3A). There was no difference in IgG antibody levels between old and young felines on any day post challenge (Figure 3B).

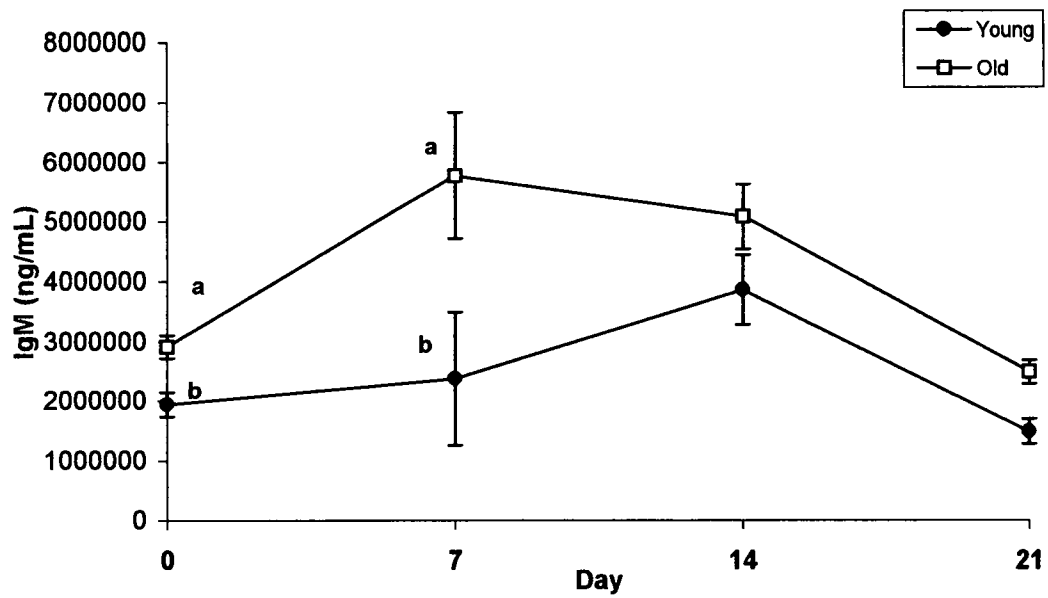
PGE₂ is an important lipid inflammatory mediator intimately involved in immune function, specifically impacting cell-mediated immunity. PBMCs from old and young felines were stimulated with 2.5 µg Con A in order to measure the production of PGE₂. Younger cats produced significantly higher levels of PGE₂ than old cats (Figure 4).

Influence of Age on Intracellular Glutathione Levels and Lipid Peroxidation

The intracellular antioxidant glutathione was measured using erythrocyte lysates obtained from old and young felines. Levels of intracellular glutathione in young felines were significantly elevated compared to those in old felines (Figure 5A). Serum samples from young and old felines were used to quantify lipid hydroperoxide production, which correlates with oxidative stress and cellular damage. Compared to old felines, young felines produced significantly higher amounts of lipid peroxides (Figure 5B).

Figure 3: Effect of age in antibody levels in young and aged felines. Young and old felines were vaccinated with the Fel-O-Vax® vaccine, and 2 weeks later challenged with the same vaccine. Serum was collected from all animals on days 0, 7 14, and 21. Serum was analyzed for IgM (3A) and IgG (3B) levels using Cat IgG and IgM ELISA Quantitation Kits. Values are expressed as ng/ml of antibody. Different letters denote a statistically significant difference between groups ($p \leq 0.05$).

A



B

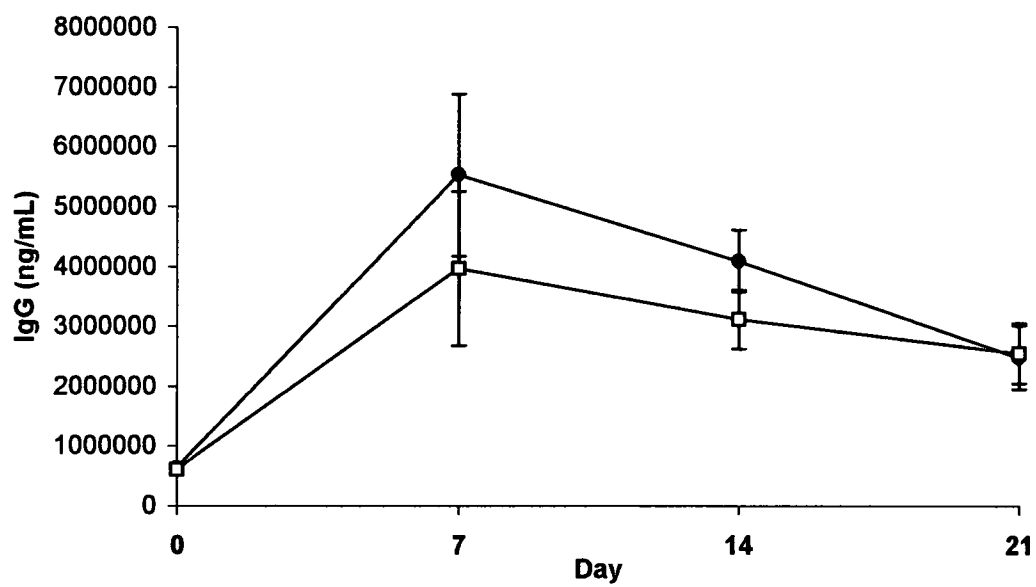


Figure 4: Prostaglandin E₂ (PGE₂) levels in young and aged felines. PBMCs from old and young felines were stimulated with 2.5 µg Con A for 18-20 hours. The supernatants from each culture were collected and levels of PGE₂ were measured. Different letters denote a statistically significant difference between groups ($p \leq 0.05$).

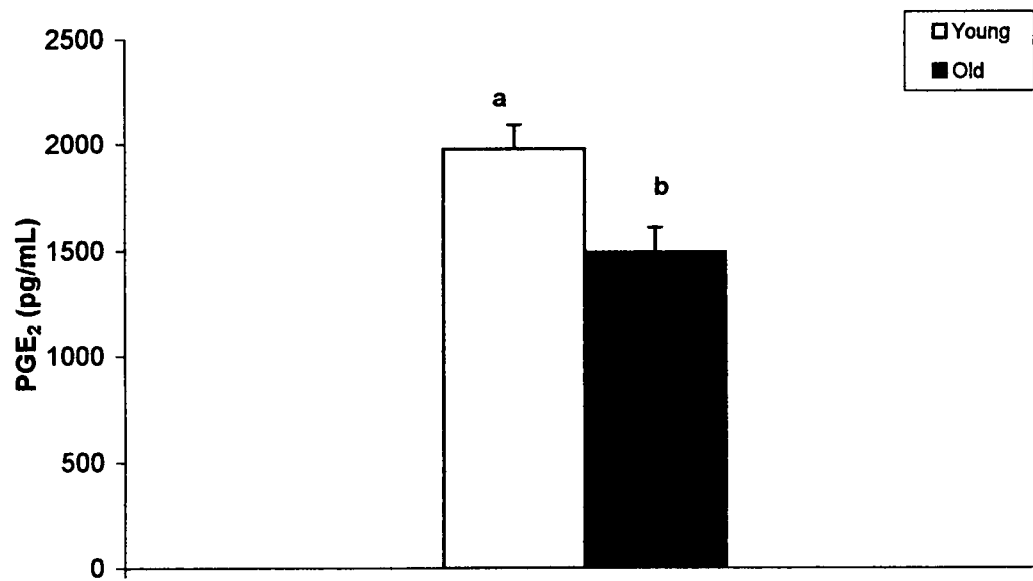
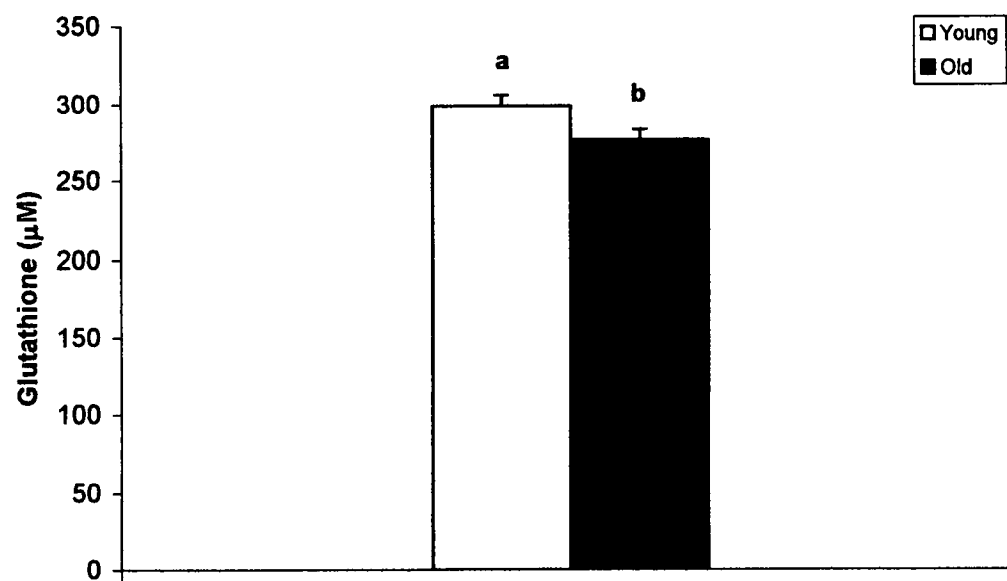


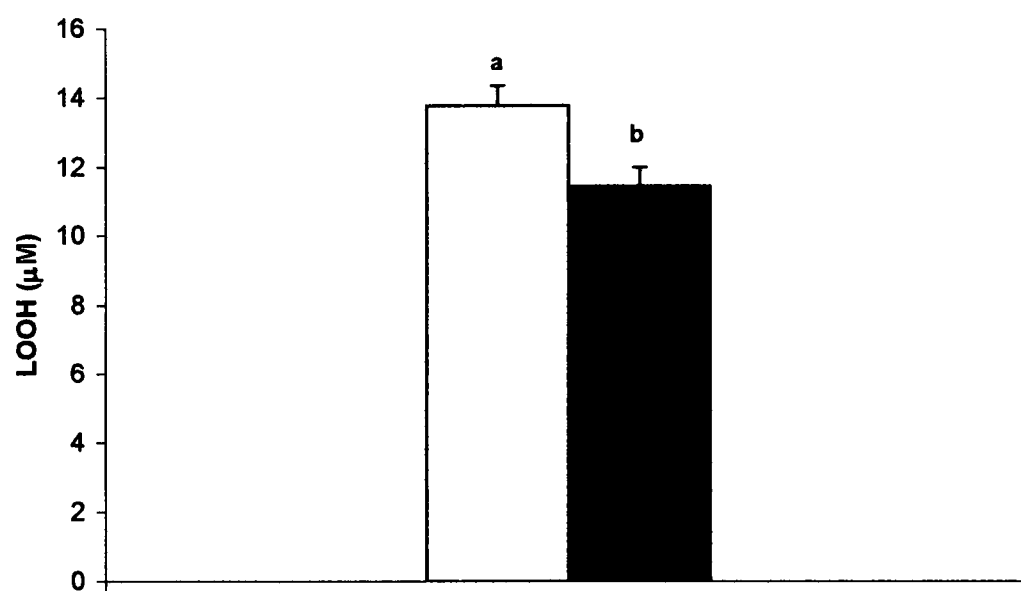
Figure 5: Effect of age on intracellular glutathione levels and lipid peroxidation.

Intracellular glutathione from red blood cell lysates of both young and old felines was analyzed (5A). Serum samples from young and old felines were used to measure the levels of lipid peroxides, correlating to actual lipid peroxidation using a colorimetric assay (5B). Values were expressed as μM amounts per sample. Different letters denote a statistically significant difference between groups ($p \leq 0.05$).

A



B

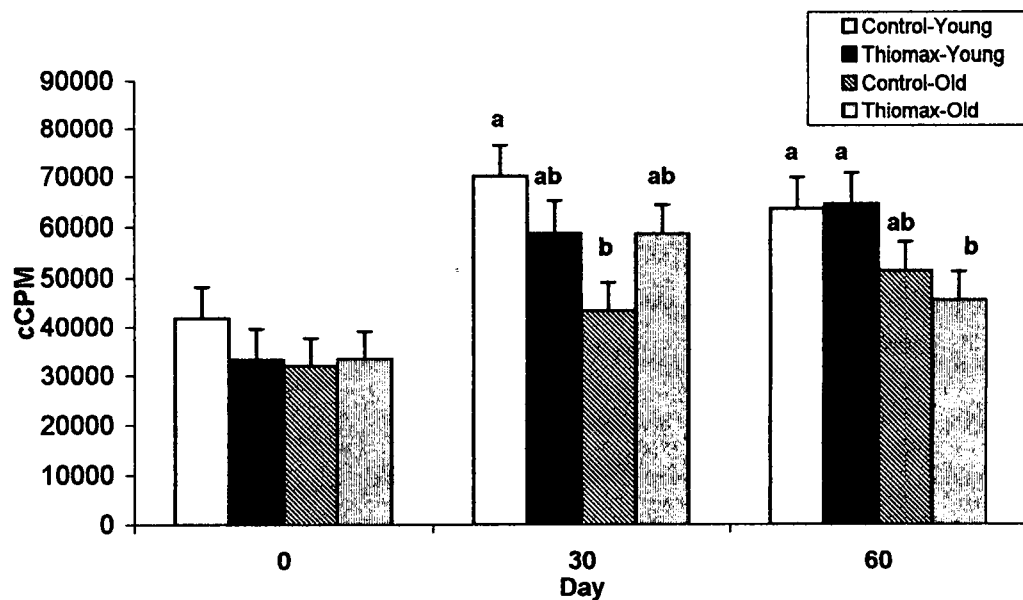


Effect of ThiomaxTM supplementation on Selected Immune Parameters

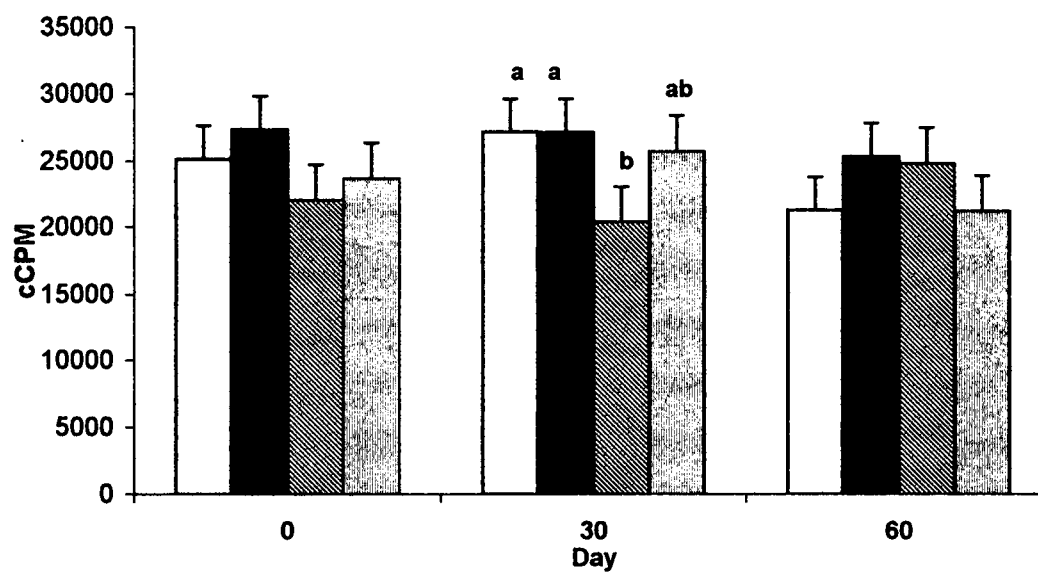
In order to assess the impact of ThiomaxTM supplementation on cell-mediated immune function, lymphocyte proliferation assays were performed on isolated peripheral blood mononuclear cells (PBMCs) from young and aged felines. The mitogens Con A, PHA, and PWM were used to stimulate lymphocyte proliferation *in vitro*. On day 30 young felines maintained on the control diet had significantly higher proliferation than old feline PBMCs on the control diet when stimulated with 2.5 µg/ml Con A (Figure 6A). On day 30, ThiomaxTM supplementation increased proliferation of old feline PBMCs stimulated with Con A to levels comparable with young feline values, however these values were not significantly different from old controls (Figure 6A). ThiomaxTM supplementation had no effect on young felines at day 30 (Figure 6A). On day 60 of the treatment period, there was no effect of ThiomaxTM supplementation on either young or old feline PBMCs stimulated with Con A (Figure 6A). When stimulated with 2.5 µg/ml PWM on day 30, young feline PBMCs in either the control or ThiomaxTM supplemented group had significantly higher proliferation than old feline PBMCs on the control diet. However, on day 30, ThiomaxTM supplementation increased proliferation of old feline PBMCs to levels comparable with young control and ThiomaxTM supplemented felines when stimulated with PWM, however these values were not significantly different from old controls (Figure 6B). There was no effect of ThiomaxTM supplementation on days 0 and 60 for any treatment group stimulated with PWM (Figure 6B). In order to assess the impact of ThiomaxTM supplementation on T lymphocyte subsets in young and old felines, flow cytometric analysis was performed to measure T lymphocyte subsets. When total T cells (CD3⁺), T helper cells (CD4⁺), and cytotoxic T

Figure 6: Effect of ThiomaxTM supplementation on mitogen-induced proliferation in young and aged felines. PBMCs from young and old felines fed either a control or ThiomaxTM supplemented diet were stimulated with 2.5µg/ml Con A (**6A**) or 2.5 µg/ml PWM (**6B**) on days 0, 30 , and 60 of the treatment period. After 72 hours, ³H-thymidine incorporation was assessed as a correlate of cellular proliferation. Values were expressed as corrected counts per minute (cCPM). Different letters denote a statistically significant difference between groups ($p \leq 0.05$), where as groups that share a letter are not significantly different from each other.

A - Concanavalin A (2.5 $\mu\text{g/ml}$)



B - Pokeweed Mitogen (2.5 $\mu\text{g/ml}$)



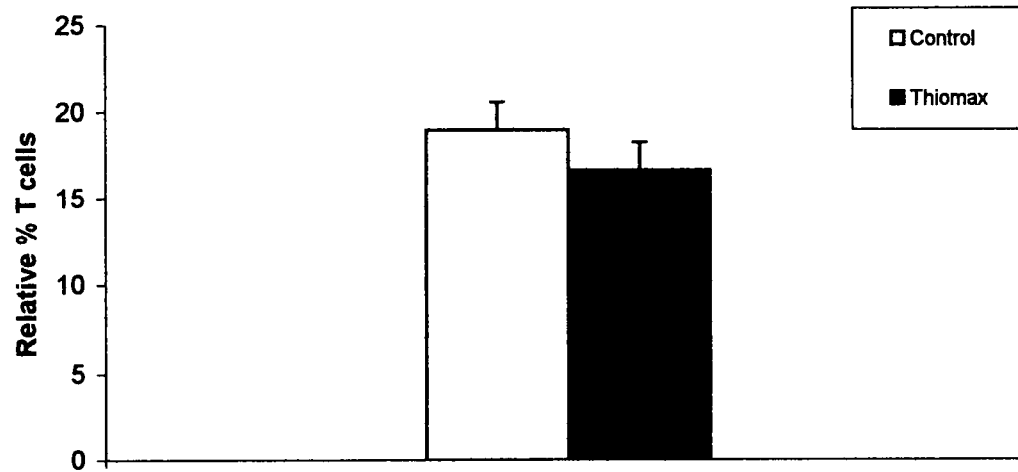
cells (CD8⁺) were measured there was no significant difference between young and old felines on the ThiomaxTM supplemented diet compared to young and old felines on the control diet during the whole sampling period (Figure 7A-C).

The effect of ThiomaxTM supplementation on humoral immunity was assessed in young and old felines by measuring IgM and IgG antibody levels in response to antigenic challenge with Fel-O-Vax®. On day 0 post challenge, old felines fed the ThiomaxTM supplemented diet had significantly higher levels of IgM antibodies than the old control group or the young felines, and on day 7, old felines supplemented with ThiomaxTM had significantly higher values than young felines in either group (Figure 8A). On day 21 post challenge, old felines on either the control or ThiomaxTM supplemented diet produced significantly higher IgM levels compared to young felines (Figure 8A). There was no effect of ThiomaxTM supplementation on IgG antibody levels in any groups on any day post challenge (Figure 8B).

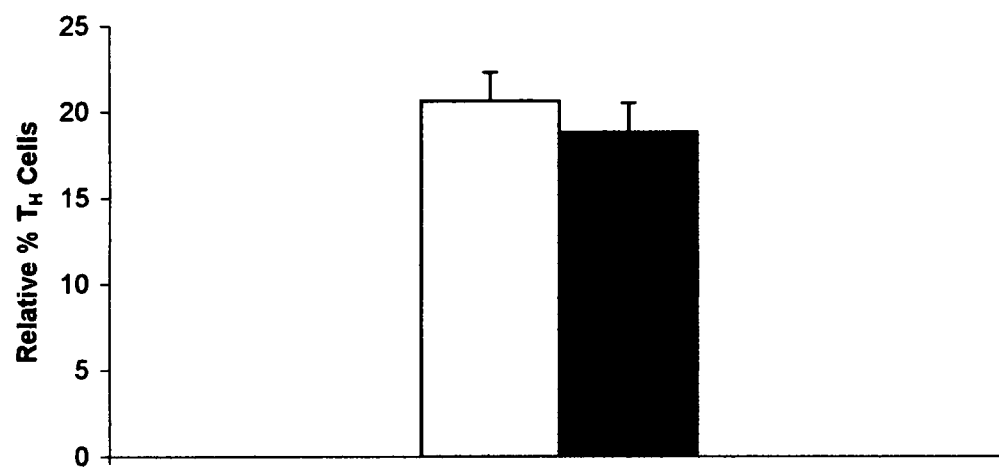
PBMCs from old and young felines fed either control or ThiomaxTM supplemented diet were stimulated with 2.5 µg Con A in order to measure the production of PGE₂. On day 0 young felines exhibited elevated levels of PGE₂ compared to old felines (Figure 9). However, animals maintained on ThiomaxTM exhibited no difference when compared to controls (Figure 9). On day 60 ThiomaxTM supplementation significantly increased PGE₂ production in old feline PBMCs compared to those on the control diet, but supplementation did not affect PGE₂ levels in young animals (Figure 9). No effect was seen at day 30 (Figure 9).

Figure 7: Impact of ThiomaxTM supplementation T cell subsets in young and aged felines using single color flow cytometry. PBMCs from young and old felines on either the control or ThiomaxTM supplemented diet were labeled with monoclonal antibodies (mAb) to CD3 (total T cells) (7A), CD4 (T-helper cells) (7B), and CD8 (cytotoxic T cells) (7C). Cells stained with the respective mAb were labeled with fluorescein isothiocyanate (FITC)-conjugated goat F(ab')² anti-mouse IgG+IgM (H+L)^d. Labeled cells were run on a flow cytometer to obtain relative numbers of the respective T cell populations. Values for young and old felines from day 30 and 60 were grouped together for analysis. Different letters denote a statistically significant difference between groups ($p \leq 0.05$).

A - Total T cells (CD3⁺)



B - Helper T (T_H) Cells (CD4⁺)



C - Cytotoxic T (T_C) Cells (CD8⁺)

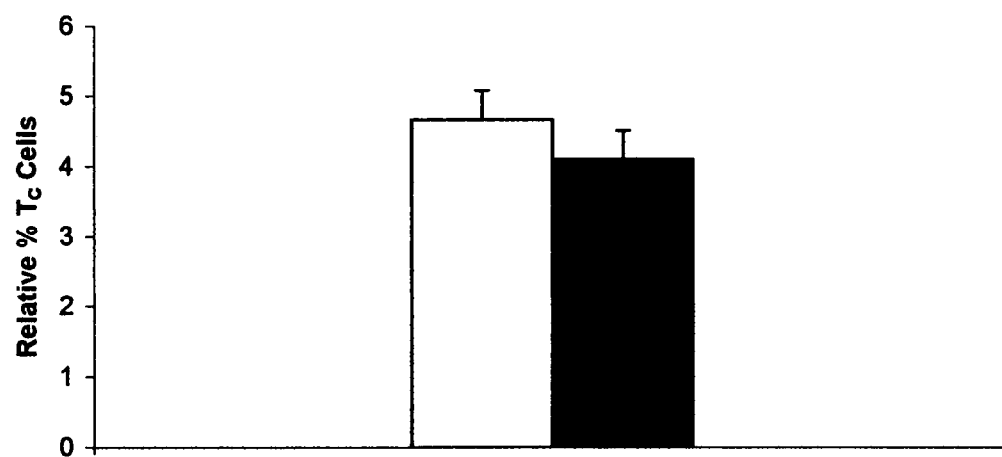
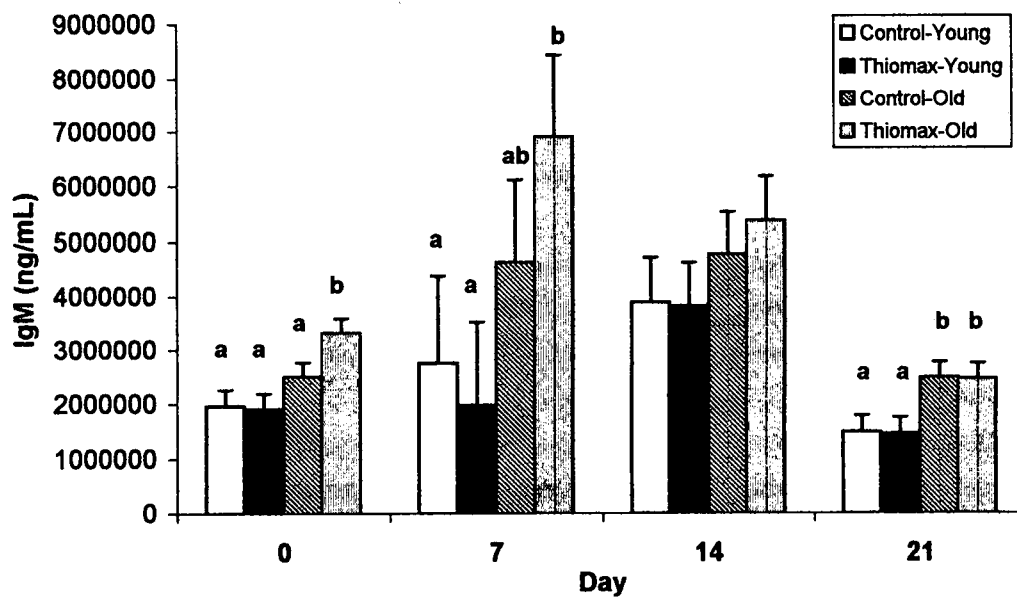


Figure 8: Effect of ThiomaxTM supplementation on IgM and IgG levels in young and aged felines. Young and old felines on either a control or ThiomaxTM supplemented diet were vaccinated with the Fel-O-Vax® vaccine, and 2 weeks later challenged with the same vaccine. Serum was collected from all animals on days 0, 7 14, and 21. Serum was analyzed for IgM (**8A**) and IgG (**8B**) levels. Values are expressed as ng/ml of antibody. Different letters denote a statistically significant difference between groups ($p \leq 0.05$), where as groups that share a letter are not significantly different from each other.

A



B

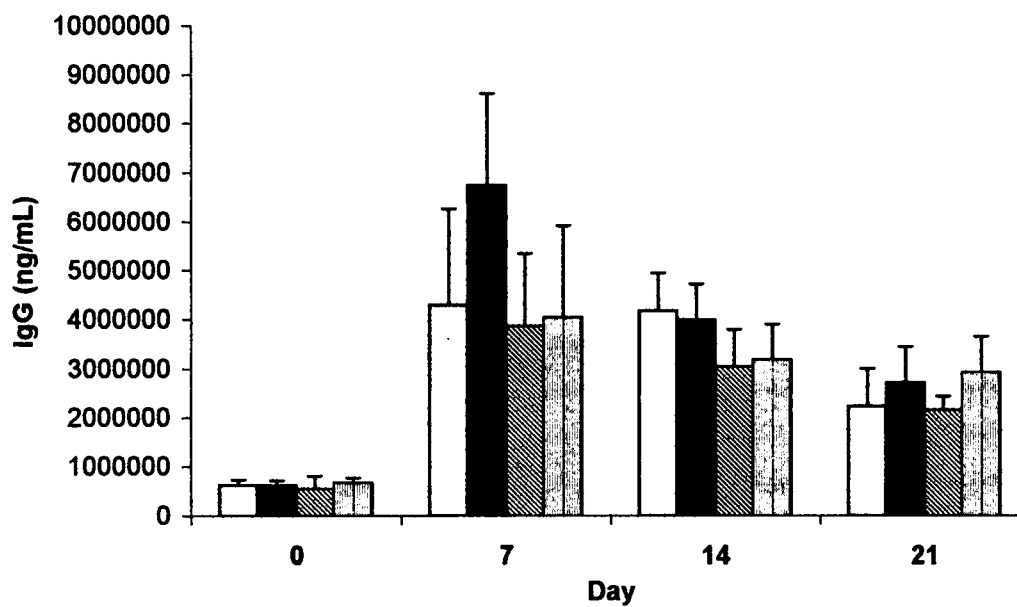
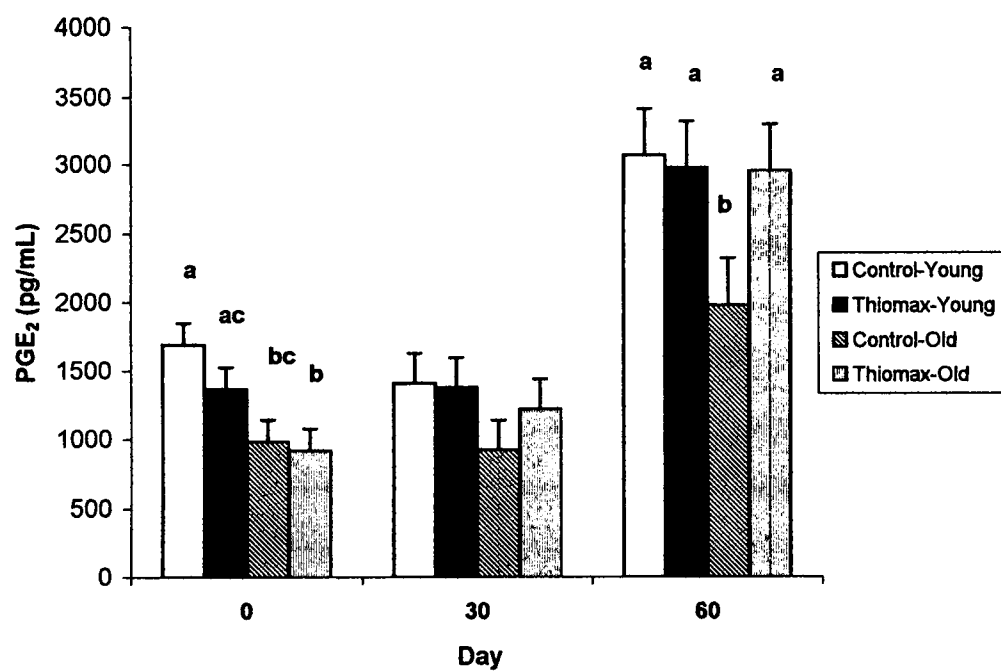


Figure 9: Influence of ThiomaxTM supplementation on prostaglandin E₂ (PGE₂) production in young and aged felines. PBMCs from old and young felines were stimulated with 2.5 µg Con A for 18-20 hours. The supernatants from each culture were collected and levels of PGE₂ were measured. Different letters denote a statistically significant difference between groups ($p \leq 0.05$), where as groups that share a letter are not significantly different from each other.

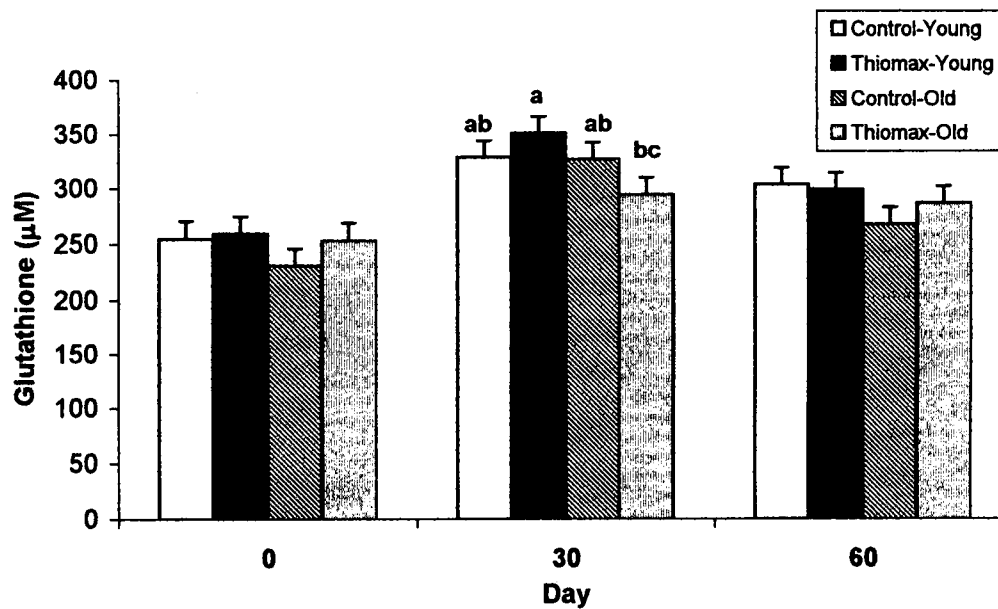


Influence of ThiomaxTM supplementation on Intracellular Glutathione Levels and Lipid Peroxidation

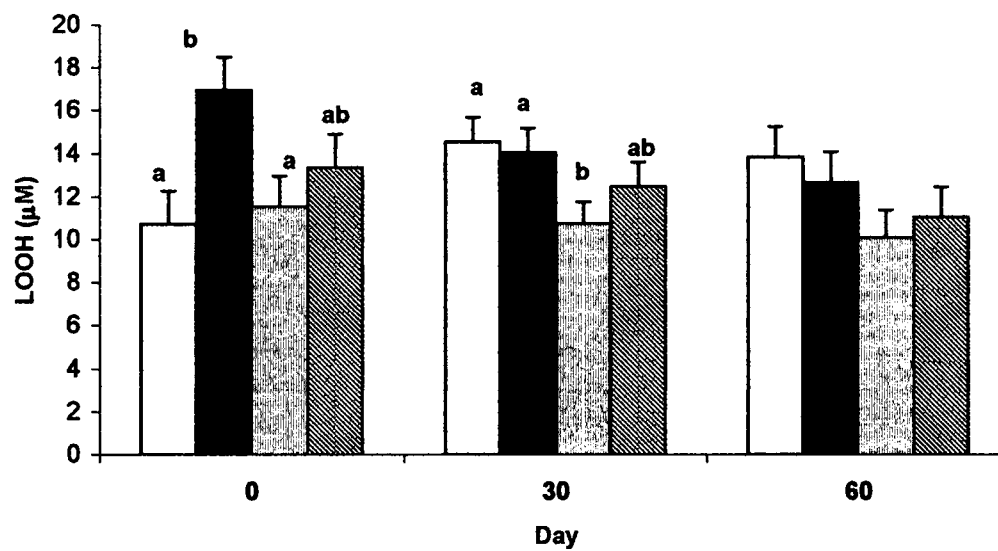
On day 30 of the treatment period, ThiomaxTM supplementation resulted in an increase in glutathione production in young felines, however, in aged animals there was a reduction in glutathione (Figure 10A). However, these values were not statistically different (Figure 10A). There was no significant difference in intracellular glutathione levels between groups on day 0 and 60 (Figure 10A). In order to assess oxidative stress, serum samples from young and old felines were used to measure lipid hydroperoxide production. On day 0 of the treatment period, young felines fed a ThiomaxTM supplemented diet, had significantly elevated levels of lipid hydroperoxide when compared with young control and old felines (Figure 10B). On day 30, young felines on either the control or ThiomaxTM supplemented diet had higher serum levels of lipid hydroperoxides than old felines, however old felines on the ThiomaxTM supplemented diet had slightly elevated levels compared to old control and similar to young felines (Figure 10B).

Figure 10: Impact of ThiomaxTM supplementation on intracellular glutathione levels and lipid peroxidation in young and aged felines. Intracellular glutathione from red blood cell lysates of both young and old felines from days 0, 30, and 60 of the treatment period were analyzed (10A). Serum samples from young and old felines on days 0, 30, and 60 of the treatment period were used to measure the levels of lipid peroxides (10B). Values were expressed as μM amounts per sample. Different letters denote a statistically significant difference between groups ($p \leq 0.05$), where as groups that share a letter are not significantly different from each other.

A



B



DISCUSSION

The objective of this study was to determine whether age and/or diet had any effect on immune function, through measurement of mitogen-induced lymphocyte proliferation, lymphocyte subsets, antibody production, and PGE₂ production in young and old felines. Similarly, the effects of ThiomaxTM on glutathione status, and oxidative stress, as measured by serum lipid peroxidation, were measured in these animals.

In order to assess the effect of age on cell-mediated immune function and lymphocyte responsiveness, mitogen-induced lymphocyte proliferation was assayed in PBMCs obtained from young and old felines. Data from these experiments demonstrate that following stimulation with 0.25µg of ConA, old feline PBMCs had significantly lower lymphocyte proliferation than PBMCs from young felines (Figure 1A).

Stimulation with 0.25µg PWM produced a similar trend of reduced lymphocyte proliferation in old feline PBMCs compared to young feline PBMCs, though not significant (Figure 1B). These findings are consistent with a study in which old felines had significantly reduced mitogen-induced lymphocyte proliferation in response to Con A and PHA compared to young felines (83). In a study by Meydani et al., young canine PBMCs stimulated with the mitogens Con A and PHA had significantly higher proliferation than old canine PBMCs stimulated with the same mitogens (82). In a similar study, mitogen-induced proliferation in response to Con A, PHA, and PWM was significantly lower in old canines compared to young canines (81). This reduced proliferative response to mitogen stimulation is also seen in humans, highlighted by a study in which PBMCs from elderly subjects had significantly reduced lymphocyte

proliferation in response to PHA (34). This trend of reduced proliferative response to mitogenic stimulation in the aged is also seen in the murine model (27, 31, 33).

Another parameter used to assess the impact of age on immune function was the enumeration of lymphocyte subsets. In order to delineate relative numbers of T lymphocyte subsets in old and young felines, flow cytometric analysis was performed on young and old feline PBMCs stained with antibodies for specific T lymphocyte antigens, including CD3 (total T cells), CD4 (helper T cells), CD8 (cytotoxic T cells). We found that there was a significant decrease in CD3⁺ and CD8⁺ T cells, as well as a strong trend towards a decrease in CD4⁺ T cells in old felines compared to young felines (Figure 2A-C). In companion animals, there seems to be some discrepancy as to the aged related alterations in these T cell subsets. Kearns et al. reported decreased percentages of total T cells and CD4⁺ cells, but not CD8⁺ cells, in old canines compared to young canines (80). Another study found similar levels of CD3⁺ T cells and CD8⁺ T cells in young and old canines, but a decrease in CD4⁺ cells in old canines compared to young canines (91). Conversely, Greeley et al. reported decreased total T cells in young canines compared to old canines, whereas CD4⁺ and CD8⁺ values were similar between age groups (81). While there are discrepancies in the data, there seems to be a common trend of decreased CD4⁺ T cells, which agrees with our findings of a strong trend towards decreased CD4⁺ cells in aged felines. However, in our study we also found significant decreases in CD3⁺ and CD8⁺ T cells, conflicting with some of the previous studies done in the canine model system.

A decrease of the CD4⁺ compartment of T cells may have a significant impact on immune function. CD4⁺ Helper T cells (T_H cells) produce IL-2, which is essential for T

cell growth. In humans(35) and mice (27), aged subjects produce significantly less IL-2 than young subjects. This reduction of IL-2 production seen in the aged may be a consequence of decreased CD4⁺ T cell subsets. The decrease in IL-2 may contribute to a decrease in CD8⁺ T cells, and this decrease may impact the ability of an individual to fight infections and cancer. CD4⁺ T_H cells also secrete cytokines such as IL-4, which play an integral role in humoral immunity through activation and differentiation of B cells (42). Therefore a decrease in CD4⁺ T cells could negatively impact both cell-mediated and humoral immune function. CD4⁺ T_H cells also aid in the activation of macrophages by production of interferon- γ (IFN- γ). Activated macrophages act as potent antigen presenting cells as well as facilitating the clearance of bacterial pathogens through phagocytic and cytotoxic mechanisms. There is a dearth of information concerning aged related changes in lymphocyte subsets in companion animals, especially in the feline model, and further studies need to be conducted to find consistent results.

Humoral immunity was assessed in young and old felines by measuring IgM and IgG antibody levels in response to antigenic challenge with Fel-O-Vax®. Old felines had significantly higher IgM antibody levels at days 0 and 7 than young felines (Figure 3A), demonstrating an age difference in B cell responses and IgM production. IgM is the first antibody secreted in a primary immune response, whereas upon secondary challenge IgG is the primary antibody produced. This elevated IgM production seen in aged felines post-challenge may be due to an inadequate or depressed memory B cell response. In a study by Brietbart et al., elderly human subjects had significantly lower percentages of memory B cells(32). Although percentages of memory B cells were not measured in this study, alterations in the memory B cell compartment may have led to the elevated IgM

production seen in these aged felines. $CD4^+ T_H$ cells secrete cytokines which influence the class of antibody produced during an immune response (24), and a change in the $CD4^+ T_H$ cell compartment in aged individuals may impact antibody production. However, it seems in felines that the production of IgG in response to antigenic challenge is not influenced by age. In this study, however, only total IgG and IgM antibody production were measured, and antigen specific antibody responses were not assessed. In a study by Gardner et al., elderly subjects had significantly decreased antibody titers to three different influenza vaccines compared to young subjects (36). Measurement of antigen specific antibody responses in future studies may elucidate potential differences in humoral immune function in young and aged felines.

PGE_2 is an important lipid inflammatory mediator with numerous and diverse biological functions. PGE_2 is involved in bronchodilation, modulation of inflammatory responses, and wound repair(92). PGE_2 is intimately involved in immune function, impacting cell-mediated immune responses as well as the production of cytokines(93). In T cells PGE_2 can inhibit proliferation and IL-2 production, regulate T cell maturation and activation, and induce $CD4^+ T_H$ cells to secrete T_H2 cytokines, such as IL-4 and IL-10 (93, 94). In this study, younger cats produced significantly higher levels of PGE_2 than old cats (Figure 4). Based on several murine macrophage experiments in which aged murine macrophages produced significantly higher levels of PGE_2 than young murine macrophages (47-49), the expected result would be an increased production in aged felines and not in young felines. The feline model system is one that has not been studied as exhaustively as the rodent or human system, and felines may respond differently in their secretion and response to PGE_2 . The elevated levels of PGE_2 produced by young

felines did not negatively impact mitogen-induced lymphocyte proliferation. Therefore, the young felines may not be as sensitive to PGE₂ as old felines.

Glutathione (GSH) is an important intracellular antioxidant with potent free radical scavenging capabilities. Levels of intracellular GSH in young felines were significantly elevated compared to those in old felines (Figure 5A). Studies have confirmed the age related decrease in intracellular glutathione in aged humans (95), and rodents (10, 51, 75). In the murine model, this decrease in glutathione in the aged is accompanied by decreased *in vivo* (delayed-type hypersensitivity (DTH)) and *in vitro* (mitogen-induced lymphocyte proliferation) cell-mediated immune responses (75). In *in vitro* studies in which intracellular GSH has been depleted, there is a marked decrease in lymphocyte proliferation in human PBMCs (54). In an *in vitro* study using human skin fibroblast and human umbilical vein endothelial cells, depleting cells of intracellular GSH significantly increased mitogen-stimulated PGE₂ production(55). Reduced glutathione levels in the aged may contributed to reduced immune function through reduced lymphocyte activation and proliferation as well as increasing the secretion of PGE₂, which at high levels exerts an immunosuppressive effect. In our study, old felines showed significantly reduced mitogen-induced lymphocyte proliferation to Con A than young felines, which may be due in part to significantly reduced levels of intracellular glutathione in old felines.

Lipid peroxidation can occur as a consequence of free radical attack, or oxidative stress, on lipids of the cell membrane resulting in disruption of membrane integrity and fluidity. In this study, serum samples from young and old felines were used to quantify lipid hydroperoxide production, which correlates to oxidative stress and cellular damage.

Compared to old felines, young felines produced significantly higher amounts of lipid peroxides (Figure 5B). Aging is associated with increased lipid peroxidation (6) and studies with rat hepatocytes showed significantly increased levels of lipid hydroperoxides, a by product of lipid peroxidation, in aged rat hepatocyte plasma membranes than those in young rats. However, in our study we found significantly higher lipid peroxidation in young verses old felines. This may be due to several factors. Collecting blood by veinipuncture in felines is a very stressful and invasive event for these animals, compared to canines or humans. At the time of blood collection these animals are undergoing extreme stress, especially the younger felines who may not be conditioned for this procedure. Serum samples are a snap shot in time for these animals, reflecting their condition right at the time of collection, not necessarily their overall condition. Therefore these younger felines might have been experiencing a load of stress (increased respiration and production of free radicals) greater than the older felines, reflected in higher lipid peroxidation values, translating into oxidative stress. Although the young felines had higher levels of lipid peroxides than old felines, they may be able to cope better with oxidative stress. It has been shown in rodents that several exogenous antioxidant enzymes decrease with age including superoxide dismutase, catalase, and glutathione peroxidase, as well as the aforementioned glutathione (51). It may be that younger felines are better equipped to deal with oxidative stress since their endogenous antioxidant defense system is more robust than those in old felines. PGE₂ was significantly elevated in young felines compared to old felines, and this may be a consequence of increased lipid peroxidation seen in young felines. Since prostaglandin production is initiated by peroxidation of membrane polyunsaturated fats (5), the

increased lipid peroxidation seen in young felines may contribute to the significantly higher levels of PGE₂ compared to old felines.

As well as discerning age related changes in several parameters looked at in this study, we also assessed the impact of ThiomaxTM supplementation on immune function. ThiomaxTM, manufactured by Health Span Sciences, is a proprietary antioxidant cocktail composed of α -lipoic acid and n-propyl gallate. α -Lipoic acid has a naturally occurring form, the R-enantiomer, and a synthetic form composed of a 1:1 mixture of the R- and S-enantiomers, which is used as nutritional supplements (88). α -Lipoic acid is taken into the cell and rapidly converted to its reduced form, dihydrolipoic acid (DHLA), which is a more potent antioxidant than α -lipoic acid (89). α -Lipoic acid and its reduced form DHLA have potent free radical scavenging properties against several reactive oxygen intermediates (60). In a study by Jones et al., pre-incubation of an endothelial cell line with α -lipoic acid neutralized H₂O₂ in culture produced by menadione, which is a known H₂O₂ generator (89). Since mitochondria produce H₂O₂ as a consequence of respiration, α -lipoic acid could therefore help to reduce the amount of oxidative stress in the cell (89). α -Lipoic acid also participates in important redox interactions with other endogenous antioxidants (88). The α -lipoic acid/DHLA redox couple seem to be able to regenerate several important endogenous antioxidants including ascorbate (vitamin C) and vitamin E, supported by evidence that α -lipoic acid supplementation prevented symptoms of vitamin C and E deficiencies (60). Another important interaction of α -lipoic acid is that α -lipoic acid supplementation increases intracellular glutathione concentrations both *in vivo* and *in vitro* (88, 89). In a study by Hultberg et al., the addition of 100 and 500 μ M α -lipoic acid to HeLa cells for 3 days significantly increased

intracellular glutathione concentration compared to the controls (96). Cysteine is essential for glutathione synthesis, but when cysteine is extracellular it can be rapidly oxidized to cystine, thereby limiting glutathione synthesis (96). α -Lipoic acid is taken up into the cell and rapidly reduced to dihydrolipoic acid (DHLA), which is released out of the cell and reduces extracellular cystine to cysteine, thereby allowing cysteine uptake and glutathione synthesis (96). It is because of α -lipoic acid's many antioxidant properties that it is dubbed "a universal antioxidant".

n-Propyl gallate is a synthetic antioxidant used extensively in fats and oils to prevent spoilage, as a food preservative to prevent lipid peroxidation, and in cosmetics. n-Propyl gallate is a chain breaking antioxidant, which stops lipid peroxidation through prevention of propagation of further radicals and can function to inactivate reactive oxygen intermediates before they can initiate the lipid peroxidation process (97). n-Propyl gallate also exerts a protective effect by enhancing the antioxidant enzymes catalase, which reduces H_2O_2 to water and oxygen, and glutathione reductase, which regenerates reduced glutathione (GSH)(98). n-Propyl gallate has been shown to protect glutathione depleted lens epithelial cells from oxidative injury when exposed to H_2O_2 (90). In another study, mice supplemented with n-propyl gallate were exposed to phosgene, which caused lung injury, and supplementation with n-propyl gallate increased survival, reduced lipid peroxidation, and prevented phosgene-induced glutathione depletion in the lungs compared to control mice (98). The components of ThiomaxTM have potent antioxidant properties and it was our aim to assess if dietary supplementation with this antioxidant cocktail would affect immune function in young and aged animals.

The effect of ThiomaxTM on mitogen-induced lymphocyte proliferation was measured using PBMCs from young and old felines. ThiomaxTM supplementation increased proliferation of PBMCs from old felines stimulated with 0.25 µg of Con A or PWM on Day 30 (Figure 6A-B). This increase in proliferation suggests there may be a beneficial effect of ThiomaxTM supplementation in old felines, however the same may not hold true for young felines since ThiomaxTM had no effect on proliferation in young felines. While there was a possible trend of increased proliferation with ThiomaxTM supplementation, there was no effect of ThiomaxTM supplementation on lymphocyte subsets in old or young felines.

In contrast to our results, several studies have confirmed that antioxidant supplementation increased mitogen-induced lymphocyte proliferation and modulated lymphocyte subsets in companion animals. β-carotene supplementation has been shown to increase mitogen-induced proliferation of PBMCs in response to Con A, PHA, and PWM in both young and old canines, and also increased the percentage of CD3⁺ and CD4⁺ T cells in old canines compared to old canines on a control diet (80). In another study, β-carotene supplementation of 20 or 50 mg/day significantly increased the percentage of CD4⁺ T cells on week 8 of treatment (84). Similarly, dietary lutein supplementation in young canines and felines significantly increased mitogen-induced lymphocyte proliferation (86, 87). In canines, 20 mg/day of dietary lutein increases the percentage of total T cells and CD8⁺ T cells at 8 weeks of treatment, whereas 5 mg/day of dietary lutein increased the percentage of total T cells and CD4⁺ T cells at 12 weeks of supplementation (87). In felines, 10 mg/day of dietary lutein significantly increased CD4⁺ T cells at 12 weeks of supplementation (86). In old felines, vitamin E

supplementation significantly increased mitogen-induced proliferation in response to Con A stimulation compared to old felines on a control diet (83).

The effect of ThiomaxTM supplementation on humoral immunity was assessed by measuring IgM and IgG antibody levels in response to antigenic challenge with Fel-O-Vax®. On day 0 and 7 post-challenge, old felines on the ThiomaxTM supplemented diet had significantly higher IgM levels than young felines while on day 21 old felines had higher IgM levels than young cats regardless of treatment group (Figure 8A). In either young or old felines, ThiomaxTM supplementation did not increase IgM or IgG production compared to the age matched controls (Figure 8A-B). There was no significant difference between groups, either old or young, control or ThiomaxTM supplemented, in IgG levels in response to vaccine challenge (Figure 8B).

Several studies have shown that antioxidant supplementation can increase antibody production in companion animals in response to antigenic challenge. Increased antibody production would be beneficial since antibodies serve an important protective function against bacterial and viral infections in which they serve to neutralize infectious particles and toxins and aid in the recruitment of immune cells and clearance of microorganisms. In canines, dietary lutein increased IgG production (87) and β -carotene supplementation increased IgG and IgM production (84, 85) in response to antigenic challenge. Similarly, dietary lutein increased IgG production in felines (86).

In evaluating the effects of ThiomaxTM on PGE₂ levels, we demonstrated that ThiomaxTM supplementation did not have an effect on PGE₂ in young or old felines. In fact, ThiomaxTM supplementation significantly increased PGE₂ production in old felines compared to old felines on the control diet on day 60 (Figure 9). As stated before, aged

animals have higher PGE₂ production than younger animals, which can be immunosuppressive. Several studies have shown that supplementation with antioxidants, especially vitamin E, has an suppressive effect on inappropriate secretion of PGE₂. With murine macrophages, supplementation with the antioxidants vitamin E and melatonin significantly reduced the spontaneous release of PGE₂ compared to control animals (4), while in another study vitamin E supplementation significantly reduced the amount of PGE₂ produced by old murine macrophages when stimulated with LPS (48).

As noted, several studies have documented the immunomodulatory effect of antioxidants on companion animals. In our study, we did not see a significant effect of ThiomaxTM supplementation on mitogen-induced lymphocyte proliferation, lymphocyte subsets, antibody production, or PGE₂ production. There seems to be a trend towards increased proliferation in old felines, but not young felines, on the ThiomaxTM supplemented diet, but only on day 30. The sample size for our experiments were 10 felines per treatment group, so perhaps a larger sample size may be needed to determine the significance of ThiomaxTM supplementation on these selected immune parameters. At the conclusion of the study, it was also determined that the dose of ThiomaxTM, back-calculated from human experiments, was 3 times higher than it should have been. This overestimation of the dose of ThiomaxTM may have contributed to suppression or lack of response since the optimal dose may not have been achieved. Further testing with the correctly calculated concentration and increased numbers in treatment groups may yield more consistent results. In addition, α -lipoic acid, a component of ThiomaxTM, has been shown to have some prooxidant properties (88). Since the dose of ThiomaxTM was higher than was optimal for humans, ThiomaxTM may have exerted a prooxidant effect, creating

an environment of oxidative stress, thereby not having the antioxidant effect ThiomaxTM was purported to have.

As stated previously, α -lipoic acid, a component of ThiomaxTM, has been shown to increase intracellular glutathione levels. There was no effect of ThiomaxTM supplementation on young or old cats in this study; ThiomaxTM did not increase intracellular glutathione (Figure 10A). In addition, ThiomaxTM did not reduce lipid peroxidation in the serum of young or old felines. Ironically, felines maintained on the ThiomaxTM supplemented diet had higher lipid peroxidation than old felines on the control diet (Figure 10B). Again, the overestimation of the dose of ThiomaxTM may have contributed to the lack of effect seen in these felines in regards to glutathione and lipid peroxidation.

In summary, this study confirmed the age related decrease in mitogen-induced lymphocyte proliferation, lymphocyte subsets, and intracellular glutathione. Age related changes in PGE₂ production and lipid peroxidation were atypical of results in other animal models, therefore more testing needs to be done to determine normal levels of PGE₂ and lipid peroxidation in old and young felines. We were not able to find any effect of ThiomaxTM supplementation in young and old felines in any of the parameters measured. Subsequent studies need to be conducted in which the optimal ThiomaxTM concentration is used with increased sample sizes.

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