An Assessment of the Effects of Oxidative Stress and Dietary Antioxidants on Toxin-Induced Dilated Cardiomyopathy in the Turkey (*Meleagris gallopavo*)

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In

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Keywords: Turkey, Dilated cardiomyopathy, Oxidative stress, Antioxidants, Biomarkers

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ABSTRACT

Dilated cardiomyopathy (DCM) or round heart disease is a muscle disease of the heart characterized by left ventricular dilatation and abnormal systolic and diastolic ventricular function. In animals, including turkeys and humans, DCM is a major cause of morbidity and mortality that results in heart failure. In the turkey, DCM can be idiopathic or induced. Since idiopathic or spontaneous DCM occurs in about 2-4% of normal turkeys, it is of significant concern to the poultry industry. This dissertation was designed to increase our understanding of the pathophysiology of DCM in commercial turkeys. Specific objectives included: evaluating the influence of dietary selenium (Se) and vitamin E on pouls fed toxic levels of furazolidone (Fz). Evaluating differences among reciprocal crosses of turkey varieties in susceptibility to a toxic level of Fz that induces DCM were used to assess the role of genetics in DCM. Using glutathione (GSH), glutathione peroxidase (GPx), malondialdehyde (MDA), and plasma uric acid (PUA) as biomarkers, oxidative stress (OS) levels were evaluated. Oxidative stress was also evaluated in pouls fed diets containing varying concentration and combinations of vitamin E (0, 50 and 100 IU/kg) and Se (0.0, 0.3 and 0.5 mg/kg). Results from echocardiography measurements at four weeks of age, for pouls fed toxic levels of Fz, showed the Narragansett x Bourbon Red reciprocal cross had the lowest internal-diastolic (LVIDd) and systolic dimensions (LVISd), while the Bourbon Red x Narragansett reciprocal cross had the largest LVIDd and LVISd. Left ventricular internal-diastolic and systolic dimension were lower for cross bred than parental pouls. In treatment pouls, heterosis for ventricular dilation was most significant for Bourbon Red x Narragansett cross. The data suggest that reciprocal crosses respond differently to toxin
that induces DCM and genetics may influence a turkey’s response to toxic levels of Fz that causes DCM. Results from OS measurements in poults fed normal and those fed normal diets with Fz at two weeks of age, showed no significant differences in MDA and GPx levels. PUA and GSH levels were however significantly increased for poults fed Fz-containing diets. At four weeks of age, no differences were observed for MDA and GPx measurements between poults fed normal and Fz-containing diets. PUA levels increased for poults fed normal diets with Fz, while GSH levels increased only for those fed normal diets. Differences between poults fed normal and Fz-containing diets were significant for GPx measurements. Results of this study showed that, feeding diets with Fz does not increase OS. Measure of the influence of feeding diets supplemented with different concentrations and combinations of Se and vitamin E to poults fed either normal or normal diets with Fz at two and four wks of age, showed higher MDA levels for poults fed Fz-containing diets supplemented with 0.3 mg/kg Se and 100 IU/kg vitamin E. For antioxidant biomarkers, GPx activity were increased for poults fed normal diets with Fz supplemented with 0.5 mg/kg Se and those fed 100 IU/kg vitamin E. Poults fed normal diets supplemented with 100 IU/kg vitamin E had the highest GPx. PUA levels were higher for poults fed normal diets with Fz supplemented with 0.5 mg/kg Se at two wks of age. At four wks of age, PUA concentrations were higher for poults fed Fz-containing diets supplemented with 100 IU/kg vitamin E. Increased PUA were also observed for poults fed diets supplemented with 0.5 mg/kg Se and 50 IU/kg vitamin E and 0.5 mg/kg and 100 IU/kg vitamin E. Poults fed diets supplemented with 50 and 100 IU/kg vitamin E had the highest GSH at two wks of age. Measurements taken at 2 wks of age, for poults fed normal diets supplemented with different concentrations and combinations of Se and vitamin E had increased GSH levels when compared with those fed diets with Fz at four wks of age. In this study, we showed that supplementation of
poults fed normal diets with Fz with different concentrations and combinations of Se and vitamin E did not reduce DCM at 2 wks of age. However, at 4 wks of age, though DCM was not decreased by feeding diets supplemented with different concentrations and combinations of Se and vitamin E, reduced oxidant and antioxidant biomarkers were observed.

**Keywords:** Turkey, Furazolidone, Dilated Cardiomyopathy, Genetics, Oxidative Stress, Vitamin E and Selenium
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DEDICATION

To my grandfather, the late Emanuel A. M. Tackie.
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ARVC</td>
<td>Arrhythmogenic right ventricular cardiomyopathy</td>
</tr>
<tr>
<td>BR</td>
<td>Bourbon Red</td>
</tr>
<tr>
<td>BS</td>
<td>Blue Slate</td>
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<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CHF</td>
<td>Congestive heart failure</td>
</tr>
<tr>
<td>DCM</td>
<td>Dilated cardiomyopathy</td>
</tr>
<tr>
<td>ECHO</td>
<td>Echocardiography</td>
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<tr>
<td>FR</td>
<td>Free radicals</td>
</tr>
<tr>
<td>Fz</td>
<td>Furazolidone</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HCM</td>
<td>Hypertrophic cardiomyopathy</td>
</tr>
<tr>
<td>HOO$^\cdot$</td>
<td>Hydrogenperoxyl</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>LVIDd</td>
<td>Left ventricular internal-diastolic dimension</td>
</tr>
<tr>
<td>LVISd</td>
<td>Left ventricular internal-systolic dimension</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondiladehyde</td>
</tr>
<tr>
<td>N</td>
<td>Narragansett</td>
</tr>
<tr>
<td>NO$^\cdot$</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO$_2$</td>
<td>Nitrogen dioxide</td>
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<tr>
<td>Symbol</td>
<td>Term</td>
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<td>--------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>$\text{O}_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>$\cdot\text{OH}$</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>OS</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>PGH</td>
<td>Prostaglandin endoperoxide</td>
</tr>
<tr>
<td>PUA</td>
<td>Plasma uric acid</td>
</tr>
<tr>
<td>RO$^.$</td>
<td>Alkoxyl</td>
</tr>
<tr>
<td>ROO$^.$</td>
<td>Peroxyl</td>
</tr>
<tr>
<td>RP</td>
<td>Royal Palm</td>
</tr>
<tr>
<td>RDH</td>
<td>Round heart disease</td>
</tr>
<tr>
<td>SB</td>
<td>Spanish Black</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TIDCM</td>
<td>Toxin-induced dilated cardiomyopathy</td>
</tr>
<tr>
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CHAPTER 1

Introduction

Dilated cardiomyopathy (DCM) or round heart disease (RHD) is a disease condition of the myocardium that is characterized by weakness of the heart muscle, left ventricular or biventricular dilation, cardiac hypertrophy, and depressed myocardial performance (Dec and Fuster, 1994). In animals, including turkeys and humans, DCM is a major cause of morbidity and mortality that results from heart failure. Economic losses caused by DCM, estimated to be millions of dollars in the turkey, are due to 1 to 4% mortality rate and reduced body weights of birds that survive after 4 wks of age (Lax et al., 1990).

Two types of DCM have been described in turkeys: idiopathic or spontaneously occurring (IDCM), and toxin-induced. Toxin-induced DCM mimics the physiological characteristics of IDCM and can therefore be used as a model to define the genetic and molecular basis of this abnormality. Over the years, continued interest in understanding and finding the potential etiology of this disease has led to the investigation of causal factors including morphology, physiology, and genetic.

Physiological factors are one of the most investigated causes of DCM in animals. Myofibril loss has been described as one of the primary physiological changes in DCM-affected animals (Sakia et al., 1996). In some animals, this loss is accompanied by sarcomeric disarray (Schaper et al., 1991). Beltrami et al. (1995) reported the myocardial alterations in DCM-affected hearts were due to myocyte loss, slippage of myocytes within the wall, segmental replacement, and interstitial fibrosis with hypertrophy of residual myocytes. Tagawa et al. (1996) previously suggested that the cytoskeleton, together with contractile proteins and the excitation-contraction coupling mechanisms of the heart, represents major determinants of the intrinsic
function of the heart’s myocyte. In bovine with DCM, Furuoka et al. (2001) reported physiological changes that included cardiomyocyte hypertrophy, interstitial fibrosis, vacuolation of the cardiac muscle fibers, and severe overall fibrosis. Other physiological changes associated with DCM that have been reported in animals include dilation of the chambers, thickening of the ventricular wall, pulmonary edema, and, occasionally, ascites (Currie, 1999). In Holstein-Friesian cattle, Nart et al. (2004) reported that increased necrosis in nuclear transverse cross-sectional area and cardiomyocyte length were the primary morphological characteristics observed in DCM-affected animals. Though investigations into physiological and morphological changes associated with DCM in turkey have been limited, Marian and Roberts (1994) reported problems with the membrane transport mechanism in furazolidone (Fz)-induced DCM-affected turkeys.

Biochemical factors have also been investigated as possible causes of DCM. Weekes et al. (1999) identified proteins with altered expressions in the left ventricles of DCM-affected bovine crossbreds. Of the 35 proteins with altered expressions, 24 decreased and 11 increased. Two of these proteins, ubiquitin c and alpha 1 anti-proteinase, were significantly altered in DCM-affected hearts. Similar investigations in humans by Pleissner et al. (1997) identified myosin light chain 2, ventricular (MLC2) and heat shock protein HSP 27 by protein microsequencing and gel map comparison to those with altered expression in DCM-affected individuals. Using 2-D gels, Heinke et al. (1999) also described several differentially expressed proteins in DCM affected canine hearts. The differentially expressed proteins including creatine kinase M and cytochrome b5 are known to function in energy metabolism and to be associated with mitochondria. This data and others from diverse proteomic investigations support earlier work in multiple species which showed that genetic differences significantly affect the incidence
and severity of DCM. Previous investigations into the inheritance of DCM by Hunsakar (1971) showed that commercial turkeys from different genetic backgrounds respond differently when fed diets containing toxic levels of Fz. Differences among the genetic lines were especially significant for percent mortality.

Durand (1999) reviewed previous reports of factors that influence DCM including genetic and environment. Though the consensus is that DCM is a heterogeneous disease, several single genes have been shown to be associated with cardiomyopathy in animals including humans. The genes include actin and desmin (Olson and Keating, 1996), α-tropomyosin, Troponin T, and Troponin I (Kamisago et al., 2000). Autosomal dominant inheritance is reported to be the predominant pattern of transmission of DCM with X-linked, autosomal recessive, and mitochondrial inheritance being less common (Mestroni et al., 1999).

The ability to treat and/or prevent the incidence and progression of DCM is dependent, in part, on determining the mechanisms responsible for the development of the disease. Recent studies suggest that because free radicals (FRs) increase in the failing myocardium, they may be important contributors to the deterioration of the myocardium and development of DCM (Dhalla et al., 1996). Increase in myocardial free radicals that cause oxidative stress (OS) has been discussed as capable of causing sub-cellular abnormalities leading to cardiomyopathic changes and depressed contractile function and failure (Gupta and Singal, 1989, and Prasad, 1989). Further, elevated levels of OS have been proposed to be associated with the pathogenesis of DCM (Gupta and Singal, 1989; Kirshenbaum and Singal, 1992, and Kirshenbaum et al., 1994). In a study by Pitkanen and Robinson (1996), it was observed that increased mitochondrial free radical production was a potential cause of familial DCM. Recently, plasma biochemical markers
for OS (MDA) have been reported to be increased in patients with congestive heart failure (CHF) including DCM (Keith et al., 1998).

It is known that the effects of FRs on lipids, DNA, and proteins can be influenced by a spectrum of exogenous dietary antioxidants including vitamin E, C, phenolic compounds, and by endogenous antioxidants including the scavenger enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx), (Gutteridge, 1995). In humans and animal models, antioxidants such as beta-carotene, ascorbic acid (vitamin C), and alpha-tocopherol (vitamin E) have been shown to reduce OS and slow the deterioration of left ventricular mechanical function (Shine et al., 2002). In other studies, increased OS (MDA) along with decreased activities of antioxidant enzymes such as GPx-1 and SOD have been shown to cause myocardial infraction (Hasenfuss, 1998). When the hearts of these animals showed signs of failure, SOD decreased and subsequent further decline was linked with progressive heart failure. This led to the hypothesis that a maladaptive decrease in antioxidant enzyme expression or activity may contribute to net OS and the development of heart failure (Hill and Singal, 1996).

The role of OS in the development of toxin-induced DCM in the turkey remains unclear. This dissertation project had as its primary objective, to determine the effect of OS on the incidence and severity of toxin-induced DCM in the turkey. It involved comparing OS levels in birds fed normal and Fz-containing diets and evaluated the effect of feeding synthetic antioxidants that are known to influence antioxidant status on DCM. The research tested the hypothesis that OS influences the incidence and severity of DCM. The work for the first time, determined if OS contributes to toxin-induced DCM in vertebrates. The specific aims of this dissertation research were:
Specific Aim 1: Evaluate the nature of the inheritance of toxin-induced DCM in the turkey.

This aim will extend the work of my Master of Science research, which showed that differences among five turkey varieties for the incidence and severity of toxin-induced DCM were significant. For this, I compared the incidence and severity of toxin-induced DCM in F\textsubscript{1} crosses to those in the parental varieties.

Specific Aim 2: Evaluate of oxidative stress levels in turkey poults fed normal and Fz-containing diets.

Because there is no consensus biomarker for OS, to achieve this aim, I will look at both oxidant and antioxidant levels in blood samples. Measurements of OS levels were based on diverse biomarkers reported to be good indicators for OS (Ames et al., 1981; Spanier and Traylor 1991).

Specific Aim 3: Evaluate the effects of dietary Vitamin E and Selenium on the incidence and severity of toxin-induced DCM in the turkey.

Supplements of selenium (Se) and vitamin E were fed to turkeys on toxic-levels of Fz. The effect of these dietary supplements on the incidence and severity of DCM as well as on OS will be evaluated. Changes in endogenous antioxidant levels have been reported to significantly cause degeneration of cardiac myocyte and cardiovascular diseases (Yucel et al., 1998). Additionally, imbalance in the levels of antioxidant or oxidant has been shown to interfere with homeostasis of the heart, which causes free radicals to activate subcellular changes that progress to cardiomyopathy and cardiac failure (Singal et al., 1998). Thus, as proposed in this dissertation, it is logical to evaluate the influence of vitamin E and Se, two consensus antioxidants, on toxin-induced DCM.
CHAPTER 2

Review of Literature

Introduction

This literature review described published work related to dilated cardiomyopathy. The review described cardiomyopathies, causes, and factors that may influence the incidence and severity of this disorder that is devastating to both the agricultural and biomedical industries through the effect on humans, livestock, and poultry. In addition to the genetics of the disease, the review discussed the effect free radicals have on the incidence of various cardiomyopathies and the role, if any, of vitamin E and selenium in reducing or increasing the incidence.

2.1 Cardiomyopathy

Cardiomyopathies are heterogeneous disease conditions of the myocardium that are often characterized by cardiac dysfunction which causes it to lose its efficiency in pumping blood to the body. In humans, cardiomyopathies represent a major cause of morbidity and mortality in both children and adults and are a frequent reason for cardiac transplantation (Towbin and Bowles, 2001). In the United States alone, cardiomyopathies are known to cause about $200 million per year in healthcare costs (Richardson, 1996). According to the World Health Organization, cardiomyopathies can be classified as hypertrophic, restrictive, arrhythmogenic right ventricular and dilated (O’Connell and Bristow, 1994). This classification system also includes specific cardiomyopathies that can be associated with ischemia, valve dysfunction, hypertension, myocarditis, metabolic disorders, systemic diseases, muscular dystrophies, neuromuscular disorders, toxic agents, and late pregnancy.
2.1.1 Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is reported to be an autosomal dominant genetic disorder which affects about 0.2% of the population (Maron, 2002). Clinical presentation of HCM ranges from symptomless to sudden cardiac death resulting from either mechanical or electrical anomalies in young adults. The diseased condition can be characterized pathologically by the asymmetrical thickening of the left ventricular wall and inter-ventricular septum and exaggerated pump function along with poor relaxation of the heart (Davies, 2000). The onset of typical HCM symptoms such as dyspnoea, angina, syncope, embolism, myocardial ischemia, mitral valve regurgitation, arrhythmias, and outflow tract obstruction have been reported to be highly variable in diseased individuals. Obstruction of blood flow from the left ventricle to the aorta has also been shown to be indicative of HCM. Diagnosis of affected individuals is often made on the basis of heart murmur, abnormal electrocardiogram, or abnormal results from echocardiogram.

Studies have proposed that HCM affected patients without a family history of the disease have sporadic mutations or mild forms of familial HCM in which phenotypic changes are difficult to identify. Moreover, studies involving genetic analyses have causally linked nine different chromosomal loci to HCM. Of these loci, eight have been shown to code for sarcomere proteins, and the newly discovered \textit{PRKA-2} which codes for a subunit of AMP-activated protein kinase (AMPK), (Bing et al., 1997). All 8 proteins have been shown to have different roles within the contraction-relaxation cycle and contribute significantly to energy homeostasis of the myocardium: for instance, $\alpha$ and $\beta$-myosin heavy chains have been reported to interact with actin filaments through hydrolysis of ATP to develop mechanical force; troponin T, troponin I, $\alpha$-tropomyosin, myosin light chain-1, and myosin light chain-2 have regulatory functions; myosin
binding protein C has a structural role, and the largest cardiac protein, titin, helps to transmit force. About 35% of familial HCM has been attributed to mutations in the gene that codes for β-myosin heavy chain, 20% to mutations in myosin binding protein C, 15% to mutations in troponin T, and less than 3 percent to mutations of the α-tropomyosin gene (Watkins et al., 1995). Another potential mechanism that causes cardiac hypertrophy might be increased cardiac contractility. It has been proposed to occur with rare defects of myosin light chain-1, myosin light chain-2, and α-tropomyosin genes causing an increase in Ca\textsuperscript{2+} sensitivity and force (Potter et al., 1996).

2.1.2 Restrictive Cardiomyopathy

Unlike HCM, restrictive cardiomyopathy is the least common type of cardiomyopathy. It can be associated with ventricular filling and with normal or decreased diastolic pressure of either or both ventricles with a normal systolic function (Richardson et al., 1996). In humans, restrictive cardiomyopathy has been proposed to be due to increased stiffness of the myocardium leading to an increase in ventricular pressure and a slight increase in volume (Davies, 2000). The increase of the ventricular wall reduces the ability of the ventricle to relax and fill during diastole. The presence of elevated jugular venous pressure, peripheral and pulmonary edema, and ascites have been observed in affected individuals (Schoenfeld et al., 1987).

2.1.3 Arrhythmogenic Right Ventricular Cardiomyopathy

Arrhythmogenic right ventricular cardiomyopathy (ARVC) was first recognized in the late 1970s (Corrado et al., 1997). The incidence and prevalence of this disease are not well documented. Clinical presentation is characterized by arrhythmias of the right ventricular origin ranging from premature beats to sustained ventricular fibrillation leading to sudden death (Carrado et al., 1997). It has also been reported to be the second most common cause of
unexpected mortality in young individuals especially athletes. In addition to its effect on the right ventricle of the myocardium, ARVC is also characterized by progressive loss of myocytes. The loss of myocytes is attributed to either massive or partial replacement of myocardium by fatty or fibro-fatty tissue advancing from the epicardium into the endocardium (Basso et al., 1996). ARVC is believed to be a familial disease in about 30% of affected individuals thus leading to an increased interest in the role of genetics in its incidence and severity.

Linkage studies have shown hereditary transmission of ARVC to be autosomally dominant (Carrado et al., 1997). The ryanodine receptor plays a crucial role in the electromechanical coupling through the control of calcium released from the sarcoplasmic reticulum into the cytosol (Tiso et al., 2001). Consequently, defects in this receptor could initiate an imbalance of calcium homeostasis that might trigger apoptosis. Mutations in the cardiac ryanodine receptor gene have been reported in four separate families from northern Italy to be associated with ARVC (Tiso et al., 2001).

2.1.4 Dilated Cardiomyopathy

The most studied and debilitating form of all cardiomyopathies is dilated cardiomyopathy (DCM). Dilated cardiomyopathy is a disease of the myocardium characterized by left ventricular dilatation and abnormal systolic and diastolic left ventricular function. Abnormal rhythms, known as arrhythmias, which causes disturbances in the heart’s electrical conduction have also been reported to be associated with DCM (Marian and Roberts, 1994). In DCM, myocardial muscle mass is increased and ventricular wall thickness is reduced (Hughes and McKenna, 2005). It has been reported that DCM-affected hearts assume a globular shape, with diffuse endocardial thickening, and atrial enlargement often with thrombi in the appendages. A study by Durand, (1998) reported the heart muscle to become thin and weakened and is unable to pump
the blood efficiently. The heart muscle stretches and dilates so that it can hold more but in time becomes even weaker, leading to symptoms of heart failure (Hughes and McKenna, 2005). These structural changes decrease the amount of blood ejected from the ventricle with systole and allow more blood in the ventricle after contraction. DCM has been reported to be prevalent in both human and animal species, thus making it of significant importance to both the agricultural and biomedical industries. A smaller volume of blood enters the ventricle during diastole and increases end-diastolic pressure and pulmonary pressures. The enlarged stretched ventricle alters valvular function, usually resulting in regurgitation. Cardiomyopathy occurs as a consequence of dilatation of the atria and ventricles (Dec and Fuster, 1994).

For instance, in humans, DCM prevalence has been reported to be about 40-50 cases per 100,000 individuals and account for more than 10,000 deaths annually in the United States (Manolio et al., 1992). In commercial turkey production, DCM has been reported to account for about 2-4 % mortality (Fram et al., 1999). DCM has also been recorded in many large and giant breed dogs (Sisson and Thomas, 1995). For instance, in Portuguese Water Dogs a form of DCM has been reported which develops within the first weeks or months of life (Dambach et al., 1999; Sleeper et al., 2002). In dogs, the dystrophin gene has been identified as being responsible for some cases of X-linked DCM (Towbin et al., 1993). Moise et al. (1991) reported canine X-linked muscular dystrophy as part of a syndrome, include severe cardiac involvement, and deletion of the entire dystrophin gene has been demonstrated in German Short-haired pointers with skeletal myopathy and dilated cardiomyopathy (Schatzberg et al., 1999).
2.2 Animal Models of DCM

During the last decade, considerable advances in our understanding and management of cardiovascular diseases have been made. These advances have been made possible in-part by the use of model systems. The use of animal models affords us a unique opportunity to extensively study diverse factors and conditions that may cause heart failure. Despite the advances, many of the animal models of heart failure developed to date, including canine and bovine are reported to have a number of shortcomings (Hasenfuss, 1998). One of the most important limitations is that the pathophysiological changes and characteristics of the failing human myocardium are not mimicked by these animal models (Hasenfuss, 1998).

A widely used animal model for DCM is the rat, \textit{(Rattus norvegicus)}, (Bers et al., 1991). As an animal model for human DCM, the rat’s myocardium exhibits a very short action potential which normally lacks a plateau phase (Bers et al., 1991). Additionally, normal rat myocardium has significantly more myosin heavy-chain isoform that shift towards the $\beta$-myosin isoform with hemodynamic load or hormonal changes which do not conform to that of humans (Swynghedauw, 1986). Further, the resting heart rate of the rat is five-fold higher in humans and the force–frequency relation is inverse (Bers et al., 1991).

The limitations of rats as DCM models have led to the use of dogs. The dog’s myocardium allows for the study of left ventricular function and volume that resembles that of humans. Like the human myocardium, the dogs contain a higher proportion of the $\beta$-myosin heavy-chain isoform that has similar excitation–contraction coupling processes (Lompre et al., 1981). Despite these physiological benefits of using the dog as a model for DCM, dogs are costly and require substantial resources with respect to housing, care, and breeding.

The turkey has been proposed as a model for human DCM (Genao et al., 1996). Unlike
many animal models, the cardiomyopathic turkey heart is strongly similar to that of human in terms of gross morphology, myocardial energetic, muscle physiology, myofilament properties, $\text{Ca}^{2+}$ metabolism, and the beta-receptor-adenylyl cyclase signaling system (Genao et al., 1996). With these similarities to human cardiomyopathy, the turkey appears to be an effective animal model.

2.3 Mechanism of Fz-Induction of DCM in the Avian Model

Furazolidone (Fz) or N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone is a nitrofuran that has been in use for the past decades in both humans and animals for the treatment of certain bacterial and protozoa infections (Calla, 1983). For veterinary services and poultry production in the Middle and Far East, Fz is still widely used to improve carcass quality (Graham and Quresci, 2000). However, in the United States and some European countries, Fz has been discontinued as a drug for food-producing animals, primarily due to its potential toxicity and observations of residues in catfish (Rupp et al., 1993), poultry meat and eggs (Kumar et al., 1994), and in pork (Hoogenboom et al., 1992). In animals, the 3-amino-2-oxazolidone ring of Fz has been found as an intact chemical compound tightly bound to proteins (Lu et al., 1998). In fact, at least 75–80 % of the protein-bound residues in the case of microsomes and cells, and 15–25 % in the case of livers of animals treated with Fz, still harbor intact 3-amino-2-oxazolidone rings (Hoogenboom et al., 1991). Making it of particular interest for consumer and animal safety since 3-amino-2-oxazolidone ring might be released not only from the parent compound, but also from protein-bound metabolites of the drug which can be present as residues in the edible tissues of farm animals (Vroomen et al., 1988).

The use of Fz as a research tool has, however, continued in the United States and elsewhere especially to induce toxicity that causes DCM. Understanding the mechanism that
underlines this toxicity remains of strong research interest. One effort towards this has involved the use of paramagnetic resonance (EPR) spectroscopy (Lax and Kukolich, 1992) to show that Fz is reduced to its corresponding nitro-anion radical by ascorbate and hypoxanthine. Glutathione (GSH) prevents the formation of nitro-anionic radical. It was suggested from their study that the generation of nitro-anion radical may be involved in the induction of cardiomyopathy by Fz, and that GSH might have a role in its prevention.

Some studies have shown that Fz reduces antioxidants in biological systems. For instance, in a study by Ali (1992), when Fz was given to rats as a single oral dose of 75, 150 or 300 mg/kg of body weight, it reduced glutathione (GSH) and ascorbic acid level, and increased lipid peroxidation activity were seen. In an in vitro study by Stroo and Schaffer (1989), Fz stimulated lipid peroxidation activity, reduced superoxide dismutase and, catalase, and stimulated oxygen consumption. Because myoglobin is known to protect the mechanical function of the heart from hypoxia by acting as a sarcoplasmic oxygen reservoir, O'Brien et al. (1992) investigated myoglobin's role in Fz-induced DCM. The study showed a 50 % decrease in myocardial myoglobin concentration in cardiomyopathic hearts. The decrease in myoglobin concentration in the heart correlated with changes in the biochemical and physiological indicators of myocardial performance.

Feeding diets containing Fz has also been shown to affect ATP production (Liao et al., 1996). Isolated hearts from turkeys with Fz-induced cardiomyopathy have been reported to have a 73 % decrease in their baseline isovolumic contractile performance. Biochemically, in the myopathic heart, there were significant decreases in the ATP (23 %) and phosphocreatine (42 %) concentrations and in creatine kinase activity 34 % (Liao et al., 1996). The results suggested that the decrease in energy reserve via the creatine kinase system contributes to reduced cardiac
function in the failing heart. Webb et al. (1991) showed that Fz cardio-toxicity in ducklings given 700 mg/kg affected the levels of creatine kinase isoenzymes.

Lax et al. (1994) evaluated the effect of Fz on Ca\textsuperscript{2+} homeostasis in cardiac muscle cells. They reported increased activity in chick cardiac myocytes of thapsigargin-sensitive Ca\textsuperscript{2+}-ATPase that did not affect Na+/Ca\textsuperscript{2+} exchange. This finding supported studies by O’Brien et al. (1991, 1993) which showed that defective Ca\textsuperscript{2+} channel function that results from abnormal Ca\textsuperscript{2+} homeostasis in cardiomyopathy.

Nevertheless, the mode of action of Fz remains generally without a consensus. Rossi et al. (1996) discussed the metabolic pathway affected by Fz which includes the progressive reduction of a nitro group that produce a variety of intermediates and end products. During the reduction, the nitro-anion radical generated, is re-oxidized rapidly to the parent compound by molecular oxygen in a process known as redox cycling (Rossi et al., 1996). Additionally, reduction is favored by hypoxic conditions and has been proposed to initiate formation of the nitroso and hydroxylamine-acrylonitrile intermediates as well as the formation of stable products that induce open-chain cyano-derivatives (Vroomen et al., 1987). Although the cytotoxic mechanism of action of Fz still remains largely unknown, it has been ascribed to either covalent binding of reactive intermediates to biological macromolecules or damage to cells caused by the redox cycling process, which produces a flux of superoxide’s and other toxic FR intermediates (Boyd 1980).

2.4 Free Radicals

Free radicals (FRs) are formed during a variety of biochemical reactions and cellular functions. By definition, FRs are molecules or macromolecular fragments with one or more unpaired electrons in their outer orbitals. The unpaired electron causes the chemical reactivity of
that FR (Slater, 1984). A compound is designated as a FR by either gaining or losing an electron. Free radicals typically have unpaired electrons which are represented by a superscript bold dot: R·. They can be either positively or negatively charged. Below are examples of FR reactions: (1) electron is donated from a reducing radical while an electron is accepted for an oxidizing radical; (2) hydrogen abstraction is then initiated; (3) after which, addition reactions occur; (4) upon completion of the addition reaction, self-annihilation reactions, and (5) disproportionateness occur respectively (Slater, 1984; Pryor, 1966).

(1) \( \text{CO}_2^- + \text{O}_2 \rightarrow \text{CO}_2 + \text{O}_2^- \)
(1) \( \text{OH}^- + \text{RS} \rightarrow \text{OH}^- + \text{RS}^- \)
(2) \( \text{CCl}_3^- + \text{RH} \rightarrow \text{CHCl}_3^- + \text{R}^- \)
(3) \( \text{CCl}_3^- + \text{CH}_2 = \text{CH}_2 \rightarrow \text{CH}_2 \text{(CCl}_3 \text{)} - \text{CH}_2 \)
(4) \( \text{CCl}_3^- + \text{CCl}_3^- \rightarrow + \text{C}_2\text{Cl}_6 \)
(5) \( \text{CH}_2\text{CH}_2^- + \text{CH}_3\text{CH}_2^- \rightarrow \text{CH}_2 = \text{CH}_2 + \text{CH}_3\text{-CH}_3 \)

In biological entities, FRs have been reported to cause damage to cells. For instance, covalent binding of FRs to membrane enzymes or receptors can modify activities of membrane components through covalent binding thus changing the structure and function of membranes (Nohebel, 1979). Through in vitro studies of both biological materials and model systems, FRs have been proposed to cause chemical modifications and damage to proteins, lipids, carbohydrates, and nucleotides (Poter, 1980). In animals, FRs is formed from the body's natural metabolic activity and as a byproduct of the immune system’s response to foreign organisms. Production of these FRs in vitro, or in cells and tissues in high amounts has been reported to have deteriorative effects. In biological systems previously studied, (Halliwell, 1993) have reported FRs to be generated from either endogenous including (mitochondria, phagocytes, xanthine oxidase, arachidonate pathways, peroxisomes, inflammation, and bimolecular
oxidation) or exogenous (cigarette smoke, radiation, ultraviolet light, pollution, chemical reagents and Industrial solvents) (Halliwell, 1993). Examples of these FRs include alkoxyl (RO·), superoxide (O2·−), hydrogen peroxyl (HOO·), hydroxyl (OH·), peroxyl (ROO·), nitric oxide (NO·), and nitrogen dioxide (NO2·). Superoxide anion radical is the primary radical produced in animals and humans during normal respiration in the mitochondria and from autoxidation reactions. A previous study by Chaudière and Ferrari-Iliou (1999) showed O2·− to form unstable complexes with transition metals of prosthetic groups which leads to inactivation of enzymes and the initiation of apoptosis of active site. However, O2·− can act as an oxidizing or reducing agent in biological systems. Studies have shown that O2·− by itself is not very potent and does not cross the lipid membrane bilayer (Kruidenier and Verspaget, 2002). Nevertheless, it can initiate a reaction with nitric oxide leading to the formation of peroxynitrate, an oxidant which can produce reactive intermediates (Kontos, 2001). In a study by Groives (1999), peroxynitrate was shown to initiate damage to biomolecules through nitration of tyrosine, nucleic acids, and lipids. Hydroxyl radical is the most reactive but short-lived species with the ability to damage any biological molecule it encounters (Yu, 1994). Hydroxyl radical has been shown to be generated in all living species from either natural or artificial sources, leading to the initiation of a chain reaction progressing to lipid peroxidation. Unlike O2·−, which can be detoxified by superoxide dismutase, the OH· cannot be degraded through enzymatic reaction since it requires diffusion. However, diffusion is slower than the half-life of OH·, causing it to react with anything in its surroundings. To ensure protection of important biomolecules, the use of antioxidants such as glutathione has been implemented.
2.5. Sources of Free Radical Production

2.5.1. Mitochondria

Mitochondrion, with an independent genome, is a membrane-enclosed organelle found in most eukaryotic cells that is responsible for the generation of adenosine 5'-triphosphate which produces chemical energy for the body (Henze and Martin, 2003). During the production of energy generation, the mitochondrion oxidizes products of glycolysis such as pyruvate and nicotinamide adenine dinucleotide produced in the cytosol. Previous research has shown this process to be dependent on aerobic respiration. Therefore, when the production of oxygen becomes limited, the glycolytic products generated can be metabolized by anaerobic respiration. Under normal physiological conditions, most of the oxygen is metabolized in a tetravalent pathway that yields no FRs. In this pathway, oxygen receives four electrons at a time to form water with no reactive intermediates. However, about 5 % of oxygen is metabolized by the univalent pathway that can lead to the production of FRs (Frodovich, 1978).

It has been estimated that in vertebrates, more than 90 % of cellular oxygen is consumed in mitochondria for energy metabolism, and 2 % of the consumed oxygen is converted into FRs (Toren, 2002). Typical example of the endogenous FRs generated has been discussed by Toren (2002). The study showed that 90 % of oxygen consumed undergoes various steps to produce water. However, during this process the oxygen could potentially lead to the generation of short-lived intermediates such as \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \) and \( \text{O}^\cdot \). It was further shown that complex III is the major source of ROS production in the mitochondrion. During this process, electrons from complex I or II dehydrogenases are transferred to coenzyme Q (Q), also known as ubiquinone. This leads to reduced coenzyme Q, \( \text{QH}_2 \) which undergoes a stepwise one-electron reduction using oxidized and reduced forms of cytochrome \( b \) and cytochrome \( c \) in the Q cycle. The
unstable intermediate in the Q cycle (Q) leads to superoxide formation by transferring electrons directly to molecular oxygen. The superoxide is enzymatically dismutated by superoxide dismutase to form H₂O₂ which is metabolized by enzymes that will be discussed later such as catalase (CAT) and glutathione reeducates (GPx) to recycle water and molecular oxygen leading to FR formation.

The FRs generated has been shown to alter mitochondrial and non-mitochondrial macromolecules including lipids, proteins, and DNA (Lee et al., 1997). In the mitochondrion for instance, nucleotide changes in mtDNA that are not intrinsically pathogenic may predispose it to modulate the effects of deleterious mutations. In turn, deleterious mutations may promote the accumulation of somatic changes through oxidative phosphorylation-related mutagens. This phenomenon could trigger a positive feed-back loop contributing to the progression of the mitochondrial dysfunction (Luft, 1994).

Free radicals generated in the mitochondria have been shown to lead to disease such as cancer, respiratory disorders, diabetes, neurological problems, seizures, thyroid dysfunction, and DCM. With respect to DCM, previous studies proposed the heart to be one of the most energy demanding tissues in the body. It is dependent on oxidative phosphorylation to supply the large amount of ATP required for beat-by-beat contraction and relaxation (Huss and Kelly, 2005). A study by Huss and Kelly (2005) reported that high energy is essential for maintenance of specialized cellular processes including ion transport, sarcomeric function, and intracellular Ca²⁺ homeostasis. Therefore, this high metabolism-function of the heart makes it predisposed to diseases. The progression of diseases of the heart such as DCM is associated with the consistent decline in mitochondrial respiratory pathway activity leading to diminished capacity for ATP production. Reduction in the ability of energy transduction leads to secondary dysregulation of
cellular processes essential for cardiac pump function such as, Ca^{2+} handling and contractile function, which commences in a downward spiral of increased energy demand and diminished function of the myocardium.

2.5.2 Polymorphonuclear Leukocytes (PMNLs)

Previous studies have proposed nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase system on the membrane surface of neutrophils to be a highly efficient source of FRs (Tauber et al., 1979). Although the enzyme is normally dormant, however, when activated by bacteria and/or cytokines, it exhibits a burst of oxygen consumption normally referred to as a "respiratory burst" (Babior, 1984). The respiratory burst increases FR, which are released into the external environment (Babior, 1984). As soon as the PMNLs are activated, the membrane bound NADH or NADPH-dependent oxidase enzyme is activated and oxidizes cytoplasmic NADH/NADPH to NAD^{+}/NADP^{+} and shuttles electrons to oxygen to form oxygen. The radical then undergoes dismutation to form H_{2}O_{2}. H_{2}O_{2} has been proposed either to metabolized to water or will undergo Haber-Weiss or Fenton reaction. In azurophilic granules of neutrophils, an enzyme known as myeloperoxidase is discharged into the phagocytic vacuole or extracellular fluid upon stimulation of neutrophils (Klebanoff, 1980). In the presence of H_{2}O_{2}, myeloperoxidase catalyzes the oxidation of halides by transferring two electrons from the halide ions to H_{2}O_{2} to yield highly reactive hypohalous acid (Weiss and LoBuglio, 1982).

2.5.3 Plasma Membrane

Arachidonic acid is released from membrane phospholipids by the action of phospholipase. A previous study has shown arachidonic acid to be metabolized by cyclooxygenase and lipoxygenase pathway. During metabolism by the cyclooxygenase pathway, carbon-centered FR intermediates are formed by a cyclooxygenase-mediated deletion of one
methylene hydrogen of the arachidonic acid (Freeman et al., 1982, and Weiss and LoBuglio, 1982). An oxygen-centered FR has been proposed to be generated during the breakdown of \( \text{H}_2\text{O}_2 \) to prostaglandin \( \text{G} \) (PGG), a cyclooxygenase product from arachidonic acid metabolism (Egan et al., 1976). This radical has been shown to be \( \cdot\text{OH} \). Hydroperoxidase converts PGG to \textit{prostaglandin endoperoxide} (PGH), and during this process, a number of FRs are generated (Kontos et al., 1980, and Kuehl et al., 1980). The conversion of PGH to thromboxane is also known to generate FRs (Salvador et al., 1977). Biosynthesis of prostaglandins and thromboxanes therefore results in hemoproteins, oxygen and carbon centered FRs, which react with biosynthetic enzymes to initiate damage to macromolecules (Freeman et al., 1982).

### 2.5.4 Enzymatic Production of Free Radicals

Of the enzymes that are known to catalyze FR production, xanthine oxidase is probably the most investigated and studied. It generates \( \text{O}_2^- \) during the reduction of oxygen to hydrogen peroxide (Freeman et al., 1982). Some studies have shown, however, the lack of FR intermediates produced by human xanthine oxidase (XO) since it serves as an in \textit{vivo} \( \text{NAD}^+ \) dependent dehydrogenase. During ischemia, XO is proteolytically transformed to \( \text{O}_2^- \) producing oxidase (McCord and Frodovich, 1968). McCord et al. (1982) reported that during the process of ischemia, adenosine triphosphate (ATP) is catabolized to ADP, AMP, and inosine. However, in the absence of re-phosphorylation, the adenine nucleotides are metabolized to hypoxanthine and xanthine. The enzyme xanthine dehydrogenase is then transformed into xanthine oxidase by a protease activated by increased free calcium. During reperfusion, hypoxanthine and xanthine react with \( \text{O}_2 \) in the presence of XO to form the \( \text{O}_2^- \) radical. In the presence of iron salts, \( \text{O}_2^- \) forms \( \text{OH}^- \) radicals (Chambers et al., 1985). Similar to OX, aldehyde oxidase has parallel properties as XO and has shown the ability to generate FRs (Rajgopalan, 1980). This occurs
during the catalytic cycle of dehydro-oratate dehydrogenase (Aleman and Handler, 1967), flavoprotein dehydrogenase (Massey et al., 1969) and tryptophan dioxygenase (Hirata and Hayaishi, 1971) to produce O$_2^\cdot$.

**2.5.5 Peroxysomes**

Peroxisomes are ubiquitous organelles in eukaryotic cells that participate in the metabolism and breakdown of fatty acids and other metabolites in a process called beta-oxidation. In this process, fatty acids are broken down two carbons at a time and converted to Acetyl-CoA. This product is then returned to the cytosol for further use. Some studies have suggested that divalent reduction of O$_2$ by oxidases found in peroxysomes lead to the production of H$_2$O$_2$ (Freeman and Crapo, 1982). For instance, D-amino acid oxidase has been shown as an example of a peroxysomal H$_2$O$_2^\cdot$ generating enzyme (Freeman and Crapo, 1982). The H$_2$O$_2$ generated by peroxysomal oxidases, is metabolized by peroxysomal catalases leaving the H$_2$O$_2$ to diffuse out of the peroxysomes and into the cytoplasm where it is converted to FR.

**2.6 Reactivity of Oxygen Free Radicals**

Though the sources of FR in animals is diverse, research has shown that FRs also vary in their reactivity (Pryor, 1986). While FRs can be relatively stable, those of biological interest tend to be extremely reactive and unstable and tend to have a short life span (Pryor, 1986). It has been reported that due to the highly reactive nature of FRs, they tend to exist at low concentrations ranging from $10^{-4}$ to $10^{-9}$ M and do not move far from their site of formation. Free radicals can act as either oxidizing or reducing agents. Previously it has been shown that when FRs reacts with a non-radical, other FRs generated in the process initiate a chain reaction.

**2.6.1 Superoxide**

Superoxide (O$_2^\cdot$) with a half life of $1\times10^{-6}$ is the main FR produced in biological systems.
during normal respiration in mitochondria and by autooxidation reactions throughout the rest of the cell. McCord and Fridovich (1969), showed $O_2^-$ to be a frequently encountered intermediate of $O_2$ in both biotic and abiotic systems. It is generated through radiolysis, photosensitized oxidations and autoxidation, and also enzyme catalyzed oxidations. Superoxide has been shown to initiate and propagate FR chain reactions and damage to cellular components (Weiss and LoBuglio, 1982). In aqueous medium, $O_2^-$ reacts with a variety of biological substrates as either an oxidant or a reductant (Halliwell and Gutteridge, 1990). Biologically, $O_2^-$ is toxic and can be deployed by the immune system to kill invading microorganisms. Studies have reported $O_2^-$ to be produced in large quantities by the enzyme NADPH oxidase for use in oxygen-dependent killing mechanisms of invading pathogens (Halliwell and Gutteridge, 1990). Furthermore, $O_2^-$ has been reported to undergo protonation in acidic pH to become a stronger oxidant. The protonation of $O_2^-$ can lead to the oxidization of amino acids, fatty acids, and $\alpha$-tocopherol (Weiss and LoBuglio, 1982). Superoxide has also been shown to react with nitric oxide to produce peroxynitrite which eventually decomposes to form $\text{OH}^-$. The $\text{OH}^-$ can then initiate damage to cellular substrates (Koppenol et al., 1992). Thus, regardless of limitations to $O_2^-$ reactivity, its sensitivity to biological targets makes it toxic.

### 2.6.2 Hydrogen Peroxide

Hydrogen peroxide ($H_2O_2$) is a pale blue viscous liquid which appears colorless in a dilute solution. It has been reported (Halliwell et al., 1992) to be non-toxic to cells though it has the ability to cross membranes easily at concentrations of 10-100 uM and attack cellular components and macromolecules. A study by Halliwell et al. (1992) reported that high $H_2O_2$ levels inactivate glycolytic enzymes or react with transition metal ions and inorganic or organic complexes yielding highly reactive intermediates.
2.6.3 Hydroxyl Radical

Hydroxyl radical (OH·) is a highly reactive and unstable but extremely powerful oxidant that is also short-lived. Although OH has the capability to interact with almost all biological substrates (Southern and Powis, 1988), previous study (Halliwell, 1985) has reported OH to be primarily involved in three types of reactions: addition reactions, hydrogen extractions, and electron transfers. Hydroxyl radicals usually cannot travel large distances in biological systems. Hence they react with substrates in close proximity to prolong their reactivity. Furthermore, OH has been reported to be produced from the decomposition of hydro-peroxides (ROOH) or, in atmospheric chemistry, by the reaction of excited atomic oxygen with water, thus, making it dangerous to organisms (Southern and Powis, 1988). Unlike superoxide, which can be detoxified by superoxide dismutase, OH· cannot be eliminated by an enzymatic reaction, as this would require its diffusion to the enzyme's active site. As diffusion is slower than the half-life of the molecule, it will react with any oxidizable compound in its vicinity, thereby initiating damage to macromolecules including carbohydrates, nucleic acids, lipids, and amino acids.

2.7 Oxidative Stress

In general terms, the damage done by FRs features the chemical reaction known as oxidation and FR attacks on tissue is known as oxidative stress (OS). Oxidative stress is the damage caused to protein, lipid, and DNA when the production of reactive oxygen species (ROS) or FRs exceeds the ability of the body to neutralize and/or remove it (Sies, 1991; Van Dyke, 2002). Previously, oxidative damage has been demonstrated to cause diseases such as cancer, cardiovascular diseases, Alzheimer’s, and aging (Diaz-Velez et al., 1996).
Over the years, interest in understanding the role of FRs and/or OS in cardiovascular diseases has lead to the use of both human and animal models (Singal et al., 1998). Research using such models suggests increased OS due to an increase in FRs or a relative deficit in the endogenous antioxidant reserve, in the pathogenesis of cardiomyopathies (Bendich et al., 1986; Gupta and Singal, 1989; Kirsherbaum and Singal, 1993, and Singal et al., 1998).

A study by Hill and Singal (1996) demonstrated, for the first time in myocardial infarct rats the development of congestive heart failure (CHF) that correlated with a progressive decrease in antioxidants and a concomitant increase in OS. Rats in the non-heart failure stage demonstrated increased levels of SOD, GPx, catalase activities, and vitamin E levels as well as significantly increased GSH (redox) indicating reduced OS (Kirsherbaum and Singal, 1993). However, rats in the moderate to severe heart failure stage had significantly decreased levels of SOD, GPx, catalase activities, vitamin E, and increased lipid peroxidation as well as depressed GSH (redox) indicating increased OS (Kirsherbaum and Singal, 1993).

Lipid peroxidation has been reported at high levels in cardiomyopathic hamsters (Kirsherbaum and Singal, 1993). Exposure of perfused rabbit interventricular myocardium to FRs such as O$_2^-$ and OH$^-$ have been shown to lead to the development of structural alterations in the vascular endothelium including the development of vacuoles and edema. Though FRs and OS appear to influence cardiomyopathies and other forms of cardiovascular diseases, research suggests that both enzymatic and non enzymatic antioxidants offer some protection against the damage caused by FRs and/or OS. Increased reactive oxygen species have been shown to be associated with diabetes (de Cavanagh et al., 2001), cancer (Neuzil et al., 2001), atherosclerosis (McQuillan et al., 2001), and cardiovascular disorders (Chen et al., 1980). In patients with IDCMDemirbag et al. (2005) showed reduced vitamin C and thiol levels.
2.8 Oxidative Stress and Dilated Cardiomyopathy

The heart is a highly metabolic organ with high energy demands. This high demand is normally met by the mitochondrion which is responsible for about 30% myocardia energy production (Graff et al., 1999). Mitochondria can cause a decline in total ATP production, which may lead to cardiac contractile dysfunction. In addition ROS and/or FRs that are generated in the mitochondria of the myocardium during oxidative phosphorylation, of xanthine oxidases, non-phagocytic NADPH oxidases, and cytochrome P450 also produced. The production of these compounds can also lead to an increased formation of ROS due to catecholamines auto-oxidation and the uncoupling of nitric oxide (NO) syntheses (Giordano, 2005).

When ROS formation exceeds cellular antioxidant production, OS results, which could cause damage to cellular lipids, proteins, and DNA leading to cell damage and death (Fosslien, 2003). OS may cause mutations in DNA by initiating strand breakage, purine oxidation, and protein-DNA cross-linking. It can also enhance changes to chromatin structure by altering gene expression and impairing cellular integrity and function (von Harsdorf et al., 1999). Studies by Giordano (2005) and von Harsdorf et al. (1999) reported that damage caused by OS is due to the ability of ROS and/or FR to induce strand breakage in DNA by initiating mutations, oxidizing purine bases, and potentially causing protein-DNA cross-linking. Modification of proteins by OS also leads to inactivation of critical enzymes. This may affect physiological processes such as function of ion channels and Ca\(^{2+}\) flux fundamental for normal myocardium (Giordano, 2005). OS may also affect L-type calcium channels on the sacrolemma and suppress the amount of Ca\(^{2+}\) produced (Giordano, 2005). It also depresses the activity of the sarcoplasmic reticulum Ca\(^{2+}\) ATPase, a membrane calcium pump essential in cardiac calcium handling and contractility.

The myocardium is able to handle and survive excess FRs and ROS due to a delicate
balance between oxidants and antioxidant defense mechanisms (Ferrari et al., 1991). In the heart, antioxidant defense mechanisms include the enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSHPx) and non-enzymes vitamin E, ascorbic acid, and cysteine (Ferrari et al., 1991). Pathological processes disrupt this cellular balance between oxidant and antioxidant by increasing the production of FRs. Research using animals as well as humans suggest that increased OS as indicated by biological markers (biomarkers) contributes to heart failure (Gupta and Singal, 1989; Kirshenbaum and Singal, 1992; Kirshenbaum et al., 1994). Increased lipid peroxidation has been reported in the case of cardiomyopathic hamsters (Kobayashi et al., 1987). Evaluating OS activity has been done primarily with the use of biomarkers.

2.9 Biomarkers of Oxidative Stress

Oxidative stress has been proposed as a significant factor in many diseases. However, definitive evidence for its association with diseases has often been lacking due, in part to the lack of biomarkers and/or reliable methods available to assess OS levels (Mak and Newton, 2001). For instance, previous research has proposed ROS to be short-lived and highly reactive making the accuracy of their in vivo measurement questionable.

A biomarker for OS is a biological molecule that changes when ROS and/or FRs change and can be objectively measured and evaluated (Offord et al., 2000). Biomarkers have been shown to yield important information on the nature of radical damage and antioxidant action in vivo, particularly regarding the nature of pro-oxidant effects, compartmentalization, and bioavailability (Bartosz and Bartosz, 1999). For a biomarker to be a good predictor of OS, it must be validated. The criteria for validation comprises intrinsic qualities such as specificity, sensitivity, degree of inter- and intra-individual variability, and knowledge of confounding and
modifying factors (Griffiths et al., 2002). Other characteristics of the sampling and analytical procedures of relevance include speed of the analytical method. Biomarkers of biological importance have been discussed by various studies indicating the progress or genesis of pathological conditions (Bartosz and Bartosz, 1999, and Asensi et al., 1994). Only a few of these biomarkers will be discussed here as they pertain to this dissertation research.

2.9.1 Lipid Peroxides

Lipid peroxides are one of the most common biomarkers used to indicate OS levels in animals. Masuda and Yamamori (1991) reported increased concentrations of lipid peroxidation end products in some diseases associated with OS. For example, peroxidation appears to be important in diseases such as atherosclerosis (Durak et al., 2000) and tissue injury (Ilhan et al., 2001) caused by ischemic or traumatic brain damage (Peker et al., 2004). Though several assays have been proposed to measure lipid peroxidation no single assay is considered an accurate measure. Lipid peroxidation yields a group of aldehyde products that are stable and can be measured in plasma. Malondialdehyde (MDA) is one of the most common lipid peroxidation products that are combined predictive of OS (Griffiths et al., 2002). It is a physiologic ketoaldehyde produced mainly by peroxidative decomposition of unsaturated lipids as a by-product of arachidonic acid metabolism. The product generated from MDA due to tissue injury can combine with free amino groups of proteins yielding modified protein adducts. The modification of proteins could potentially alter biological properties.

In a previous study (Halliwell and Chirico, 1993), MDA has been reported to be measured in plasma using the thiobarbituric acid-reactive substances (TBARS) assay. The product of the reaction of TBARS with MDA is measured using spectrophotoscopy or high-performance liquid chromatography (HPLC). Despite HPLC simplicity, numerous errors have
been associated with data interpretation. For instance, previous research has shown MDA to have some instability due to the rapid \textit{in vivo} metabolization into carbon dioxide and acetate by aldehyde dehydrogenase. Despite the disadvantages associated with use of MDA as a biomarker, it has been shown to be highly effective predictor and indicator of certain diseased conditions. In a study by Slatter et al. (2000), increased in plasma MDA were observed and atherosclerotic plaques promoted by diabetes.

Another product of lipid peroxidation used extensively as a sensitive and specific marker of OS is F$_2$-isoprostane (Morrow et al., 1990). Isoprostanes are oxidation products of arachidonic acid. A study by Roberts and Morrow (1997) reported that there are two separate routes of isoprostane formation, endoperoxide and a dioxethane. During isoprostane formation, 8-iso-IPF2-α is used as a marker for OS (Morrow and Roberts, 1999). Additionally, in plasma, studies have reported isoprostanes to have a short half-life of approximately 18 minutes and to be excreted rapidly. To maintain a steady-state, it must be formed constantly in biological substances (Morrow and Roberts, 1999). F$_2$-isoprostanes from biological sources can only be measured as free compounds using gas chromatography-mass spectrometry (Li et al., 1999). The disadvantage associated with measuring isoprostanes in plasma is that it is not possible to measure them over a period of time since the half-life is short. In a study by Yucel et al. (2002), higher lipid peroxidation activity was expressed in patients with dilated cardiomyopathy. Similarly, Castro et al. (2003) reported higher lipid peroxidation activity was observed in patients with chronic heart failure secondary to DCM or ischemia.

Conjugated dienes have also been proposed to be a product of \textit{in vitro} lipid peroxidation. Conjugated dienes are primary products of the breakdown of fatty acids. Nevertheless, they are considered to be specific products of FR reactions. Conjugated dienes can be measured
spectrophotometrically in the UV-region at 230–235 nm. However, a disadvantage associated with the diene assay is that many other biological substances are absorbed in the same UV-region (Corongiu et al., 1986), thereby making conjugated diene assay not suitable for determination of lipid peroxidation in vivo.

2.9.2 Amino Acid Oxidation

Lipid peroxidation consequences including amino acid oxidation, denaturation of proteins, and loss of function to the proteins are also used as indicators. For the analysis of oxidation products proteins, urine, plasma, cell, nucleus, mitochondria, and cytoplasm can be used. In a study by Griffiths et al. (2002), it was shown that modifications to histidine and lysine in low density lipoproteins lead to an alteration in the receptor lipoproteins. Protein oxidation stimuli lead to new functional group formation that includes hydroxyls and carbonyls (Dean et al., 1997).

Carbonyls have emerged as excellent biomarker for protein oxidation (Berlett and Stadtman, 1997). They are generated in response to oxidizing stimuli including alkoxy and peroxy radicals. As a biomarker, carbonyls measured by either immunodetection using ELISA or Western blot. Protein carbonyls are a generic marker of oxidation (Pompella et al., 1996). In a review by Chevion et al. (2000) alterations in protein carbonyls were shown to be most strongly linked with aging. Previous research has described protein thiols, aliphatic amino acids, oxidized tryptophan and tyrosine as biomarkers for OS (Robinson et al., 1998; Griffiths et al., 1992, and Shigenaga, 1999). Semi-quantitative analysis of thiols immunohistochemically by Wardman and von Sonntag (1995), showed a correlation with tumor growth (Wardman and von Sonntag, 1995).

Regardless of the changes in protein structures and diseases, studies have shown that
supplementation of diets with antioxidants can reverse impact on some of those modifications in protein carbonyls. Supplementation of a rat diet with flavonoid rutin for 18 days caused a reduction in protein carbonyl content (Funabiki et al., 1999). Srigiridhar and Nair (2000) supplemented the diet of rats with α-tocopherol or a combination of α-tocopherol (40 mg/day) and ascorbic acid (24 mg/day) for 15 days and showed that protection against protein carbonyl formation in iron-deficient rats during iron repletion increased. Tocotrienols reduced the protein carbonyl levels in the aging nematode, Caenorhabditis elegans (Adachi and Ishii, 2000). These limited studies are consistent in demonstrating a protective effect against plasma protein oxidation through dietary intervention.

2.10 Antioxidants

Animals need antioxidants to minimize the damage caused by ROS by delaying or reducing oxidation of cellular substrates (Halliwell et al., 1990). Antioxidants act as a chemical trap and physical quencher for FR and ROS (Bendich et al., 1986). Antioxidants show health-promoting properties in biological systems because they are easily oxidized (Stephens et al., 1996). Antioxidants can be either enzymatic or non-enzymatic (Fridovich, 1983). Non-enzymatic antioxidants which have been studied extensively in association with cardiovascular disease consist of Vitamin E, Vitamin C (ascorbic acid), flavonoids, and carotenoids. Selenium, a trace mineral that combines with proteins to form selenoprotein, has also been shown to have antioxidant properties.

2.10.1 Enzymatic Antioxidants

Enzymatic antioxidants provide the first line of defense against the indiscriminate damage that FRs and ROS cause cellular components (Chaudiere and Ferrari-Iliou, 1999).
2.10.2 Catalase

Catalase is a major enzymatic antioxidant that catalyses the two-stage conversion of hydrogen peroxide to water and oxygen. In animals, catalase is present in all major body organs and within cells in peroxisomes that have most of the enzymes capable of generating $\text{H}_2\text{O}_2$. Its activity in the liver and erythrocytes is known to be high. In erythrocytes catalase has been reported to provide protection against $\text{H}_2\text{O}_2$ generated through the dismutation of $\text{O}_2$ generated by hemoglobin autoxidation. Some organs including the brain, skeletal muscles, and heart contain lower levels of catalase, making them vulnerable to the damage caused by FR (Griffiths et al., 1992).

2.10.3 Glutathione Peroxidases

Another type of enzymatic defense to free radicals and ROS is glutathione peroxidase (GPx). Glutathione peroxidases catalyse the oxidation of glutathione (Ursini et al., 1995). Though it is widely distributed in almost all tissues, GPx is believed to be synthesized mainly in the kidney, though the highest concentrations are found in liver. The predominant subcellular distribution of GPx is in the cytosol and mitochondria (Rotruck et al., 1973).

2.10.4 Superoxide Dismutases

Superoxide dismutase (SOD) catalyses the dismutation of $\text{O}_2^-$ to $\text{H}_2\text{O}_2$. Three different forms of SOD have been described in mammalian tissues, each with a specific subcellular location and unique tissue distribution (Fridovich, 1997). Copper zinc superoxide dismutase (CuZn-SOD) is found in the cytoplasm and organelles of virtually all mammalian cells, it has a molecular weight of approximately 32000 kilodalton (KDa) and two protein subunits, each containing a catalytically active copper and zinc atom. Manganese superoxide dismutase (MnSOD) is found in the mitochondria of most cells with a molecular weight of 40000 kDa. It is
believed to contain four protein subunits each with an individual manganese atom. Fridovich (1997) reported that the amino acid sequence of MnSOD is different from that of CuZnSOD. The last SOD is extracellular superoxide dismutase (ECSOD). This is a secretory copper and zinc containing SOD distinct from the CuZnSOD described above. Previously, it has been reported to be synthesized by only a few cell types such as fibroblasts and endothelial cells, and is expressed on the cell surface where it is bound to heparin sulphates. A study by Karlsson et al. (1993) reported ECSOD to be the major SOD detectable in extracellular fluids and is released into the circulation from the surface of vascular endothelium following the injection of heparin. Enzymatic activity of SOD in the body consists of cell repair and reduction of damage done to cells by superoxide. Studies have shown that SOD acts both as an antioxidant and anti-inflammatory

2.11 Antioxidants and Dilated Cardiomyopathy

Numerous antioxidants have been discussed in relation to cardiovascular and other diseases of economic importance. Most investigations looked at the role antioxidants directly or indirectly play in ameliorating diseases. Here, my interest is in the role antioxidants may or may not have in reducing the toxic effects of furazolidone, which causes dilated cardiomyopathy. However, in this review focus was on two antioxidants which have been discussed extensively in relation to dilated cardiomyopathy.

2.11.1 Vitamin E

Vitamin E was discovered by Evans and Bishop in 1922, during an investigation into the relationship between fertility and nutrient intake in rats (Evans and Bishop, 1922). Vitamin E-deficient diet, when fed to rats, caused premature termination of pregnancies and in some cases fetal resorption. Over the years, structural analysis has revealed that vitamin E consists of eight
molecules of related structures including four tocopherols, alpha (α), beta (β), gamma (γ), and delta (δ) (Evans et al., 1936) and four tocotrienols, also α, β, γ, and δ (Pennock et al., 1964). A review by Schneider (2005), discussed the biochemical makeup of these eight vitamin E structural components. The four tocopherols have a chromanol ring with a saturated phytol chain. The tocotrienols consist of three chiral centers present at carbons 2, 4, and 8 and a natural occurring isomer which have an R-configuration at all three positions.

Previously, the biological activity of vitamin E has been shown to be dependent on the chemical configuration of its stereochemistry (Traber et al., 1990; Ingold et al., 1987; Burton et al., 1990). There are two forms of vitamin E, natural (RRR-α-tocopherol or α-tocopherol) and synthetic (all-rac-α-tocopherol). In natural vitamin E the methyl groups in 2, 4, and 8 positions have an R configuration while in the synthetic it can either be R or S. The way and manner in which the two tocopherol act in biological systems has been shown to be significantly different during post absorption in the rat's liver (Traber et al., 1990). Ferslew et al. (1993) reported of α-tocopherol preferentially taken up by plasma and red blood cells as compared with the synthetic form, thus indicating the bioavailability and ability to retain it in plasma and body tissue. Traber et al. (1998) also reported of all-rac-α-tocopherol as being preferentially excreted in urine as compared to the natural form.

A review by Pryor (2000), showed vitamin E to prevent certain types of chronic diseases, mainly those believed to be affected by OS. Li et al. (1997) showed reduced levels of α-tocopherol, both of bimolecular endogenous antioxidant levels, early in DCM-affected Syrian hamsters. Administration of α-tocopheryl acetate at higher concentrations restored α-tocopherol, GPx and protein oxidation levels to normal. Similarly, Hill et al. (2003) reported that Se and vitamin E deficiency resulted in decreased levels of GPx and α-tocopherol activity and increased
levels of creatine phosphokinase in guinea-pigs. In 1996, the Cambridge Heart Antioxidant Study (CHAOS) reported that supplementation of \( \alpha \)-tocopherol (400–800 IU/day) for slightly under 2 yrs significantly \((P<0.005)\) reduced cardiovascular death and non-fatal myocardial infarction by 77\% (Stephens et al., 1996). Meydani (1997) reported enhanced immune response as people aged when given supplements of \( \alpha \)-tocopherol. The daily administration of 100 mg of \( \alpha \)-tocopherol for several months increased the formation of antibodies in response to hepatitis B vaccine and tetanus vaccine in elderly adults. In a different study, Numes et al. (2005) investigated the molecular events involved in cell death caused by OS and the potential role of vitamin E and glutathione on cell death in chicken skeletal muscle cells and bovine fibroblasts. Results of the study suggested that OS activates caspase and promotes apoptosis. The presence of vitamin E and glutathione, however, provided protection against cell death from low levels of OS. Further showing the importance of vitamin E neutralizing OS, Avanzo et al. (2001) reported increased OS in chicken superficial pectoralis muscle due to deficiency in \( \alpha \)-tocopherol and Se leading to multiple alterations in the antioxidant system.

### 2.11.2 Selenium

Selenium (Se) is an essential trace mineral also referred to as selenocysteine (Ganther et al., 1976), the 21st amino acid. It is a component of certain enzymes including GPx, which enables it to function in catabolism of peroxidase, hence its role as an antioxidant. Selenium functions as a redox center, during DNA synthesis for selenoenzyme, thioredoxin reductase, reduces nucleotides and helps control the intracellular redox state (Allan et al., 1999). The best-known example of this redox function is the reduction of hydrogen peroxide and damaging lipid and phospholipid hydroperoxides to harmless products such as water and alcohols by GPx (Allan et al., 1999). Diplock (1994) reported this function to help maintain
membrane integrity, protect prostacyclin production, and reduce the likelihood of propagation of further oxidative damage to bimolecules such as lipids, proteins, and DNA with the associated increased risk of conditions such as cardiomyopathy, atherosclerosis and cancer (Néve, 1996).

In both humans and animals, Se is an essential trace element that is obtained from dietary sources including cereals, grains and vegetables. Selenium bioavailability has been reported to be influenced by endogenous factors, including growth, pregnancy or lactation, the efficiency of digestion, gut transit time and the presence of gastrointestinal disorders or disease (Jackson, 1997). Although the proportion of the nutrient absorbed from the gastrointestinal tract is a major determinant, tissue utilization of the absorbed nutrient and renal conservation of Se are also important factors influencing its bioavailability (Jackson, 1997). Waschulewski and Sunde (1988) reported Se bioavailability to be dependent on the conversion of absorbed Se into biologically active form and tissue retention. However, in animals, human’s inclusive, previous study has reported an association between Se deficiency and diseases of economic importance (Ganapathy and Thimaya, 1985). For instance, Keshan disease, an endemic cardiomyopathy appearing in women of childbearing age and pre-school children has been observed in people residing in low Se areas including China (Cheng and Qian, 1990). In an attempt to understand the etiology of Keshan disease, Cong et al. (2008) evaluated gene–environment interaction in the pathogenesis of Keshan disease by assessing the association of low blood Se and polymorphisms in GPx-1 gene. They reported lower blood concentrations of Se and GPx-1 activity in DCM-affected. Genetic analysis revealed a single nucleotide polymorphism (Pro198Leu) in the GPx-1 gene to be associated with Se deficiency as well as impaired GPx-1 activity. In a different though similar study, Oster et al. (1983) reported significant differences between serum Se levels in
congestive cardiomyopathy patients versus healthy individuals. Congestive cardiomyopathy patients had serum Se levels of 47.8 µg Se/ml while healthy individuals had 81.1 µg Se/ml.

Additionally Thomson et al. (1977) evaluated differences in blood Se concentrations in New Zealanders residing at two different geographical areas. Result of the study showed a close relationship between blood GPx activity, a biomarker for OS, and blood Se concentrations. Similarly, Vijaya et al. (2000) reported decreased levels of serum Se in DCM-affected patients. A study by Chou et al. (1998), reported significantly reduced serum Se concentration and blood GPx activity in IDCM-affected Taiwanese.

In animals, Se deficiency has been reported to result in hepatosis dietetica and mulberry heart disease in pigs, encephalomalacia and exudative diathesis in poultry, and myopathies in ruminants and horses (Blood et al., 1983). In cows, pigs, lambs and ducklings, combined vitamin E and Se deficiency leads to a number of conditions that affect heart function and causes myocardial damage (Van Fleet and Ferrans, 1977). Cawley and Bradley (1978) described Se deficiency in 40 Friesian calves in Australia that died suddenly over an 8-year period due to myocardial degeneration. Further investigations showed low blood Se content and red blood cell Se-GPX activities. In my dissertation research, emphasis was on vitamin E, and selenium which are non-enzymatic antioxidants and enzymatic antioxidants.
CHAPTER 3

Genetic Variation in Turkey Poults Fed Normal and Furazolidone-Containing Diets.

3.1 Abstract

Dilated cardiomyopathy (DCM) is characterized by an increase in myocardial mass and volume as well as thinning and stretching of the walls of the myocardium. It is believed to be the most common cardiomyopathy in both livestock and humans. In the turkey, about 5% of spontaneous mortality has been attributed to idiopathic DCM. Despite this economic loss, the etiology of DCM remains little understood. In an earlier study, we reported differences in the incidence and severity of DCM among five varieties of the domesticated turkey. In this research, we sought to extend that study by evaluating differences among reciprocal crosses of the 4 varieties that included Bourbon Red (BR), Spanish Black (SB), Narragansett (N) and Royal Palm (RP) for the incidence and severity of furazolidone (Fz)-induced DCM. Echocardiographic measurements at four weeks of age for birds fed toxic levels of Fz showed that NxBR reciprocal cross had the lowest (P<0.05) internal-diastolic (LVIDd) and systolic dimensions (LVISd), while the BRxN reciprocal cross had the largest (P<0.05) LVIDd and LVISd. The LVIDd and LVISd were not significantly different from SBxRP birds fed diets with Fz. Myocardial dilation, as measured by echocardiographic LVIDd and LVISd appeared to be lower for cross-bred than parental varieties. In birds fed Fz-containing diets, heterosis for LVIDd and LVISd was most significant for BRxN cross. This data suggest that reciprocal crosses respond differently to toxin that induces DCM. The present investigation provides additional evidence that genetics may significantly influence a turkey’s response to toxic levels of Fz that causes DCM.

Key Words: Turkey, Dilated cardiomyopathy, Genetics, Heritage turkey varieties
3.2 Introduction

The growth in the turkey industry over the past 50 years is partly due to increased consumption that arises from consumer preference for turkey meat and meat products (National Turkey Federation-Statistic, 2003). In addition to turkey consumption in the U.S, export of turkey meat has also increased. In 2008, the United States exported about 605 million pounds of the total turkey produced (National Turkey Federation-Statistic, 2008). To meet this increased demand, selection of turkeys for traits such as growth rate and body weight has been done using genetic and non-genetic approaches. This may have contributed to the increase in dilated cardiomyopathy (DCM) or round heart disease (Frame et al., 1999).

Dilated cardiomyopathy in poultry was identified in turkeys in 1962 (Magwood and Bray, 1962). It is characterized by dilatation of the ventricles, hypertrophy of the cardiac muscles, round heart with or without accompanying ascites, and hydro pericardium (Magwood and Bray, 1962; Jankus et al., 1973). In the turkey, mortality is highest at the end of the second week post-hatch (Sautter et al., 1968). Slowing the growth rate of birds through reduction of protein intake has been proposed to reduce the incidence (Breeding et al., 1994). The effect of diet is just one of the several factors that have been implicated in the incidence of DCM, other factors including age, stress, diet, and altitude (Czarnecki, 1984). Further supporting the environmental effect, cardiomyopathy can also be induced in turkeys using alcohol and drugs such as furazolidone (Fz) (Czarnecki, 1984). Though these factors play a role in the incidence of DCM, they do not appear to be as significant as genetics (Czarnecki, 1984; Genao et al., 1996).

Knowledge of whether genetics affects the incidence of DCM in turkeys is limited. In a classic study, Hunsaker (1971) used two commercial lines that originated from diverse stock to show that the two lines respond differently when fed diets containing toxic levels of Fz. Durand
reviewed several studies that reported single gene mutations which altered expression of different proteins in DCM-affected animals.

The genetics of DCM in other animals has also been investigated (Hasenfuss, 1998). In humans, hamsters, and mice, several genes have been identified as causal for DCM (Hasenfuss, 1998). These genes have been reported to encode proteins that form cytoskeleton of the cardiac myocyte including \( \alpha \)-cardiac actin (Olson et al., 1998), cysteine-rich protein 3 (Knoll et al., 2002), LIM-domain binding factor 3 (Arimura et al., 2004), myosin heavy polypeptide 7 (Kamisago et al., 2000), titin cap (Hayashi et al., 2004), \( \alpha \)-tropomyosin (Olson et al., 2001), troponin I (Murphy et al., 2004) and troponin T (Kamisago et al., 2000). Other genes implicated in DCM code for extra sarcomeric proteins including caveolin 1 (Zhao et al., 2002), desmin (Li et al., 1999), lamin A/C (Burke and Stewart, 2002) and phospholamban (MacLennan and Kranias, 2003). Werner et al. (2008) identified a region on chromosome 8 associated with juvenile DCM in Portuguese Water dogs.

In commercial turkey production, genetics have made major improvements in the growth characteristics of the modern turkey from crosses of sire and dam lines selected for improved growth and reproduction (Havenstein et al., 2004). Genetic gains arising from these mating could thus be due to improvements within the primary lines (additive genetic variation) and/or heterosis (non-additive genetic variation) in the crosses used to produce the commercial turkey. The possible effect of non-additive genetic variation on the incidence and severity of DCM has been previously investigated. Gyenai, 2005, evaluated five unique turkey varieties, including Bourbon Red (BR), Spanish Black (SB), Narragansett (N) and Royal Palm (RP) to ascertain whether there are differences in the incidence and severity of DCM when fed diets containing toxic levels of Fz. The Spanish Black and Bourbon Red varieties were shown to be most
susceptible and less susceptible to DCM, respectively. As a follow-up to that study, it was hypothesized that a turkey’s response to toxic levels of Fz is affected by its breed. The primary objective of this research was to evaluate the differences among reciprocal crosses of heritage turkey varieties for the incidence and severity of DCM induced by feeding diets containing Fz.
3.3 Materials and Methods

Animals

Turkey varieties used to generate the reciprocal crosses included BR, SB, N and RP. These varieties had been previously shown to differ in the incidence and severity of DCM when fed Fz-containing diet, a toxin that induces DCM (Gyenai, 2005). From each cross, one day-old poults were obtained from mating of 6 hens to a tom from another variety. The poults were randomly divided into treatment and control and raised according to standard management practices (Nesheim et al., 1986). Poults in the treatment group were fed a standard commercial starter-diet containing 700 parts per million of Fz ad libitum from one day-of-age to 4 wks of age (Czarnecki et al., 1973; Gyenai, 2005).

DCM diagnosis

Both poults fed normal and Fz-containing diets were gently restrained in left lateral recumbence and quality two-dimensional long-axis imaging planes were obtained from the right parasternal location using a portable Aloka Echocardiography (ECHO) machine with a 7.5MHz phased array transducer (Kienle and Thomas, 1995). Left ventricular internal-diastolic dimension (LVIDd) and internal-systolic dimension (LVISd) heart measurements were measured as the largest and smallest chamber diameters immediately below the mitral valve and parallel to the mitrial annulus at 4 wks of age on poults fed normal and Fz-containing diets (Wu et al., 2004). The ECHO readings were made in the M-Mode. This mode generated a one-dimensional view of small portions of the heart which allowed for the detection of axial motion of structures parallel to the beam (Kienle and Thomas, 1995).
**Percent heterosis**

Percent heterosis was calculated according to Nestor et al. (2001) based on the following:

\[
\% \text{ heterosis} = \frac{\text{crossbred mean} - \text{purebred mean}}{\text{purebred mean}} \times 100
\]

**Statistical analysis**

The data was analyzed using the General Linear Model procedure (SAS Inst., Inc., Cary, NC, 2002). Differences between treatment and control poults were tested for significance (P<0.05) using Duncan’s Multiple Range Test. Analysis of variance (ANOVA) was used to evaluate differences between the crosses and the parental varieties. Results were reported as least squares means (LSMeans) ± standard error (SE).
3.4 Results

At 4 wks of age, mortality was highest for BRxSB cross fed Fz-containing diets. No data was reported for LVIDd and LVISd for BRxSB cross due to high mortality. Within the control group there were no differences among crosses for LVIDd and LVISd (Figures 3.1 and 3.2.). Differences between control and poults fed Fz-containing diets were significant for LVIDd and LVISd (Figures 3.1, 3.2, 3.3 and 3.4). Comparison of left ventricular measurements showed about 40 % to 95 % increase in ventricular dilation for poults fed Fz-containing diets as compared with control group for LVIDd and LVISd (Figures 3.1, 3.2, 3.3 and 3.4). Among poults fed Fz-containing diet, NxBR and SBxRP crosses had the largest (P<0.05) LVIDd measurements (Figures 3.1 and 3.2). The BRxN cross had significantly the smallest LVIDd and LVISd measurements, about 55 % better when compared to others with the exception of SBxBR cross (Figures 3.1-3.2).

No significant differences were observed among reciprocal crosses within the control group. Differences among the crosses were significant for some comparisons for poults fed Fz-containing diets (Figures 3.1 and 3.2). Comparison of NxBR and BRxN crossbred showed NxBR to have the smallest ventricular measurements among poults fed Fz-containing diets. Comparison of RPxSB and SBxRP reciprocal crosses showed SBxRP to have much smaller ventricular measurements as than any other. No comparison was made for SBxBR reciprocal crosses due to its high mortality. Similar observation was made for LVISd measurement as discussed for LVIDd (Figures 3.1 and 3.2).

At 4 wks of age, comparison of crossbred with parental variety control group birds showed about 30 % better myocardial dilation (Figures 3.1). The BRxN cross had the smallest LVIDd though not statistically different from other reciprocal crosses. Between the crossbred
and parental variety birds fed Fz-containing diets, the NxBR cross had the largest for LVIDd though not different from crossbred SBxRP and parental variety SB and N, respectively (Figures 3.1). LVISd measurements showed rather reduced left ventricular measurement for parental varieties with the SB variety having the smallest (Figures 3.2). Poults fed Fz-containing diets LVISd showed a much different trend with crossbred SBxRP and NxBR having the largest and RP of the parental variety the smallest (Figures 3.2).

Percent heterosis for echocardiographic measurement was improved for control group birds as compared to those fed Fz-containing diets and parental varieties (Table 3.1). Among those receiving Fz-containing diets, NxBR and SBxBR cross had significantly improved left ventricular measurements, respectively, and when compared to the parental varieties (Table 3.1). However, differences were not observed for the LVISd measurements (Table 3.2). No significant differences were observed in either control or birds fed the Fz-containing diets for LVISd measurements.
3.5 Discussion

In this study, we found increased left ventricular measurements LVIDd and LVISd for crossbred birds fed Fz-containing diets compared to control group birds at 4 wks of age. The toxic levels of Fz added to the diets of birds may have resulted to a combination of myocyte injury and necrosis associated with myocardial fibrosis, which led to impaired mechanical function with ventricular dilation (Goldstein et al., 1998). Also, addition of Fz may have led to direct toxicity or mechanical insults such as; chronic volume overload in mitral valvular regurgitation with myocyte failure and cytoskeletal uncoupling, resulting in to dilation (Goldstein et al., 1998). The differences observed between LVIDd and LVISd for birds fed Fz-containing diets and those in the control group are similar to that of Wu et al., (2004) and Gyenai, (2005), which reported increased left ventricular dimension. A review by Vlahović and Popović, (1999) reported impaired left ventricular diastolic function to be one of the abnormalities associated with cardiac diseases accompanied by changes in filling patterns in humans, from delayed relaxation through pseudo-normalization, and finally, to restrictive filling. Left ventricular diastolic dysfunction has also been reported in patients with hypertensive or valvular heart disease, hypertrophic and DCM (Mandinov et al. 2000). Andrade et al. (2005) tested the surgical technique plication of the left ventricular free wall, to reduce left ventricular area and volume and improve left ventricular systolic function, without using a cardiopulmonary bypass in crossbred dogs. They reported plication of the left ventricular free wall reduced left ventricular area and volume and improved left ventricular systolic function in crossbred dogs with doxorubicin induced cardiomyopathy morbidity and mortality were reduced which is similar to the findings in our study.
We observed reduced left ventricular dilation for crossbred BRxN and increased ventricular dilation for NxBR fed Fz-containing diets. Our results are similar to findings of Sussman et al. (1999) which investigated combined effects of hypertrophy and dilation in cross-breeding of two cardiomyopathic transgenic mouse lines which develop either hypertrophy or dilation. Results of that study showed alteration in the intensity of signals driving hypertrophy and dilation in cross-bred litters resulting in novel disease phenotypes different from either parental line. In another study, Distl et al. (2007) analyzed the mode of inheritance for DCM in Irish wolfhounds using regressive logistic models by testing for mechanisms of genetic transmission. They reported that male dogs were affected more often by DCM than female dogs. Segregation analysis showed a mixed monogenic-polygenic model including a sex-dependent allele effect.

We also observed differences in reciprocal crosses birds fed Fz-containing diets NxBR when compared with BRxN LVIDd and LVISd measurements and compared with ventricular measurements of the parental varieties. Similar to our findings, in specific dog breeds, DCM has been reported to have a heritable basis as well as in certain families within breeds (Dukes-McEwan and Jackson, 2002). Autosomal modes of inheritance for DCM have been suggested for Irish wolfhound, Newfoundland and Portuguese water dog (Cobb et al., 1996; Dambach et al., 1999; Dukes-McEwan and Jackson, 2002; Sleeper et al., 2002).

In summary, results of our study showed the BRxN crossbred fed Fz-containing diets to have reduced left ventricular LVIDd and LVISd measurement when compared to other crosses and parental varieties. Although differences were not observed among control crossbred birds, LVIDd and LVISd was significantly reduced when compared to that of parental varieties. Thus,
based on our current findings it is evident that reciprocal crosses fed Fz-containing diets respond differently to toxin that induces DCM, indicative of a genetic effect.
Figure 3.1. Left ventricular internal-diastolic dimension of 4 week-old crossbred pouls and parental varieties fed normal (CTL) and furazolidone-containing diet (TRT).
Figure 3.2. Left ventricular internal-systolic dimension of 4 week-old crossbred poults and parental varieties fed normal (CTL) and furazolidone-containing diet (TRT).
Figure 3.3. M-mode echocardiographic image of left ventricle from a bird fed normal diet at 4 weeks-of-age. The numbers shown on the right hand side used for diagnosis are the left ventricular internal-diastolic and systolic dimension, 0.95 and 0.90 cm, respectively.
Figure 3.4. M-mode echocardiographic image of left ventricle from a bird fed Fz-containing diet at 4 weeks-of-age. The numbers shown on the right hand side used for diagnosis are the left ventricular internal-diastolic and systolic dimension, 2.43 and 2.17 cm, respectively.
Table 3.1. Percent (%) heterosis for left ventricular internal-diastolic and systolic dimensions of crossbred heritage turkey poults.

<table>
<thead>
<tr>
<th>Cross†</th>
<th>LVIDd*</th>
<th>LVISd*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL</td>
<td>TRT</td>
</tr>
<tr>
<td>BRxN</td>
<td>-43.0</td>
<td>-25.0</td>
</tr>
<tr>
<td>NxBR</td>
<td>-37.3</td>
<td>51.0</td>
</tr>
<tr>
<td>RPxSB</td>
<td>-41.0</td>
<td>5.5.0</td>
</tr>
<tr>
<td>SBxRP</td>
<td>-31.2</td>
<td>15.4</td>
</tr>
<tr>
<td>SBxBR</td>
<td>-35.0</td>
<td>-16.5</td>
</tr>
</tbody>
</table>

*Where LVIDd and LVISd represent left ventricular internal-diastolic and systolic dimensions, respectively.

†Where BR, N, RP and SB represent Bourbon Red, Narragansett, Royal Palm and Spanish Black, respectively.
CHAPTER 4
Evaluation of Oxidative Stress Levels in Turkey Poults Fed Normal and Furazolidone-Containing Diets

4.1 Abstract

Extensive research has provided evidence that implicates an increased generation of free radicals or oxidative stress in cardiomyopathy in diverse animals including humans. In turkeys, dilated cardiomyopathy (DCM) is a major cause of early bird mortality resulting in deaths of about 2-4%. In this study, it was hypothesized that DCM is associated with increased oxidative stress levels in a random population of turkeys. Specifically, feeding toxic levels of furazolidone (Fz), which causes DCM, may be related to changes in oxidative stress in turkeys. Changes in oxidant and antioxidant levels in blood were used at two and four wks of age as biomarkers for oxidative stress. Malondialdehyde (MDA) was used as a measure of lipid peroxidation. Glutathione (GSH), glutathione peroxidase (GPx), and plasma uric acid (PUA) were used as biomarkers for antioxidant levels. At two wks of age, there were no significant differences in levels of MDA and GPx between control and those fed Fz-containing diets. PUA and GSH levels were however significantly increased for poults fed Fz-containing diets. At four wks of age, no differences were observed between control and those fed diets with Fz for MDA and GPx. PUA concentrations however increased significantly for poults fed normal diets with Fz as compared with controls, while significant increase in GSH concentrations were observed for controls. Differences between controls and poults fed Fz-containing diets were significant for GPx. Left ventricular internal diastolic and systolic dimension correlated positively with each other at two and four weeks of age. Positive correlations were also observed for PUA and GPx at two wks of age. Results of the current study showed that, feeding poults diets with Fz does not increase
oxidative stress, rather antioxidant levels are increased. Thus, DCM caused by Fz is independent of oxidative stress.

**Keywords:** Furazolidone, Dilated Cardiomyopathy, Oxidative Stress, Plasma Uric Acid, Glutathione, Malondialdehyde, and Glutathione Peroxidase.
4.2 Introduction

Dilated cardiomyopathy (DCM) is a disease condition of the myocardium which affects diverse animals including human (Hasenfuss, 1998), dog (Wilson et al., 1987), rat (Sakai et al., 1996), pig (Muders and Elsner, 2000) and the turkey (Genao et al., 1996). Dilated cardiomyopathy can either be idiopathic (IDCM) or toxin-induced (TIDCM), (Gwathmey and Hamlin, 1983). Idiopathic or spontaneous DCM has been most widely investigated in humans and other mammals (Hasenfuss, 1998). TIDCM has been most widely investigated in the turkey as a model for human IDCM because of similarities of the disorders between the two species (Hajjar et al., 1993). An understanding of DCM in birds will therefore provide a strong foundation for further defining the etiology of this disease in humans and other animals.

Avians have been shown to have a high average body and metabolic rate that is approximately 2-2.5 times higher than that of animals of similar size (Holmes et al., 1995). This leads to higher levels of reactive oxygen species (ROS) and/or free radicals (FR) in birds (Lindstedt et al., 1976). Plasma glucose concentration has also been reported to be 2-6 times higher in birds than in mammals (Ku et al., 1993). Glucose is reported to be a limiting factor in maillard reaction formation, resulting in protein glycosylation and tissue cross-linking (Ku et al., 1993). Glycosylation disables proteins which may lead to aging and diseases such as cardiomyopathy (Cerami, 1985).

Over the years, there has been a growing recognition of the importance of ROS or FR intermediates in the pathogenesis of DCM (Demirbag et al., 2005). Earlier studies reported evidence linking degeneration of the myocardium and related diseases to an increased production of ROS and/or FRs (Halliwell and Gutteridge, 1990). Ide et al. (1999) used electroparamagnetic resonance (EPR) with an oxygen radical (O$_2^-$) spin trap to show a 2.8 fold increase in the
formation of superoxide anion in sub-mitochondrial particles from failing myocardium. Elevated levels of lipid peroxides and 8-isoprostaglandin F2α, which are the major indicators of the biochemical consequences of ROS and/or FR formation in plasma and the pericardial fluid of patients with heart failure, have also been reported (Belch et al., 1991).

Studies using isolated perfused hearts have shown that brief exposure to FRs result in a loss of contractile function and structural abnormalities of the heart (Gupta and Singal, 1989 and Ytrehus et al., 1986). In the rat, FR-induced reduction of contractile function was correlated with decline in myocardial SOD, GSH, and α-tocopherol content and with an increase in H2O2 and lipid peroxidation (Gupta and Singal, 1989 and Vaage et al., 1997). Also in rats, Singal et al. (1983) reported that FRs react with unsaturated lipids to initiate self-perpetuating chain reactions of lipid peroxidation in membranes. Oxidation of sulphydryl groups in proteins and strand scission in nucleic acids have also been reported to be facilitated by FRs (Kaul et al., 1993 and Singal et al., 1988). *In vivo* studies of stress-induced hearts have provided evidence that cardiac dysfunction may be due to the production of FRs that causes autooxidation of catecholamines (Singal et al., 1982; Meerson, 1980). Using a canine model, Kramer et al. (1984) showed that FRs affect the activity of Na+/K+ ATPase, the Na+/Ca++ exchanger, and Ca++ binding of the myocardium, all of which affect Ca++ movements across the sarcolemma. Free radicals have also been reported to alter the coupling of sarcoplasmic reticular Ca++ transport with ATP hydrolysis (Hess et al., 1983)

Though FRs are deleterious, Ferrari et al. (1991) reported that the myocardium has developed a mechanism for ameliorating these effects. The myocardium is able to tolerate the continuous FR production by maintaining a delicate balance between oxidants and antioxidants (Traber et al., 1990). In the heart, the antioxidant defense mechanisms include enzymes such as
superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), and non-enzymatic antioxidants including vitamin E, ascorbic acid, and cysteine (Pryor, 2002). Various pathologic processes have been reported to disrupt this balance by increasing the production of FRs in proportion to the available antioxidants leading to OS (Sies, 1991). Oxidative stress in the myocardium, as in other organs and tissues, is indicated by elevated or reduced levels of biomarkers including plasma uric acid (PUA), malondialdehyde (MDA), and SOD and reduction oxidation (redox) state of glutathione (Gupta and Singal, 1989; Kirshenbaum and Singal, 1992, and Kirshenbaum et al., 1994).

As an antioxidant, uric acid has been shown to react with several FRs including superoxide, hydrogen peroxyl, and hypochlorus (Becker, 1993). In humans, Ames et al. (1981) reported that urate is reduced by oxo-heme to uric acid which protects erythrocytes against lipid peroxidation. Similarly, uric acid has been reported to protect DNA from damage caused by a reaction between Adriamycin, iron and Hydrogen Peroxyl (Miura et al., 1993). Uric acid concentrations in blood have therefore been used as a direct indicator for OS (Yazar et al., 2003). In broilers, reduction in uric acid production using the xanthine oxidase inhibitor, allopurinol is associated with increased OS as indicated by the accumulation of markers for FR damage (Klandorf et al., 2001, and Simoyi et al., 2002).

As a biomarker for OS, MDA is a small (molecular weight) molecule produced from the fragmentation of polyunsaturated fatty acids after attack by FRs. It is a biomarker of lipid peroxidation (Draper et al., 1988). MDA has been reported to be an effective biomarker for OS in animals including humans (Draper et al., 1988). It has also been used as a biomarker for diseases associated with heart and other organs and for structural damage of tissues (Griffiths et al., 2002). Ece et al. (2006) described increased serum MDA in children with chronic renal
failure. Similarly, Kluchova et al. (2006) showed increased serum MDA in patients with chronic obstructive pulmonary disease. In broiler chickens infected with *Eimeria tenella*, Georgieva et al. (2005) reported increased MDA levels. Similar data about the onset of OS with increased MDA levels in parasitic diseases have been reported in goats and humans (Dede et al., 2002, and Pabon et al., 2003).

Considering a biomarker for OS, GPx belongs to a family of selenoproteins that function to catalyze the reduction of $\text{H}_2\text{O}_2$ and other peroxides including DNA and lipid peroxides into water and alcohols. At least five GPx isoenzymes, GPx1 - GPx5, have been described in mammals (Lustgarten et al., 2007). The cytosolic and mitochondrial GPx1 and the phospholipids hydroperoxide GPx4 are found in most tissues. GPx1 is particularly abundant in erythrocytes, kidney, and liver, and GPx4 is highly expressed in renal, epithelial, and testes. Cytosolic GPx2 and extracellular GPx3 are poorly expressed in most tissues except in the gastrointestinal tract and kidney, respectively (Lustgarten et al., 2007). It has also been reported that GPx enzymes play a major role in protecting cells against oxidative damage under both normal and abnormal conditions such as OS (Griffiths et al., 2002).

Glutathione, also used as a biomarker for OS, is a non-protein thiol found abundant in some cells. It is a tripeptide derived from glutamic acid, cysteine, and glycine with gamma linkages between the first two amino acids: $\gamma$-L-glutamyl-L-cysteinyl-glycine (Nicolet, 1930). The bonds between GSH prevent it from being hydrolyzed by most peptidases. It has been reported that compared to its precursors cysteine and g-lutamylcysteine GSH less easily oxidized (Meister and Anderson, 1983). GSH is found in most mammalian and many prokaryotic cells, and is the most abundant intracellular thiol (0.2–10 mM). Intracellular GSH is kept in its thiol form by glutathione disulfide reductase, an NADPH-dependent enzyme. Extracellular GSH
functions as a coenzyme and is involved in amino acid transport (Anderson, 1997). It is also involved in metabolism and the maintenance of the thiol moieties of proteins and low molecular weight compounds such as cysteine. GSH can react with another reactive glutathione to form glutathione disulfide (GSSG). Such a reaction is possible due to the relatively high concentration of glutathione in cells (up to 5 mM in the liver). GSH can be regenerated from GSSG by the enzyme glutathione reductase. Hydrogen peroxides and other peroxides are detoxified by GSH peroxidase (Meister and Anderson, 1983). In a study by, Campolo et al. (2007), increased GSH levels was reported with congestive heart failure.

Although several studies (Nunes et al., 2005; Prasad et al., 1996; Signal et al., 1998, and Qiao and Song, 1994) have implicated OS in the progression of cardiovascular diseases including DCM, its role in TIDCM in the turkey and other animals remains to be fully investigated and understood. In this study, we hypothesized that OS is associated with the incidence and severity of DCM in birds fed diets containing toxic levels of Fz. Oxidant and antioxidant levels of turkeys from commercial populations were used as biomarkers for OS to test this hypothesis.
4.3 Materials and Methods

Animals

A total of fifty one day-old male Hybrid turkey pouls susceptible to spontaneous DCM were obtained from a commercial hatchery (Ag Forte, Harrisonburg, Virginia). On receipt, the pouls were weighed, wing-banded and randomly divided into two groups (treatment and control; 25 pouls per cage). The pouls were raised according to standard management protocol. Poults were fed *ad libitum* till 4 wks of age standard commercial starter-diet formulated at the Virginia Tech feed mill which met the National Research Council (1994) recommendations (Table 4.1). Poults in the treatment group were fed *ad libitum* a diet that contained 700 parts per million of Fz (Gwathmey et al., 1999).

DCM diagnosis

Two-dimensional long-axis imaging planes were obtained from the right parasternal location of pouls gently restrained in left lateral recumbence using a portable Aloka Echocardiography (ECHO) machine with a 7.5 MHz phased array transducer. The two-dimensional ECHO readings were then viewed in the M-Mode for systolic and diastolic readings. The M-Mode generated a one-dimensional view of small portions of the heart which allowed for the detection of axial motion of structures parallel to the beam (Kienle and Thomas, 1995). Left ventricular internal-diastolic (LVIDd) and internal-systolic dimension (LVISd) were measured for the largest and smallest ventricular diameters immediately below the mitral valve and parallel to the mitral annulus at 2 and 4 wks of age on all pouls (Vollmar, 1999, and Wu et al., 2004).
Shortening fraction

The *in vivo* contractile performance of the heart of each poult was estimated according to Wu et al. (2004) using the equation:

\[\left(\frac{\text{LVIDd} - \text{LVISd}}{\text{LVIDd}}\right) \times 100\]

The rationale is that the shortening fraction estimates the left ventricular performance based on the ratio of the change in the diameter of the left ventricle in diastole and systole.

Necropsy

At 2 and 4 wks of age, some poults were sampled from both the control and treatment groups for standard necropsy to compare ECHO measurements to visual and pathological diagnosis. Hearts were dissected, atria and large vessels removed. Weight of the entire heart (WHO) was taken and the length of the apex to the thorax were conducted using vernier calipers.

Blood collection

Blood was collected into vacutainer tubes containing 0.5 M ethylenediaminetetraacetic acid from poults by venipuncture for each biomarker analysis as follows: Plasma was obtained by centrifuging the blood samples at approximately 10,000 rpm for 15 minutes at 4°C. Deproteinated whole blood was obtained by centrifuging at 3,000 rpm for 5 minutes with 50 micro liters (µl) of 5 % metaphosphoric acid per 50 µl ethylenediaminetetraacetic acid. The samples were first stored on ice at 4 °C, then snap frozen on dry ice before storing at 80°C until ready for use. The plasma was used for the analyses of PUA, MDA and GPx, while the deproteinated whole blood was used to determine total GSH levels as described below.
Biomarker analysis

Plasma uric acid (PUA)

The enzymatic assay for PUA has previously been described (Hartman et al., 2006). Duplicate samples were evaluated using an optimized commercial assay (Diazyme, La Jolla, CA). Briefly blood samples were spun down in 1.5 mL centrifuge tubes at 2000 rpm for 15 minutes at 4 °C to obtain plasma. If the plasma samples were not used immediately, they were stored at -80 °C. The assay for PUA determination in plasma involved conversion of uric acid in the sample into allantoin and hydrogen peroxide by the enzyme uricase. The hydrogen peroxide subsequently reacts with oxidizing N-ethyl-N-sulfoproyl-m-anizidin (ESPAS) and 4-aminoantipyrene with the help of peroxidase to form a red-violet quinoneimine dye (Hartman et al., 2006). The reaction is summarized as follows:

\[
\text{Uric acid} + \text{O}_2 + 2\text{H}_2\text{O} \xrightarrow{\text{uricase}} \text{Allantoin} + \text{CO}_2 + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + \text{ESPAS} + 4\text{-aminopyrene} \xrightarrow{\text{peroxidase}} \text{Quinine dye} + 4\text{H}_2\text{O}
\]

The amount of quinine dye formed, which is a direct proportion of the level of PUA in plasma, was estimated using a spectrophotometer at an absorbance of 600 nm (A_{600nm}).

Glutathione (GSH)

As for GSH, duplicate samples from each poult were also assayed. Whole blood total GSH was measured using a spectrophotometric assay at A_{412nm} using an adaptation of the manufacturers protocol (Northwest Life Science Specialties, Vancouver, WA). The general thiol reagent, 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB, Ellman’s Reagent) reacts with GSH to form the A_{412nm} chromophore, 5-thionitrobenzoic acid (TNB) and glutathione S-transferases (GS)-
TNB (Engle et al., 2009). The GS-TNB is subsequently reduced by glutathione reductase and β-nicotinamide adenine dinucleotide phosphate, releasing a second TNB molecule and recycling GSH; thus amplifying the response. Any oxidized GSH (GSSG) initially present in the reaction mixture or formed from the mixed disulfide reaction of GSH with GS-TNB is rapidly reduced to GSH. An adaptation made to the manufacturers protocol consisted of deproteination of whole blood prior to each enzymatic reaction by adding 5 % metaphosphoric acid per 50 µl EDTA to whole blood (Teitze, 1969, and Engle et al., 2009). To maintain consistent spectrophotometer readings, for the duplicate samples a 40-fold dilution of each blood sample and 5 fold dilutions of the calibrators were carried out. The absorbance was recorded at 60-second intervals for 10 minutes. Total GSH concentration of each sample was calculated from the absorbance reading using linear regression (Bloomer, 2007).

*Malondialdehyde (MDA)*

The enzymatic assay used to estimate MDA in each plasma sample measured lipid peroxidation according to Botsoglou (1994) and Jentzsch et al. (1996). The enzymatic assay was based on the reaction of MDA with two molecules of thiobarbituric acid (TBA) resulting in an MDA-TBA2 adduct. During the reaction, butylated hydroxytoluene (BHT) and EDTA were added to the reaction mixture to minimize oxidation of lipids that contribute artifactually to the TBA reaction (Verhaeghe et al., 2009). The temperature of the reaction mixture was reduced to (60 °C) to minimize the decomposition of lipid hydroperoxides. The pH of the reaction was optimized to obtain optimal yields of MDA product at pH 1.5 in the presences of hydrochloric acid to facilitate hydrolysis of the MDA (Gerard-Monnier et al., 1998). The reaction mixture was then subjected to spectrophotometric analysis at 532 nm (A_{532nm}).
Glutathione Peroxidase (GPx)

The assay (Northwest Life Science Specialties Vancouver, WA, USA) kit used to determine plasma GPx-3 activity was an adaptation of the method of Paglia and Valentine (1967). The theoretical basis of the assay is that GPx catalyzes the reduction of hydrogen peroxide and reduced glutathione to form oxidized glutathione. Oxidized glutathione is then reduced by glutathione reductase and β-nicotinamide adenine dinucleotide phosphate (NADP) to form NADP+ (Lee et al., 2008). Because GPx is limiting, the change in absorbance at A₃₄₀ is proportional to its concentration. GPx activity was reported as units based on the definition: 1 unit of GPx-1 = the amount of enzyme necessary to catalyze the oxidation (by H₂O₂) of 1.0 μmole GSH to GSSG per minute at 25 ºC, pH 7.0 (Lee et al., 2008).

Statistical analysis

All data were analyzed using the General Linear Model (GLM) procedures of SAS (SAS Institute, Cary, NC, 2002). The statistical model was:

\[ Y_{ij} = \mu + T_i + e_{(ij)} \]

where \( Y_{ij} \) was the observed dependent variable, \( \mu \) the grand mean, \( T_i \) the furazolidone treatment effect, and \( e_{(ij)} \) is the error. Significant treatment differences were established using the Duncan’s multiple range test and Waller-Duncan K-ratio t-test statement in SAS (\( P \leq 0.05 \)) for poultis fed normal and Fz-containing diets at 2 and 4 wks of age. Results were reported as least squares means (LSMeans) ± standard error (SE).
4.4 Results

At 2 wks of age, poult fed the normal diets, as expected, had significantly larger body weight, smaller LVIDd and LVISd than those fed normal diets containing Fz (Table 4.2). Though significant only for GSH, levels of biomarkers for poult fed normal diets were different from treatment poult (Table 4.2). At 2 wks of age, the echocardiography results suggest DCM. The OS evaluated was, however, inconsistent as both oxidant and antioxidants appeared to be different between the treatment and control group (Table 4.2).

The body weights of poult fed normal diets containing Fz were two fold lower than those on normal diets at 4 wks of age (Table 4.3). Though not as dramatic, significant differences were also observed at this age for LVIDd and LVISd measurements. Poult fed Fz-containing diets deferred from those on normal diets by 20 and 45 % for LVIDd and LVISd, respectively (Table 4.3). Though body weight was increased for poult fed control diets as compared to those fed diets containing Fz, there was no difference in OS levels (Table 4.3). Poult fed control diets had significantly higher GSH concentrations. PUA concentrations were higher for poult fed Fz-containing diets when compared to those fed control diets (Table 4.3). Change in percent shortening fraction at 2 and 4 wks of age was -72 % decreased for poult fed normal diets with Fz compared with those fed normal diets (Table 4.4).

Positive correlations were found between LVIDd and LVISd for control poult and those fed Fz-containing diets at both 2 and 4 wks of age (Table 4.5). PUA and GPx were also shown to positively correlate with each other (Table 4.5).

At 2 wks of age, weight of whole heart (WHO) and the length of the apex to thorax were lower for poult fed normal than poult fed Fz-containing diets (Table 4.6). At 4 wks of age, the average WHO was larger for poult fed normal diets, though the length of the apex to thorax...
measurements were larger for those fed Fz-containing diets (Table 4.6). Differences observed between poult s fed diets with and without Fz may have been due in part to the stretching of the myocardium, due to degeneration of cardiac myocytes and loss of elasticity.
4.5 Discussion

Differences between control and poult s fed Fz-containing diets for oxidative stress were investigated. The commercial strain used in this study is known to have a high prevalence of DCM that mimics the disease observed in humans (Gwathmey et al., 1991, and Hajjar et al., 1993). The use of the turkey also allows for a range of experimental approaches from in vivo echocardiography on the intact animal to biomarker analysis. Results from the current study showed significantly increased echocardiography LVIDd and LVISd measurements for poult s fed Fz-containing diets compared with control. This finding is consistent with the findings of Hajjar et al. (1993), Wu et al. (2004), and Gyenai (2005) which showed increased left ventricular dilation in DCM-affected poult s. One explanation previously advanced for the effect of Fz feeding is the increased concentration of myocardial collagen concentration, cross-linking and changes in collagen observed in DCM-affected poult s (Gwathmey et al., 1999). The over-expression of collagen in combination with cross-linking affects the extracellular matrix which consists of type I and III collagens. The collagens are responsible for the formation of large, well-structured fibers that resist extension of the myocardium leading to increased ventricular dilation (Medugorac, 1982).

The oxidant biomarker MDA and antioxidant biomarkers PUA, GPx and GSH in two groups of poult s fed normal diets with and without Fz. No changes in MDA levels associated with feeding diets with Fz were observed at 2 and 4 wks of age. This was inconsistent with previous reports that demonstrated increases in various oxidant biomarkers of OS in patients and animal models cardiovascular disease when plasma and urinary MDA concentrations (McMurray et al., 1990, and Diaz-Velez et al., 1996), plasma lipid peroxides (Belch et al., 1990), and urinary and pericardial isoprostanes were measured (Cracowski et al., 2000).
An increased MDA concentration in humans however, with congestive heart failure and in patients with ischemic chronic heart failure has been reported (Keith et al., 1999, and Serdar et al., 2001). Similarly, significant increases in plasma MDA in patients with congestive heart failure induced through exercise have been reported (Nishiyama et al., 1998). Parvin and Akhter (2008) investigated n-hexane extract of tomatoes for its protective action against OS in experimental myocardial infraction induced by administration of adrenaline in rats. They reported adrenaline to produce significant elevation of MDA content in the heart. Increased blood concentration MDA produced by FRs reaction with unsaturated fatty acids was found in DCM patients. Increased MDA concentration has been associated with a decrease in the activity of GPx and Se concentration (Li and Nan, 1989). Several explanations can be suggested for the negative findings observed here with respect to MDA and DCM. Including differences in methods used in diagnosis (McMurray et al., 1990; Diaz-Velez et al., 1996, and Belch et al., 1990), duration of the onset of DCM, and the timing of lipid peroxidation and that of assessment (Keith et al., 1998; McMurray et al., 1990, and Singal and Kirshenbaum, 1990). Differences in analytical methods could also important since several earlier studies that showed a relationship between MDA and DCM used high performance liquid chromatography (Diaz-Velez et al., 1996). It’s also possible that the concentrations of MDA may vary in different body fluids, making it more difficult to detect increased plasma levels. Such differences were observed between plasma and urinary isoprostane and MDA by Feillet-Coudray et al. (2002). The difference between plasma and urine was not observed for MDA.

A substantial body of epidemiological and experimental evidence suggests that uric acid, an antioxidant biomarker, is an important independent factor for cardiovascular and renal diseases. Elevated plasma uric acid is reported to be highly predictive of mortality in patients
with cardiovascular and coronary artery diseases. Uric acid is a degradation product of purine metabolism (Becker, 1993). Though originally considered to be just a waste product, its antioxidant properties are well recognized (Becker et al., 1991; Sevanian et al., 1991, and Becker, 1993). It is a powerful scavenger of peroxyl and hydroxyl radicals (Becker, 1993, and Regoli and Winston, 1999), and radicals derived from the reaction between peroxynitrite and carbon dioxide (Squadrito et al., 2000). In humans, Squadrito et al. (2000) reported enhanced production or retention of urate to cause urate arthritis, nephrolithiasis or urate nephropathy. In the current study, PUA was higher in poults fed Fz-containing diets at 2 and 4 wks of age compared with those fed normal diets. A positive correlation between PUA with GPx at 2 wks of age was also observed. It is has been reported that PUA concentrations are influenced by the balance between synthesis, degradation and excretion. Thus, the increase in uric acid concentration may have been caused by an increased activity of xanthine oxidoreductase and/or by an increased level of hypoxanthine/xanthine; for example, during massive cytolytic events, impaired renal function or decreased hepatic degradation of uric acid. However, the increased production of uric acid can also lead to higher production of superoxide. Previously, a stoichiometric study showed that for every molecule of uric acid formed, at least four molecules of superoxide or two molecules of hydrogen peroxide are produced (Becker et al., 1991). Uric acid does not effectively scavenge either superoxide or hydrogen peroxide. Experiments in vitro showed that uric acid reacts preferentially with stronger oxidants such as with peroxyl radical or hydroxyl radical which can potentially influence levels of other enzymatic antioxidants (Becker, 1993).

Measurement of GPx activity is one of the most accepted means of assessing Se bioavailability. Selenium has a number of important biological roles, including regulation of the
family of GPx enzymes and immunocompetence. GPx enzymes, remove hydrogen peroxide and harmful lipid hydroperoxides generated in vivo by oxygen-derived species. Glutathione is a substrate for GPx which neutralizes hydrogen peroxide for GSH S-transferase which converts electrophilic centers of various, potentially toxic compounds to thioether bonds. GSH is synthesized solely in the cytoplasm, in a translation-independent reaction from glutamine, glycine and cysteine (Lomaestro and Malone, 1995). GSH is then transported into the mitochondria which are the major intracellular source of reactive oxygen intermediates (Reed, 1990). Mitochondria, however, lack catalase and depend on GSH and superoxide dismutase to decompose the superoxide radicals that are constantly generated during cell respiration (Meister, and Anderson, 1983). A decrease of GSH therefore diminishes the capacity of cells to compensate for OS and could contribute to the pathogenesis of diseases importance in which OS is considered a possible pathogenic factor. Changes in GSH levels are supposed to determine the vulnerability of many cells towards a wide array of attacks. Here, our results were inconsistent for GSH, increased levels were observed at 2 wks of age and decreased levels at 4 wks of age for poults fed normal diets with Fz compared to those fed normal diets.

In summary, we evaluated the link between toxin-induced DCM and OS as determined by several biomarkers. Echocardiographic measurements LVIDd and LVISd showed significant difference between poults fed Fz-containing and normal diets from 2 to 4 wks of age leading to the progression of DCM and impaired myocardial contractility as expected. Levels of MDA, an oxidant, and GPx, an antioxidant, were the same at 2 and 4 wks of age in both poults fed normal and Fz-containing diets. The antioxidant PUA was higher in poults fed diets with Fz compared with those fed normal diets. GSH levels were higher for poults fed normal diets with Fz at 2 wks of age however, decreased at 4 wks as compared with those fed normal diets. The reduced level
in GSH at 4 wks of age could be a result of increased requirement of GSH as compared with other antioxidants to fight off different free radicals. In conclusion feeding pouls diets containing Fz causes increased left ventricular dilation however, did not increase oxidative stress levels. Thus, DCM caused by Fz may be independent of OS.
Table 4.1. Composition and nutrient content of basal diet

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn meal</td>
<td>39.96</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>37.97</td>
</tr>
<tr>
<td>Meat and Bone meal</td>
<td>8.00</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>2.33</td>
</tr>
<tr>
<td>Dical Phosphate</td>
<td>2.29</td>
</tr>
<tr>
<td>Ground lime stone</td>
<td>0.94</td>
</tr>
<tr>
<td>Lysine-HCL</td>
<td>0.58</td>
</tr>
<tr>
<td>D. L. Methionine</td>
<td>0.42</td>
</tr>
<tr>
<td>Salt (plain)</td>
<td>0.23</td>
</tr>
<tr>
<td>Poultry Vitamin Premix(^1)</td>
<td>0.10</td>
</tr>
<tr>
<td>Poultry Trace Mineral(^2)</td>
<td>0.10</td>
</tr>
<tr>
<td>Coban 60</td>
<td>0.06</td>
</tr>
<tr>
<td>Copper sulfate</td>
<td>0.03</td>
</tr>
<tr>
<td>Wheat, Spring, grain</td>
<td>7.00</td>
</tr>
</tbody>
</table>

Calculated nutrient content

| Metabolizable Energy (kcal/kg)      | 3,135.6290  |
| Crude protein (%)                  | 29.8226     |
| Dry matter                         | 95.771      |
| Methionine                         | 0.8831      |
| Lysine                             | 2.0450      |
| Methionine + cystine               | 1.3311      |

\(^1\)Poultry vitamin premix supplied (per kg of diet): vitamin A (retinyl acetate), 8,820 IU; vitamin D\(_3\), 2,646 ICU; vitamin E (DL-\(\alpha\)-tocopheryl acetate), 22 IU; vitamin K\(_3\) (menadione dimethylpyrimidinol bisulfite), 2.65 mg; thiamin, 3.73 mg; riboflavin, 8.82 mg; pantothenic acid (D-calcium pantothenate), 22.1 mg; niacin, 88.2 mg; folic acid, 2.21 mg; biotin, 221 µg; vitamin B\(_{12}\) (cyanocobalamin), 26 µg.

\(^2\)Poultry trace minerals supplied (per kg of diet): iron (FeSO\(_4\)·H\(_2\)O), 40 mg; zinc (ZnO), 210 mg; manganese (MnO), 120 mg; copper (CuSO\(_4\)·5H\(_2\)O), 20 mg; iodine (Ca iodate), 3 mg; cobalt (Co) 50 µg.
Table 4.2. Least Squares Means and standard error (SE) for body weight, left ventricular dimensions and biomarkers for oxidative stress in 2 week old poults.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body Weight (g)</th>
<th>LVIDd*(cm)</th>
<th>LVISd(cm)</th>
<th>MDA(µM/mL)</th>
<th>PUA(mg/dL)</th>
<th>GPxǂ(units/L)</th>
<th>GSH(µM/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL (n^1^=20)</td>
<td>271.53±33.59^a^</td>
<td>0.40± 0.05^b^</td>
<td>0.24± 0.05^b^</td>
<td>3.01±0.98^a^</td>
<td>0.03±0.01^b^</td>
<td>20.86±1.30^a^</td>
<td>9.96±1.72^b^</td>
</tr>
<tr>
<td>TRT (n^1^=12)</td>
<td>252.53±16.54^b^</td>
<td>1.02 ±0.06^a^</td>
<td>0.91±0.07^a^</td>
<td>3.13±1.29^a^</td>
<td>0.05 ±0.02^a^</td>
<td>22.41±1.71^a^</td>
<td>31.30±2.27^a^</td>
</tr>
</tbody>
</table>

*Where LVIDd and LVISd represent left ventricular internal-diastolic dimension and left ventricular internal-systolic dimension, respectively. MDA, PUA, GPx, and GSH represent malondialdehyde, plasma uric acid, glutathione peroxidase, and glutathione, respectively, and n is the number of birds analyzed. CTL and TRT for control and treatment, respectively.

ǂOne unit was the amount of enzyme necessary to catalyze the oxidation (by H_2O_2) of 1.0 µmol of reduced glutathione to oxidized glutathione/minute at 25°C, pH 7.0.

^a, bMeasurements in the same column with similar alphabetic superscript are not significantly different (P>0.05).
**Table 4.3.** Least Squares Means and standard error (SE) for body weight, left ventricular dimensions and biomarkers for oxidative stress in 4 week old poulets.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body Weight (g)</th>
<th>LVIDd (cm)</th>
<th>LVISd (cm)</th>
<th>MDA (µM/mL)</th>
<th>PUA (mg/dL)</th>
<th>GPx (units/L)</th>
<th>GSH (µM/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL (n=13)</td>
<td>937.50±43.69ª</td>
<td>0.86±0.11b</td>
<td>0.45±0.13ª</td>
<td>3.04±0.33ª</td>
<td>0.07±0.05b</td>
<td>30.90±1.86ª</td>
<td>11.25±1.46ª</td>
</tr>
<tr>
<td>TRT (n=6)</td>
<td>396.80±61.79ª</td>
<td>1.07±0.16ª</td>
<td>0.89±0.19ª</td>
<td>3.17±0.46ª</td>
<td>0.11±0.04ª</td>
<td>28.17±2.59ª</td>
<td>3.98±2.07ª</td>
</tr>
</tbody>
</table>

*Where LVIDd and LVISd represent left ventricular internal-diastolic dimension and left ventricular internal-systolic dimension, respectively. MDA, PUA, GPx, and GSH represent malondialdehyde, plasma uric acid, glutathione peroxidase, and glutathione, respectively, and n is the number of birds analyzed. CTL and TRT for control and treatment, respectively.

*One unit was the amount of enzyme necessary to catalyze the oxidation (by H₂O₂) of 1.0 µmol of reduced glutathione to oxidized glutathione/minute at 25°C, pH 7.0.

ª, b Measurements in the same column with similar alphabetic superscript are not significantly different (P>0.05).
Table 4.4. Percent shortening fraction for poults fed normal (CTL) and Fz-containing (TRT) diets.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shortening fraction (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 2</td>
<td>Week 4</td>
<td></td>
</tr>
<tr>
<td>CTL</td>
<td>40</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>TRT</td>
<td>11</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.5. Pearson Correlation Coefficients of left ventricular measurements with biomarkers for 2 (above x) and 4 (below x) week-old poults fed normal (CTL) and furazolidone containing diet (TRT)

<table>
<thead>
<tr>
<th>Dietary treatments</th>
<th>LVIDd§ (cm)</th>
<th>LVISd (cm)</th>
<th>MDA (mg/dL)</th>
<th>PUA (µM/mL)</th>
<th>GPxǂ (units/L)</th>
<th>GSH (µM/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL</td>
<td>TRT</td>
<td>CTL</td>
<td>TRT</td>
<td>CTL</td>
<td>TRT</td>
</tr>
<tr>
<td>LVIDd</td>
<td>x</td>
<td>x</td>
<td>0.66086</td>
<td>&lt;.0001</td>
<td>NS§</td>
<td>NS</td>
</tr>
<tr>
<td>LVISd</td>
<td>0.71850</td>
<td>0.98742</td>
<td>x</td>
<td>x</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MDA</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>PUA</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>x</td>
</tr>
<tr>
<td>GPx</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>GSH</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

§LVIDd, LVISd, MDA, PUA, GPx and GSH represents left ventricular internal-diastolic dimension, left ventricular internal-systolic dimension malondialdehyde, plasma uric acid, glutathione peroxidase and glutathione, respectively.

ǂOne unit was the amount of enzyme necessary to catalyze the oxidation (by H₂O₂) of 1.0 µmol of reduced glutathione to oxidized glutathione/minute at 25°C, pH 7.0.

¥NS represents non significant correlation between left ventricular measurement and biomarker.
Table 4.6. Necropsy measurements of 2 and 4 week old poults fed normal (CTL) and furazolidone (TRT) containing diet.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WHO* (g)</th>
<th>Apex-Thorax(mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 2</td>
<td>Week 4</td>
</tr>
<tr>
<td>CTL</td>
<td>1.73±1.99&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.84±0.63&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TRT</td>
<td>2.20±0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.48±0.58&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*WHO represents weight of whole heart.

<sup>a,b</sup>Measurements in the same column with similar alphabetic superscript are not significantly different (P>0.05).
CHAPTER 5
The Effects of Dietary Vitamin E and Selenium on the Incidence and Severity of Toxin-Induced DCM in the Turkey

5.1 Abstract

There is strong interest in understanding the role of antioxidants in the well-being of many animals. The aim of this study was to assess the influence of dietary selenium (Se) and vitamin E on the relationship between oxidative stress (OS) and the incidence and severity of furazolidone (Fz)-induced dilated cardiomyopathy. Pouls were fed normal and Fz-containing diets supplemented with combinations of Se (0.0, 0.3 and 0.5 mg/kg) and vitamin E (0, 50 and 100 IU/kg). Malondialdehyde (MDA), glutathione (GSH), glutathione peroxidase (GPx), and plasma uric acid (PUA) were used as biomarkers for OS. At two and four wks of age, measurements of MDA showed significantly increased concentrations for pouls fed Fz-containing diets supplemented with 0.3 mg/kg Se and 100 IU/kg vitamin E. Pouls fed Fz-containing diets supplemented with 0.5 mg/kg Se and 100 IU/kg vitamin E had the highest levels of GPx. PUA concentrations were higher for pouls fed diets with Fz supplemented with 0.5 mg/kg Se when compared with controls at two wks of age. At four wks of age, increased PUA levels were observed for pouls fed Fz-containing diets supplemented with 100 IU/kg vitamin E. Pouls fed diets supplemented with 50 and 100 IU/kg vitamin E had the highest GSH at two wks of age. In this study, we showed that no specific combination of vitamin E and selenium appeared to have a mitigatory or enhancing effect on the toxicity of furazolidone.

**Keywords:** Turkey, Dilated Cardiomyopathy, Oxidative Stress, Dietary antioxidants
5.2 Introduction

Excessive production or environmental exposure to reactive oxygen and nitrogen species via endogenous metabolic pathways cause oxidative stress (OS) and damage to biological molecules (Pryor and Godber, 1991). Orrenius et al. (2007) reported that an imbalance between oxidants and antioxidants increased the risk for peroxidation of lipids, damage to DNA and proteins which may lead to diseases of economic importance including dilated cardiomyopathy (DCM).

In birds, free radicals (FRs) can be formed in the heart by a variety of mechanisms including oxidative phosphorylation in the mitochondria and as a byproduct of normal cellular aerobic metabolism (Braunwald, 2001, and Bishop and Butler, 1995). Ku and Sohal (1993) discussed the generation of FR metabolites from oxygen metabolism under normal condition. Excess FR generation has also been found to develop from many sources including vascular nicotinamide adenine dinucleotide oxidases (Rajagopalan et al., 1996), xanthine oxidases, auto-oxidation of catecholamines (Singla et al., 1983) and nitric oxide syntheses activation (Habib et al., 1996, and Oyama et al., 1998). McMurray et al. (1992) reported increased lipid-peroxidation in the plasma of patients with heart failure. In myopathic rat, decreased \( \alpha \)-tocopherol, superoxide dismutase, catalase, and glutathione peroxidase (GPx) along with a significant increase in lipid-peroxidation have been reported (Hill and Singal, 1996). An increase in the antioxidant levels in poultry has also been proposed to enhance the defense against reactive species-mediated damage. Supplementing poultry diets with inosine, a precursor of uric acid or with vitamin E reduced tissue injury caused by FR damage and enhanced growth performance (Simoyi et al., 2002, and Villar-Patino et al., 2002). Over-production of FRs overwhelms the natural defenses of the body, leading to damage or modification of organic macromolecules such as DNA, proteins, and lipids.
However, the body is able to withstand and ameliorate the damage caused by FRs through its antioxidant defense network systems. Dilated cardiomyopathy is a disease characterized by low ejection fraction, left ventricular enlargement, and apoptosis (Winters et al., 1993, and Hajjar, 1993). DCM has been reported to affect diverse animals including humans and the turkey (Hasenfuss, 1998). In humans, DCM has been reported to account for up to 72% of 1-year mortality, in turkeys 2-4%. This makes DCM important disease both animal and human health (Frame et al., 1999).

Traber et al. (1990) discussed the antioxidant network systems (enzymatic and non-enzymatic), which ensure protection from damage caused by FRs. The enzymatic antioxidant system consists of catalase, superoxide dismutase, glutathione (GSH) and GPx, while vitamin E, C, D and selenium are a part of the non-enzymatic antioxidant system that help protect the functional and structural molecules against FR or OS mediated cytotoxicity and tissue damage (Kaul et al., 1995). Excess FR production or impaired antioxidant function in *in vitro* experiments have been proposed to adversely affect several myocyte functions (Goldhaber et al., 1989, and Kaneko et al., 1989), depress myocardial contractility (Schrier et al., 1988), cause myocardial tissue injury (Burton et al., 1984), and induce myocyte apoptosis (Gottlieb et al., 1998). A study using an *in vivo* animal model has demonstrated the significance of oxidative injury to cardiac function (Bolli et al., 1987). The best-studied example is myocardial stunning and injury due to reperfusion after a period of ischemia in rats (Bolli et al., 1987). In a different study, Singal et al. (1998) confirmed a role for OS in DCM. In dogs, the administration of α-tocopherol acutely improved cardiac function in tachycardia-induced cardiomyopathy providing evidence that, besides oxidant injury, OS may also reversibly depress cardiac function (Ukai et al., 2001). Li et al. (1997) reported reduced levels of GPx and α-tocopherol in DCM-affected
Syrian hamsters. Administration of high levels of $\alpha$-tocopheryl acetate restored $\alpha$-tocopherol, GPx and protein oxidation and increased the amount of protein oxidation by more than double in the heart (Li et al., 1997). Under experimental conditions antioxidant drugs have also been shown to prevent the occurrence of heart failure and increase survival (Dhalla et al., 1996).

Currently, there is no data about the effect of dietary antioxidants on toxin-induced DCM in the turkey. Changes in endogenous antioxidant levels proportional to oxidants have been reported to significantly increase degeneration of cardiac myocytes and cardiovascular disease in humans and other animals (Yucel et al., 1998). Additionally, an imbalance in antioxidant and oxidant levels has been shown to interfere with homeostasis of the heart, leading to FR activated sub-cellular changes progressing to cardiomyopathy and cardiac failure (Singal et al., 1998). Thus, it is rational to evaluate the influence of dietary vitamin E and selenium, as the primary objective of this study. In the current study, the effects of dietary vitamin E and Se as dietary supplements on furazolidone-induced DCM in poults were evaluated.
5.3 Materials and Methods

**Animals and Dietary treatments**

A total of 450 day-old male Hybrid birds obtained from Ag Forte (Harrisonburg, Virginia) with a high prevalence of spontaneous DCM were used. A corn-soybean meal basal diet was formulated to meet the National Research Council (1994) recommendations and fed *ad libitum* till 4 wks of age (Table 5.1). On receipt, poult's were weighed and randomly divided among 50 cages (10 poult's per cage) with 25 poult's in a 2 x 2 x 3 factorial arrangement of dietary treatments and raised according to standard management practice (Gyenai, 2005). The first factor was furazolidone (Fz) at 700 parts per million to normal diet. The second factor was Se and vitamin E supplementation to the diet. The third factor was the level of supplementation of Se: (0, 0.3 and 0.5 mg/kg) and vitamin E (0, 50 and 100 IU/kg) to the diet. Selenium and vitamin E combinations were fed in four different forms. In the vitamin E replication, 0, 50 and 100 IU/kg supplementary vitamin E and 0, 0.3 and 0.5 mg/kg Se were fed in combination to base levels in standard mash diet.

**DCM diagnosis**

Two-dimensional long-axis imaging planes were obtained from the right parasternal location of poult's gently restrained in left lateral recumbence using a portable Aloka Echocardiography (ECHO) machine with a 7.5 MHz phased array transducer. The two-dimensional ECHO readings were then viewed in the M-Mode for systolic and diastolic readings. The M-Mode generated a one-dimensional view of small portions of the heart which allowed for the detection of axial motion of structures parallel to the beam (Kienle and Thomas, 1995). Left ventricular internal-diastolic (LVIDd) and internal-systolic dimension (LVISd) were measured for the largest and smallest ventricular diameters immediately below the mitral valve
and parallel to the mitral annulus at 2 and 4 wks of age on all poults (Vollmar, 1999, and Wu et al., 2004).

**Shortening fraction**

The *in vivo* contractile performance of the heart of each poult was estimated according to Wu et al. (2004) using the equation:

\[ \frac{(LVID_d - LVIS_d)}{LVID_d} \times 100 \]

The rationale is that the shortening fraction estimates the left ventricular performance based on the ratio of the change in the diameter of the left ventricle in diastole and systole.

**Necropsy**

At 2 and 4 wks of age, some poults were sampled from both the control and treatment groups for standard necropsy to compare ECHO measurements to visual and pathological diagnosis. Hearts were dissected, atria and large vessels removed. Weight of the entire heart (WHO) was taken and the length of the apex to the thorax were conducted using vernier calipers.

**Blood collection**

Blood was collected into vacutainer tubes containing 0.5 M ethylenediaminetetraacetic acid from poults by venipuncture for each biomarker analysis as follows: Plasma was obtained by centrifuging the blood samples at approximately 10,000 rpm for 15 minutes at 4°C. Deproteininated whole blood was obtained by centrifuging at 3,000 rpm for 5 minutes with 50 micro liters (μl) of 5 % metaphosphoric acid per 50 μl ethylenediaminetetraacetic acid. The samples were first stored on ice at 4 °C, then snap frozen on dry ice before storing at 80°C until ready for use. The plasma was used for the analyses of PUA, MDA and GPx, while the deproteininated whole blood was used to determine total GSH levels as described below.
**Biomarker analysis**

**Plasma uric acid (PUA)**

The enzymatic assay for PUA has previously been described (Hartman et al., 2006). Duplicate samples were evaluated using an optimized commercial assay (Diazyme. La Jolla. CA). Briefly blood samples were spun down in 1.5 mL centrifuge tubes at 2000 rpm for 15 minutes at 4 °C to obtain plasma. If the plasma samples were not used immediately, they were stored at -80 °C. The assay for PUA determination in plasma involved conversion of uric acid in the sample into allantoin and hydrogen peroxide by the enzyme uricase. The hydrogen peroxide subsequently reacts with oxidizing N-ethyl-N-sulphoproyl-m-anizidin (ESPAS) and 4-aminoantipyrene with the help of peroxidase to form a red-violet quinoneimine dye (Hartman et al., 2006). The reaction is summarized as follows:

\[
\text{Uric acid} + \text{O}_2 + 2\text{H}_2\text{O} \xrightarrow{\text{uricase}} \text{Allantoin} + \text{CO}_2 + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + \text{ESPAS} + 4\text{-aminopyrene} \xrightarrow{\text{peroxidase}} \text{Quinine dye} + 4\text{H}_2\text{O}
\]

The amount of quinine dye formed, which is a direct proportion of the level of PUA in plasma, was estimated using a spectrophotometer at an absorbance of 600 nm (A\text{600nm}).

**Glutathione (GSH)**

As for GSH, duplicate samples from each poult were also assayed. Whole blood total GSH was measured using a spectrophotometric assay at A\text{412nm} using an adaptation of the manufacturers protocol (Northwest Life Science Specialties, Vancouver, WA). The general thiol reagent, 5-5′-dithiobis [2-nitrobenzoic acid] (DTNB, Ellman’s Reagent) reacts with GSH to form the A\text{412nm} chromophore, 5-thionitrobenzoic acid (TNB) and glutathione S-transferases (GS)-TNB (Engle et al., 2009). The GS-TNB is subsequently reduced by glutathione reductase and β-
nicotinamide adenine dinucleotide phosphate, releasing a second TNB molecule and recycling GSH; thus amplifying the response. Any oxidized GSH (GSSG) initially present in the reaction mixture or formed from the mixed disulfide reaction of GSH with GS-TNB is rapidly reduced to GSH. An adaptation made to the manufacturers protocol consisted of deproteination of whole blood prior to each enzymatic reaction by adding 5% metaphosphoric acid per 50 µl ethylenediaminetetraacetic acid to whole blood (Teitze, 1969, and Engle et al., 2009). To maintain consistent spectrophotometer readings, for the duplicate samples a 40-fold dilution of each blood sample and 5 fold dilutions of the calibrators were carried out. The absorbance was recorded at 60-second intervals for 10 minutes. Total GSH concentration of each sample was calculated from the absorbance reading using linear regression (Bloomer, 2007).

**Malondialdehyde (MDA)**

The enzymatic assay used to estimate MDA in each plasma sample measured lipid peroxidation according to Botsoglou (1994) and Jentzsch et al. (1996). The enzymatic assay was based on the reaction of MDA with two molecules of thiobarbituric acid (TBA) resulting in an MDA-TBA2 adduct. During the reaction, butylated hydroxytoluene (BHT) and ethylenediaminetetraacetic acid were added to the reaction mixture to minimize oxidation of lipids that contribute artifactually to the TBA reaction (Jentzsch et al., 1996). The temperature of the reaction mixture was reduced to (60°C) to minimize the decomposition of lipid hydroperoxides. The pH of the reaction was optimized to obtain optimal yields of MDA product at pH 1.5 in the presences of hydrochloric acid to facilitate hydrolysis of the MDA (Gerard-Monnier et al., 1997). The reaction mixture was then subjected to spectrophotometric analysis at 532 nm ($A_{532\text{nm}}$).
Glutathione Peroxidase (GPx)

The assay (Northwest Life Science Specialties Vancouver, WA, USA) kit used to determine plasma GPx-3 activity was an adaptation of the method of Paglia and Valentine (1967). The theoretical basis of the assay is that GPx catalyzes the reduction of hydrogen peroxide and reduced glutathione to form oxidized glutathione. Oxidized glutathione is then reduced by glutathione reductase and β-nicotinamide adenine dinucleotide phosphate (NADP) to form NADP+ (Lee et al., 2008). Because GPx is limiting, the change in absorbance at A340 is proportional to its concentration. GPx activity was reported as units based on the definition: 1 unit of GPx-1 = the amount of enzyme necessary to catalyze the oxidation (by H2O2) of 1.0 μmole GSH to GSSG per minute at 25 ºC, pH 7.0 (Lee et al., 2008).

Statistical analysis

All data were analyzed using the generalized linear model mixed (GLIMIX) procedures of SAS (SAS Institute, Cary, NC, 2002). The statistical model was

\[ Y_{ijk} = \mu + T_i + N_j + TN_{ij} + e_{(ij)k} \]

where \( Y_{ijk} \) was the observed dependent variable, \( \mu \) is the grand mean, \( T_i \) the furazolidone treatment effect, \( N_j \) dietary effect, \( TN_{ij} \) is the interaction between treatment and dietary effect, \( e_{(ij)k} \) is the error. Significant treatment differences were established using the least-squares means (LSMEANS) statement in SAS (P ≤ 0.05) for poult fed normal at Fz-containing diets and 2 and 4 wks of age. Results were presented as LSMEANS and standard error.
5.4. Results

As expected, poults on diets with Fz had higher mortality at both 2 and 4 wks of age (Table 5.2). At 2 wks of age, mortality was lowest for control poults fed diets supplemented with 0.3 mg/kg Se, 0.5 mg/kg Se and 50 IU/kg vitamin E, 0.5 mg/kg Se and 50 IU/kg vitamin E. Poults fed Fz-containing diets supplemented with 50 IU/kg vitamin E had the highest mortality at 2 wks of age. At 4 wks of age, control poults fed diets supplemented with 100 IU/kg of vitamin E had the highest mortality (Table 5.2). For poults fed diets containing Fz, those on diets supplemented with 50 IU/kg vitamin E and those fed 0.5 mg/kg Se had the highest mortality at 100 % mortality by 4 wks of age.

Body weight (BW) was 10 to 20 % higher for poults on diets containing Fz supplemented with combinations of Se and vitamin E with the exception of poults fed 0.5 mg/kg Se and those on 50 IU/kg vitamin E at 2 wks of age. BW was largest for control poults fed diets supplemented with 100 IU/kg vitamin E (Table 5.2). For poults fed diets containing Fz, BW was largest for those fed 0.5 mg/kg Se and 50 IU/kg vitamin E combined. At 2 wks of age, interaction effect was significantly different for controls and poults fed diets containing Fz. There was no effect for Se, vitamin E, Se x vitamin E x treatment interaction (Table 5.3). The interaction resulted from poults fed Fz-containing diets having smaller BW. Body weight measurements at 4 wks of age were 80 to 100 % increase for control group poults fed diets supplemented with different combinations of Se and vitamin E (Table 5.3). For controls, higher BW were observed for those on diets supplemented with 0.3 mg/kg Se, while those fed 0.5 mg/kg Se had the smallest BW. Among poults fed Fz-containing diets, those on 100 IU/kg vitamin E had the largest (Table 5.3). BW was significant for treatment effect and Se x vitamin E interaction at 4 wks of age (Table 5.3).
At 2 wks of age, differences in MDA for controls were significant only for those fed 0.3 mg/kg Se (Table 5.4). Pouls fed Fz-containing diets supplemented with combinations of 0.5 mg/kg Se and 100 IU/kg vitamin E, 0.3 mg/kg Se and 100 IU/kg vitamin E, 0.5 mg/kg Se and 50 IU/kg vitamin E had higher MDA at 2 wks of age. Pouls fed diets containing Fz without supplemental Se and vitamin E had the lowest MDA concentrations. Comparison of MDA levels between pouls fed Fz-containing diets and controls showed 0.5 to 220% differences. Interaction effect at this age was significant for treatment. The significant effect for treatment observed may have been due to the influence of Fz addition to diets causing high lipid peroxidation levels.

At 2 wks of age, PUA measurements for control group pouls fed diets supplemented with different combinations of Se and vitamin E were significant only for those on 0.3 mg/kg Se and 50 IU/kg vitamin E. Among pouls fed Fz-containing diets, pouls fed diets supplemented with 0.5 mg/kg Se had higher PUA levels. Comparison of PUA levels between controls and pouls fed diets containing Fz showed significant differences, except for those fed 0.5 mg/kg Se and 50 IU/kg vitamin E, 0.5 mg/kg Se and 100 IU/kg vitamin E, 0.3 mg/kg Se and 50 IU/kg vitamin E and 100 IU/kg vitamin E. Interaction effect was significant for vitamin E and for Se x vitamin E x treatment.

GPx measurements were different for control group pouls fed diets supplemented with 0.5 mg/kg Se, 0.5 mg/kg Se and 50 IU/kg vitamin E, 0.5 mg/kg Se and 100 IU/kg vitamin E at 2 wks of age, respectively (Table 5.3). Higher GPx activities were observed for pouls on Fz diets supplemented with 0.5 mg/kg Se and 100 IU/kg vitamin E, 100 IU/kg vitamin E, 0.3 and 0.5 mg/kg Se. Comparison of GPx activities between controls and pouls fed diets with Fz showed significantly higher levels for pouls on 0.3 mg/kg Se and those fed 100 IU/kg vitamin E at 2 wks of age. Interaction effect was significant for treatment and Se (Table 5.4).
GSH measurements showed a much different trend compared to that of other antioxidant biomarkers at 2 wks of age (Table 5.4). Measurements of GSH levels for control group poult showed no difference except for those fed diets supplemented with 0.5 mg/kg Se and 100 IU/kg vitamin E. Poults fed 100 IU/kg vitamin E had 18 % decreased GSH at 2 wks of age (Table 5.4). For poult fed diets containing Fz, those fed 50 and 100 IU/kg vitamin E had higher GSH concentrations (Table 5.4). Poults fed 0.5 mg/kg Se and 50 IU/kg vitamin E had decreased GSH at 2 wks of age. Differences among Se and vitamin E dietary groups for control group and poult fed Fz-containing diets showed about 90 to 600 % increase difference in GSH concentrations (Table 5.4). Interaction effect was significant for treatment, Se, Se x treatment and Se x vitamin E x treatment interaction.

Concentrations of biomarkers MDA, PUA, GPx and GSH at 4 wks of age are presented in Table 5.5. Since mortality in the treatment group was high for poult on all the diets, the numbers of poult evaluated at this age were limited, ranging from 0 to 9. For control group and poult fed diets containing Fz, MDA ranged from 2.38 to 3.85 µM/mL for those on 0 mg/kg Se and vitamin E, 100 IU/kg vitamin E and 0.3 mg/kg Se, and 50 IU/kg vitamin E, respectively. Within controls, the concentrations ranged from 2.34 to 3.90 µM/mL for poult where diet was supplemented with 0 mg/kg Se and vitamin E, 100 IU/kg vitamin E and 0.3 mg/kg Se, 50 IU/kg vitamin E, respectively. Differences between control and treatment were not significant for all Se and vitamin combinations fed as supplements. Except for treatment effect, all interactions for MDA were not significant. MDA levels decreased 50 % from 2 to 4 wks of age for poult fed diets containing Fz (Table 5.3 and 5.4).

PUA concentrations were significantly higher for controls fed diets supplemented with 0.3 mg/kg Se and 50 IU/kg vitamin E, 0.3 mg/kg Se and 100 IU/kg at 4 wks of age (Table 5.5).
For poult's fed Fz-containing diets, PUA concentrations were not different for all dietary group poult's except for poult's fed diets with 0 mg/kg Se and vitamin E, 100 IU/kg vitamin E, 0.5 mg/kg Se, and 100 IU/kg vitamin E which had decreased PUA levels (Table 5.5). Differences in PUA concentrations between controls and poult's fed diets containing Fz showed higher PUA concentrations for poult's fed diets with Fz with the exception of those on 0.3 mg/kg Se and 50 IU/kg vitamin, 0.3 mg/kg Se and 100 IU/kg vitamin E. Interaction effects were not significant at this age. PUA levels were higher for both control and treatment group poult's from 2 to 4 wks of age, except for those fed normal diets supplemented with 0 mg/kg Se and vitamin E, 50 and 100 IU/kg vitamin E.

GPx activities for controls ranged from 29.51 to 36.94 units/L for poult's fed diets supplemented with 0 mg/kg Se and vitamin E, 0.5 mg/kg Se and 100 IU/kg vitamin E, and 100 IU/kg vitamin E (Table 5.5). For poult's fed diets containing Fz, higher GPx activities were observed for controls fed diets without Se and vitamin E. Differences in GPx between controls and poult's fed diets containing Fz showed higher GPx activities for controls (Table 5.5). Interaction effects was significant for treatment, Se x vitamin E, and Se x treatment. Differences in GPx levels from 2 to 4 wks of age showed higher levels for control group poult's. For poult's fed diets containing Fz, those with higher GPx activities at 2 wks of age had either decreased or no change in GPx at 4 wks of age.

GSH concentrations were higher for control group poult's fed diets supplemented with 100 IU/kg vitamin E at 4 wks of age (Table 5.5). For poult's fed diets containing Fz, higher GSH concentrations were observed for poult's fed 0.5 mg/kg Se and 100 IU/kg vitamin E. Comparison of GSH concentrations between control and poult's fed Fz-containing diets showed higher GSH concentrations for controls, except for those fed 0.5 mg/kg Se and 50 IU/kg vitamin E (Table
5.5). There were no significant differences in GSH concentrations between 2 and 4 wks for both control and treatment group poultls, except for those fed diets supplemented with 100 IU/kg vitamin E. For poultls fed diets containing Fz, decreased GSH levels were observed for poultls on 0.5 mg/kg Se, 0.5 mg/kg Se and 50 IU/kg vitamin E, 0.5 mg/kg Se and 100 IU/kg vitamin E from 2 to 4 wks of age (Table 5.5). Interaction effects were significant for treatment, Se, Se x treatment interaction, and Se x vitamin E x treatment.

Left ventricular measurements for poultls fed diets containing Fz supplemented with different combinations of Se and vitamin E at 2 wks of age, showed 5 to 200 % increase LVIDd (Table 5.6). LVISd measurements were higher for poultls on 0 mg/kg Se and vitamin E, 0.3 mg/kg Se and 50 IU/kg vitamin E within the treatment group. For control group poultls, those fed diets without Se and vitamin E had decreased LVIDd with the exception of those fed diets supplemented with 0.3 mg/kg Se, 0.5 mg/kg Se and 100 IU/kg vitamin E (Table 5.6). Interaction effect was significant for all effects of LVIDd and LVISd with the exception of Se, and Se x vitamin E interaction for LVIDd.

At 4 wks of age, differences in echocardiography measurements LVIDd and LVISd for control group poultls were similar to that discussed at 2 wks of age (Table 5.6 and 5.7). Poultls fed diets containing Fz supplemented with 0.5 mg/kg Se and 50 IU/kg, 0.3 mg/kg Se and 50 IU/kg vitamin E had the largest LVIDd and LVISd, respectively (Table 5.7). For poultls fed diets containing Fz, larger LVIDd and LVISd were observed for poultls on 0.3 mg/kg Se and 50 IU/kg vitamin E, 0.5 mg/kg and 50 IU/kg vitamin E. Interaction effect was significant only for LVIDd and LVISd treatment effect.

Percent shortening fractions at 2 wks of age was higher for poultls fed diets without Se and vitamin E (Table 5.8). For those fed diets containing Fz, increased levels were observed for
those on 0.5 mg/kg. At 4 wks of age, differences in percent shortening fraction between controls and poults fed diets containing Fz was significantly higher for control group poults fed diets with different combinations of Se and vitamin E (Table 5.8).

Pearson correlation coefficients are reported in Table 5.9 and 5.10. Positive correlations were observed for LVIDd with LVISd at 2 wks of age (Table 5.9). LVIDd correlated negatively with BW and positively with GSH. Positive correlations were also observed for LVISd with GSH at 2 wks of age (Table 5.10). Negative correlations were observed for BW with GSH. Positive correlations were observed for GPx with GSH, PUA and GSH with MDA. At 4 wks of age, positive correlations were observed between LVIDd and LVISd (Table 5.10). LVIDd was negatively correlated with GPx. LVISd correlated negatively with BW, GPx with GSH at 4 wks of age (Table 5.10). BW correlated positively with GPx and GSH. A positive correlation was observed between GPx and GSH at 4 wks of age (Table 5.10).

Necropsy measurement at 2 wks of age showed no significant differences for weight of whole heart and measure of apex to thorax between control group and poults fed diets containing Fz supplemented with different combinations of Se and vitamin E (Table 5.11). Comparison of weight of whole heart between controls and poults fed Fz-containing diets showed larger measurements for poults fed 0.3 mg/kg Se and 50 IU/kg vitamin E, and 0.5 mg/kg Se, at 2 wks of age. Poults fed diets supplemented with 0.5 mg/kg Se however, had the smallest heart weight. Measurements of apex to the thorax showed no differences between controls and those fed diets containing Fz supplemented with 0.3 mg/kg Se, 100 IU/kg vitamin E, and 0.5 mg/kg Se and 100 IU/kg vitamin E combined at 2 wks of age (Table 5.11). Poults fed diets supplemented with combinations of 0.3 mg/kg Se and 50 IU/kg vitamin E, 0.3 mg/kg Se and 100 IU/kg vitamin E,
and those fed 0.5 mg/kg Se were different when compared to other dietary groups, but not with each other.

At 4 wks of age, significantly larger differences were observed between control groups and poult's fed Fz-containing diets supplemented with varying Se and vitamin E combinations. No differences were observed for weight of whole heart (Table 5.11). Comparisons of the two treatment groups showed control group poult's fed diets supplemented with 0.5 mg/kg Se and 100 IU/kg vitamin E to have the largest heart weight (Table 5.11). Length of apex to thorax measurement showed control group poult's fed diets supplemented with 0.3 mg/kg Se and 50 IU/kg vitamin E to have the largest heart weight at 4 wks of age. Poult's fed diets supplemented with 0.3 mg/kg Se and 100 IU/kg vitamin E, and 0.5 mg/kg Se had the smallest measurement at 4 wks of age. Poult's fed Fz-containing diets supplemented with combinations of 0.3 mg/kg Se and 100 IU/kg vitamin E, 0.5 mg/kg Se and 100 IU/kg vitamin E, had significantly larger apex to thorax measurements (Table 5.11).
5.5. Discussion

Despite the emergence of DCM as a major cardiovascular affliction of the 21st century with increasing morbidity, mortality, and cost in both animal and human lives, a unifying concept for DCM has been elusive. One strategy to gain new insight into the fundamental characteristics of DCM is the use of supplemental antioxidants. However, the use of antioxidant in the turkey, an animal model for DCM, remains limited. In this study, the influence of supplemental Se and vitamin E combinations on control and poult fed diets containing Fz a compound known to induce DCM was evaluated. Mortality at 2 and 4 wks of age was affected by treatment, where poult fed Fz-containing diets supplemented with different combinations of Se and vitamin E had significantly higher mortality rates compared with those fed control diets. At 2 wks of age, mortality was lowest for those fed control diets supplemented with 0.3 mg/kg Se, 0.5 mg/kg Se and 50 IU/kg vitamin E. Poult fed Fz-containing diets supplemented with 50 IU/kg vitamin E had highest mortality at 2 wks of age. The observation of high mortality for poult fed diets containing Fz was consistent with increased ventricular dilation LVIDd and LVISd measurements indicative of DCM. At 4 wks of age, poult fed Fz-containing diets supplemented with 50 IU/kg vitamin E and 0.5 mg/kg Se had the highest mortality 100 %. The increased mortality observed at 2 and 4 wks of age for poult fed Fz-containing diets was a result of Fz causing an increase in ventricular dilation leading to cardiac failure. Similar to our results, a study by Sautter et al. (1968) reported of high mortality in poult fed diets containing Fz. In a different study, Gwathmey et al. (1999) reported 59 % mortality in DCM affected turkeys and 22 % mortality in the group treated with carteolol.

Previous studies have demonstrated beneficial effects of supplemental Se and vitamin E on traits of economic importance (Avanzo et al., 2001; Numes et al., 2005, and Meydani, 1997).
A study by Li et al. (1997) showed that administration of α-tocopheryl acetate at higher concentrations restored α-tocopherol, GPx and protein oxidation levels to normal in DCM-affected Syrian hamsters. Similarly, Hill et al. (2003) reported Se and vitamin E deficiency to result in decreased levels of GPx and α-tocopherol activity and increased levels of creatine phosphokinase in guinea-pigs. In our study we reported larger LVIDd and LVISd measurements for poults fed diets containing Fz supplemented with different combinations of Se and vitamin E at both 2 and 4 wks of age. The increased left ventricular dilation observed could be a result of Fz decreasing enzymatic antioxidant reserves leading to an imbalance in oxidant and antioxidant level. For instance, when Fz was given to rats as a single oral dose of 75, 150 or 300 mg/kg of body weight, it reduced glutathione (GSH) and ascorbic acid level, and increased lipid peroxidation concentration (Ali, 1992). In Fz-induced cardiomyopathic hearts, O'Brien et al. (1992) reported 50 % decrease in myocardial myoglobin. The decrease in myoglobin concentration in the heart correlated with changes in the biochemical and physiological indicators of myocardial performance. Our current echocardiographic results are in agreement with previous findings related to systolic and diastolic dysfunction in humans and dogs (Ernst et al., 2001; Domanjko et al., 2002, Wu et al., 2004, and Pratali et al., 2007). Increased ventricular dilatation coupled with decreased ejection fraction in DCM-affected broad-breasted white turkeys has been reported by Gwathmey et al. (1999). In a different study, Morris et al. (1999) reported oral administration of alcohol in chickens resulted in left ventricular dilation and dysfunction. We also found that feeding poults diets without supplemental Se and vitamin E and 0.3 mg/kg Se did not affect left ventricular dilation caused by Fz while a 38 % decrease in ventricular dilation was observed for poults receiving 0.5 mg/kg Se and 100 IU/kg vitamin E. Addition of supplemental Se and vitamin E did not ameliorate changes observed for LVIDd and
LVISd in pouls fed Fz-containing diets from 2 to 4 wks of age. The observations made from 2 to 4 wks of age, suggest that supplementation of pouls diet with 0.5 mg/kg Se and 100 IU/kg vitamin E decreased ventricular dilation at 4 wks of age the protection provided by Se and vitamin E declined over time.

Polyunsaturated lipids are very susceptible to FR attack. This process, referred to as lipid peroxidation, eventually yields several relatively stable decomposition products including aldehyde compounds that can be measured in plasma as an indirect index of FR activity (Gutteridge and Halliwell, 1990). In humans and other animals, MDA, the most commonly measured index of OS, is one of many aldehyde compounds produced by lipid peroxidation. Results of our current study showed higher MDA concentrations at 2 wks of age for pouls fed diets containing Fz supplemented with different combinations of Se and vitamin E. The higher MDA concentrations were related to the increased LVIDd and LVISd observed in pouls fed diets containing Fz. Pouls on 0.5 mg/kg Se and 100 IU/kg vitamin E had highest MDA concentrations. The higher MDA observed at 2 wks of age is similar to that seen by Keith et al. (1998) who reported increased MDA concentrations in patients with congestive heart failure. Increased MDA concentrations have also been reported in cardiomyopaththic hamsters (Kirsherbaum and Singal, 1993). In diabetic rats with DCM, increased MDA levels were reported before supplementation of diets with antioxidant alpha-lipoic acid (Li et al., 2009). Ide et al., (1999) reported increased myocardial lipid peroxidation levels in cardiomyocytes after 4 wks of rapid ventricular pacing in dogs. In a different study, increased MDA levels were reported in patients with congestive heart failure (Polidori et al., 2002). Increased tissue concentrations of MDA have been also found in rats measured under oxidative stress conditions (Koksal et al., 2004).
The low concentration of MDA found in plasma samples from poults fed control diets relative to that found in poults fed diets containing Fz supports the idea that a relation must exist between DCM and MDA. In our study DCM is associated with evidence of left ventricular systolic and diastolic dysfunction. Increased MDA concentrations may be involved directly in the pathophysiology of DCM at 2 wks of age. At 4 wks of age, plasma MDA concentrations in controls and poults fed diets containing Fz supplemented with different combinations of Se and vitamin E were not different except for poults on 100 IU/kg of vitamin E. These findings are similar with Sahin et al. (2001) which reported decreased serum and liver MDA concentrations in heat stressed Japanese quails.

Vitamin E is the first line of defense against lipid peroxidation (McDowell et al., 1989). By its FR quenching activity, it breaks chain propagation and thus terminates FR attack on polyunsaturated fatty acids of biomembranes (Schneider, 2005). It functions as an antioxidant, protecting the integrity of unsaturated bonds of cellular membrane phospholipids against FRs (Tappel, 1972, and Yap et al., 2001). Vitamin E is also believed to exert regulatory actions on the immune system as well as minimizing the pathology that can result from cytotoxic immune responses (Klasing, 1998). Similar to the present study, Morrissey et al. (1997) reported that supplementation of chicken diets with \( \alpha \)-tocopherol increased tissue \( \alpha \)-tocopherol concentrations and markedly decreased MDA. According to antioxidant theory, when the concentrations of antioxidant vitamin decrease, lipid peroxidation increases in the plasma and tissues leading to the damage of cell membranes (Gallo-Torres, 1980, and McDowell et al., 1989).

Previously, PUA has been proposed as one of the most important antioxidants in plasma (Ames et al., 1981). Urate, the soluble form of uric acid in the blood, has been reported to scavenge superoxide, hydroxyl radical, and singlet oxygen and chelate transition metals (Ames et
al., 1981, and Davies et al., 1981). Hink et al. (2002) reported that uric acid prevented FR degradation of extracellular superoxide dismutase, an enzyme critical in maintaining endothelial and vascular function. In this study, PUA concentrations were not different in control group and poults fed diets containing Fz at 2 wks of age, with the exception of those fed diets supplemented with 0.3 and 0.5 mg/kg Se, 50 IU/kg vitamin E, 0.3 mg/kg Se and 100 IU/kg vitamin E. PUA concentrations increased from 2 to 4 wks of age, in poults fed Fz diets supplemented with 0.3 mg/kg Se and 50 IU/kg, 0.5 mg/kg Se and 50 IU/kg vitamin E. The increase in PUA concentrations observed for poults fed diets containing Fz could be a result of PUA been less required as an antioxidant since previously Fz has been shown to be converted to a nitro radical (Lax and Kukolich, 1992). PUA may not be essential in ameliorating the nitro radical formed. Though PUA is important in scavenging free radicals (Hare and Johnson, 2003), chelate transition metals (Rowley and Halliwell, 1985), and block peroxynitrite, a toxic product of free radical reaction with nitric oxide (Whiteman and Halliwell, 1996). Our current finding of increased PUA levels are similar to that observed by Doehner et al. (2007) who reported an association between high uric acid concentrations and increased cardiovascular risk. In a different study, Alcaino et al. (2008) reported higher uric acid levels in patients with chronic heart failure. We found that supplementation of diets with 0.5 mg/kg of Se increased plasma PUA concentrations and decreased LVIDd and LVISd measurements at 2 wks of age. Supplementation of diets containing Fz with different combinations of Se and vitamin E had no influence on left ventricular dilation at 4 wks of age, although PUA concentrations were increased for all dietary groups except poults fed 0.3 mg/kg Se/50 IU/kg vitamin E and, 0.3 mg/kg Se/100 IU/kg vitamin E.
Measurement of GPx activity is one of the most accepted measures of assessing Se bioavailability. Selenium has a number of important biological roles, including regulation of the family of GPx enzymes and immunocompetence. GPx enzymes, remove hydrogen peroxide and the harmful lipid hydroperoxides generated in vivo by oxygen-derived species. This study showed significant influence of supplemental Se and vitamin E on GPx activities for poults fed Fz-containing diets at 2 wks of age, with the exception of those fed diets containing 0.5 mg/kg Se and 100 IU/kg vitamin E. For poults fed Fz-containing diets, GPx activities were highest for those without supplemental Se and vitamin E at 4 wks of age. The differences observed for GPx at 4 wks of age was indicative of consistency and less requirement of GPx usage for poults fed diets containing Fz despite the supplementation of diet with different combinations of Se and vitamin E. Although GPx, a selenium-dependent enzyme, contains selenium as the amino acid selenocysteine located at the active site of the enzyme and has been shown by many studies to increase the expression and activity of GPx and enhanced GPx activity to potentially lower the risk of cardiovascular diseases (Oster et al., 1983, Blakenberg et al., 2003). Our findings indicated no significant difference between control and poults fed Fz-containing diets. These findings are similar to that of Li et al. (1997) who reported no significant differences in GPx activities between control and cardiomyopathic hamsters fed vitamin E containing diets in the early stages of cardiomyopathy. In a different study, Lei et al. (2009) reported increased GPx activity in patients with Keshan disease, a fatal form of DCM.

GSH plays an important role in the antioxidant defense system by scavenging free radicals and regenerating other antioxidants (Meister and Anderson, 1983). GSH, when not completely used as substrate by GPx in the GSH cycle, may increase inside cells (Blankenberg et al., 2003). Bast et al. (1991) reported GSH to be pivotal in various protective systems such as
GPx, glutathione transferase, and FR reeducates. It is also responsible for the maintenance of protein thiol status in cells. In the present study, GSH concentrations in poults fed diets containing Fz were higher at 2 wks of age for those on 50 and 100 IU/kg vitamin E. The higher GSH concentration observed at 2 wks of age is consistent with the McMurray et al. (1993) study which reported that patients with congestive heart failure had increased erythrocyte GSH levels. It was suggested that decreased SOD activity causes decreased production of hydrogen peroxide which may lead to reduced oxidation of GSH by GPx. However, intracellular hydrogen peroxide occurs not only by formation in the SOD reaction but also by diffusing across cell membranes (Gutteridge, 1995). At 4 wks of age, GSH concentrations were reduced for all poults fed the Fz diet supplemented with different combinations of Se and vitamin E. The current research findings are similar to that of Keith et al. (1998), which reported decreased erythrocyte GPx activity in patients with congestive heart failure. For poults fed diets without Fz, reduced GSH concentrations were observed except for those on 100 IU/kg vitamin E.

Results of this study showed that at 2 wks of age, supplementation of Se and vitamin E for poults fed diets that contains Fz had no influence on the incidence and severity of DCM caused by the toxin Fz. At 4 wks of age, similar observations were made with respect to supplemental Se and vitamin E. The biomarkers used to evaluate the influence of feeding supplemental Se and vitamin E on DCM were inconsistent across 2 and 4 wks of age and between dietary groups. The inconsistency of biomarkers was the rational for our use of multiple biomarkers since a consensus remains limited with previous reports that a single biomarker may yield reliable prognosis.
Table 5.1. Composition and nutrient content of basal diet

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn meal</td>
<td>39.96</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>37.97</td>
</tr>
<tr>
<td>Meat and Bone meal</td>
<td>8.00</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>2.33</td>
</tr>
<tr>
<td>Dical Phosphate</td>
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</tr>
<tr>
<td>Ground lime stone</td>
<td>0.94</td>
</tr>
<tr>
<td>Lysine-HCL</td>
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</tr>
<tr>
<td>D. L. Methionine</td>
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</tr>
<tr>
<td>Salt (plain)</td>
<td>0.23</td>
</tr>
<tr>
<td>Poultry Vitamin Premix(^1)</td>
<td>0.10</td>
</tr>
<tr>
<td>Poultry Trace Mineral(^2)</td>
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</tr>
<tr>
<td>Coban 60</td>
<td>0.06</td>
</tr>
<tr>
<td>Copper sulfate</td>
<td>0.03</td>
</tr>
<tr>
<td>Wheat, Spring, grain</td>
<td>7.00</td>
</tr>
</tbody>
</table>

Calculated nutrient content

| Metabolizable Energy (kcal/kg) | 3,135.6290 |
| Crude protein (%)             | 29.8226    |
| Dry matter                    | 95.771     |
| Methionine                    | 0.8831     |
| Lysine                        | 2.0450     |
| Methionine + cystine          | 1.3311     |

\(^1\) Poultry vitamin premix supplied (per kg of diet): vitamin A (retinyl acetate), 8,820 IU; vitamin D\(_3\), 2,646 ICU; vitamin E (DL-\(\alpha\)-tocopheryl acetate), 22 IU; vitamin K\(_3\) (menadione dimethylpyrimidinol bisulfite), 2.65 mg; thiamin, 3.73 mg; riboflavin, 8.82 mg; pantothenic acid (D-calcium pantothenate), 22.1 mg; niacin, 88.2 mg; folic acid, 2.21 mg; biotin, 221 \(\mu\)g; vitamin B\(_{12}\) (cyanocobalamin), 26 \(\mu\)g.

\(^2\) Poultry trace minerals supplied (per kg of diet): iron (FeSO\(_4\)·H\(_2\)O), 40 mg; zinc (ZnO), 210 mg; manganese (MnO), 120 mg; copper (CuSO\(_4\)·5H\(_2\)O), 20 mg; iodine (Ca iodate), 3 mg; cobalt (Co) 50 \(\mu\)g.
Table 5.2. Percent mortality of poults fed diets with and without furazolidone and supplemented with selenium (Se) and vitamin E (VE) at 2 and 4 weeks-of-age

<table>
<thead>
<tr>
<th>Dietary supplements (Se mg/kg)</th>
<th>(VE IU/kg)</th>
<th>Mortality (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Week 2</td>
<td>Week 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTL</td>
<td>TRT</td>
<td>CTL</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>8</td>
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<td>36</td>
</tr>
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<td>36</td>
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<tr>
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<td>0</td>
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<tr>
<td>0.5</td>
<td>50</td>
<td>0</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>0.5</td>
<td>100</td>
<td>0</td>
<td>36</td>
<td>28</td>
</tr>
</tbody>
</table>

†Where CTL and TRT represent poults fed normal and furazolidone-containing diets
Table 5.3. Least Squares Means ± standard error of body weight measurements of poults fed diets with and without furazolidone supplemented with selenium (Se) and vitamin E (VE) at 2 weeks-of-age

<table>
<thead>
<tr>
<th>Dietary supplements (Se mg/kg)</th>
<th>(VE IU/kg)</th>
<th>Body Weight (g)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Week 2</td>
<td>Week 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTL† TR</td>
<td>CTL TRT</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>273.3±12.59(^{a1}) (n = 19)</td>
<td>253.2±16.54(^{b2}) (n = 7)</td>
</tr>
<tr>
<td>0</td>
<td>50</td>
<td>280.8±12.59(^{a1}) (n = 19)</td>
<td>241.1±24.50(^{b2}) (n = 10)</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>307.0±12.59(^{a1}) (n = 20)</td>
<td>245.9±16.55(^{b2}) (n = 11)</td>
</tr>
<tr>
<td>0.3</td>
<td>0</td>
<td>293.7±11.97(^{a1}) (n = 19)</td>
<td>236.6±16.55(^{b2}) (n = 12)</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>279.2±13.31(^{a1}) (n = 23)</td>
<td>174.8±20.74(^{b2}) (n = 14)</td>
</tr>
<tr>
<td>0.3</td>
<td>50</td>
<td>272.0±12.58(^{a2}) (n = 19)</td>
<td>222.6±19.40(^{b2}) (n = 8)</td>
</tr>
<tr>
<td>0.3</td>
<td>100</td>
<td>293.3±12.27(^{a1}) (n = 23)</td>
<td>231.2±18.29(^{b2}) (n = 14)</td>
</tr>
<tr>
<td>0.5</td>
<td>50</td>
<td>238.4±22.40(^{a3}) (n = 22)</td>
<td>280.72±11.44(^{a3}) (n = 13)</td>
</tr>
<tr>
<td>0.5</td>
<td>100</td>
<td>292.9±12.27(^{a1}) (n = 20)</td>
<td>238.4±22.40(^{b2}) (n = 11)</td>
</tr>
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</table>

Interaction effects

<table>
<thead>
<tr>
<th>Interaction effects</th>
<th>Probabilities (P-values)</th>
</tr>
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<tbody>
<tr>
<td>trt</td>
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<tr>
<td>Se</td>
<td>NS</td>
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<tr>
<td>Ve</td>
<td>NS</td>
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<td>Se x Ve</td>
<td>NS</td>
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</tr>
<tr>
<td>Ve x trt</td>
<td>NS</td>
</tr>
<tr>
<td>Se x Ve x trt</td>
<td>NS</td>
</tr>
</tbody>
</table>

†Where CTL and TRT represent poults fed normal and furazolidone-containing diets, respectively, and n is the number of birds analyzed.

\(^{a,b}\)Measurements in the same row with similar alphabetic superscript are not significantly different (\(P<0.05\)).

\(^{1,2}\)Values in the same column with similar numeric superscript are not significantly different (\(P<0.05\)).
Table 5.4. Least Squares Means ± standard error of MDA, PUA, GPx and GSH measurements of poults fed diets with and without furazolidone supplemented with selenium (Se) and vitamin E (VE) at 2 weeks-of-age

<table>
<thead>
<tr>
<th>Dietary supplements (Se mg/kg)</th>
<th>MDA (µM/mL)</th>
<th>PUA (mg/dL)</th>
<th>GPx (units/L)</th>
<th>GSH (µM)</th>
</tr>
</thead>
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<tr>
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<td>CTL¹</td>
<td>TRT</td>
<td>CTL</td>
<td>TRT</td>
</tr>
<tr>
<td>0 0</td>
<td>3.09±0.98²b²</td>
<td>3.13±1.29³a²</td>
<td>0.03±0.01b²</td>
<td>0.05±0.02a²</td>
</tr>
<tr>
<td>0 50</td>
<td>3.61±0.98³b²</td>
<td>4.64±1.92³b²</td>
<td>0.07±0.01a²</td>
<td>0.04±0.02b³</td>
</tr>
<tr>
<td>0 100</td>
<td>3.71±0.98³b²</td>
<td>4.41±1.29³a²</td>
<td>0.04±0.01a²</td>
<td>0.03±0.01a³</td>
</tr>
<tr>
<td>0.3 0</td>
<td>4.85±0.94³b²</td>
<td>6.28±1.29³a²</td>
<td>0.08±0.01a¹</td>
<td>0.05±0.02b³</td>
</tr>
<tr>
<td>0.5 0</td>
<td>3.11±1.04³b²</td>
<td>4.68±1.62³a²</td>
<td>0.03±0.02b²</td>
<td>0.12±0.01a²</td>
</tr>
<tr>
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<td>2.89±0.98³b²</td>
<td>4.03±1.52³a³</td>
<td>0.04±0.01a²</td>
<td>0.03±0.02a³</td>
</tr>
<tr>
<td>0.3 100</td>
<td>3.01±0.95³b²</td>
<td>7.65±1.43³a²</td>
<td>0.03±0.01b²</td>
<td>0.07±0.02a²</td>
</tr>
<tr>
<td>0.5 50</td>
<td>3.11±0.89³b²</td>
<td>8.16±1.75³a¹</td>
<td>0.04±0.01a²</td>
<td>0.03±0.02a³</td>
</tr>
<tr>
<td>0.5 100</td>
<td>3.12±0.95³b²</td>
<td>9.11±1.75³a¹</td>
<td>0.04±0.01a²</td>
<td>0.03±0.02a³</td>
</tr>
</tbody>
</table>

Interaction effects | Probabilities (P-values) |
<table>
<thead>
<tr>
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<th></th>
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<tbody>
<tr>
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<td>Se</td>
<td>NS</td>
</tr>
<tr>
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</tr>
<tr>
<td>Ve x trt</td>
<td>NS</td>
</tr>
<tr>
<td>Se x Ve x trt</td>
<td>NS</td>
</tr>
</tbody>
</table>

$MDA$, PUA, GPx and GSH are malondialdehyde, plasma uric acid, glutathione peroxidase and glutathione, respectively, and n is the number of birds analyzed.

†Where CTL and TRT represent poults fed normal and furazolidone-containing diets, respectively.

One unit was the amount of enzyme necessary to catalyze the oxidation (by H₂O₂) of 1.0 µmol of reduced glutathione to oxidized glutathione/minute at 25°C, pH 7.0.

Measurements in the same row with similar alphabetic superscript are not significantly different (P<0.05).

Values in the same column with similar numeric superscript are not significantly different (P<0.05).
Table 5.5. Least Squares Means ± standard error of MDA, PUA, GPx and GSH measurements of poults fed diets with and without furazolidone supplemented with selenium (Se) and vitamin E (VE) at 4 weeks-of-age.

<table>
<thead>
<tr>
<th>Dietary supplements</th>
<th>MDA$^1$ (µM/mL)</th>
<th>PUA (mg/dL)</th>
<th>GPx$^1$ (units/L)</th>
<th>GSH (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL$^2$</td>
<td>TRT</td>
<td>CTL</td>
<td>TRT</td>
</tr>
<tr>
<td>0 0</td>
<td>3.04±0.33$^{a1}$ (n = 19)</td>
<td>3.17±0.46$^{a1}$ (n = 7)</td>
<td>0.05±0.03$^{a3}$</td>
<td>0.07±0.05$^{a2}$</td>
</tr>
<tr>
<td>0 50</td>
<td>2.75±0.27$^{b1}$ (n = 17)</td>
<td>–</td>
<td>0.09±0.03$^2$</td>
<td>–</td>
</tr>
<tr>
<td>0 100</td>
<td>2.34±0.36$^{a1}$ (n = 9)</td>
<td>3.59±0.43$^{a1}$ (n = 7)</td>
<td>0.04±0.04$^{b3}$</td>
<td>0.09±0.04$^{a2}$</td>
</tr>
<tr>
<td>0.3 0</td>
<td>3.15±0.27$^{b1}$ (n = 17)</td>
<td>3.63±0.39$^{a1}$ (n = 7)</td>
<td>0.09±0.02$^{a2}$</td>
<td>0.11±0.04$^{a1}$</td>
</tr>
<tr>
<td>0.5 0</td>
<td>2.59±0.29$^{a1}$ (n = 16)</td>
<td>–</td>
<td>0.04±0.02$^{a3}$</td>
<td>–</td>
</tr>
<tr>
<td>0.3 50</td>
<td>3.90±0.34$^{a1}$ (n = 12)</td>
<td>3.85±0.65$^{a1}$ (n = 5)</td>
<td>0.15±0.03$^{a1}$</td>
<td>0.14±0.07$^{a1}$</td>
</tr>
<tr>
<td>0.3 100</td>
<td>2.95±0.29$^{a1}$ (n = 17)</td>
<td>2.49±0.57$^{a1}$ (n = 5)</td>
<td>0.13±0.03$^{a1}$</td>
<td>0.12±0.06$^{a1}$</td>
</tr>
<tr>
<td>0.5 50</td>
<td>2.42±0.27$^{a1}$ (n = 18)</td>
<td>3.16±0.51$^{a1}$ (n = 5)</td>
<td>0.05±0.03$^{b3}$</td>
<td>0.13±0.05$^{a1}$</td>
</tr>
<tr>
<td>0.5 100</td>
<td>2.57±0.30$^{a1}$ (n = 15)</td>
<td>2.38±0.57$^{a1}$ (n = 6)</td>
<td>0.05±0.03$^{b3}$</td>
<td>0.09±0.06$^{a2}$</td>
</tr>
</tbody>
</table>

Interaction effects  | Probabilities (P-values) |
<table>
<thead>
<tr>
<th></th>
<th></th>
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<tbody>
<tr>
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<td>&lt;0.0001</td>
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<tr>
<td>Se</td>
<td>NS</td>
</tr>
<tr>
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</tr>
<tr>
<td>Ve x trt</td>
<td>NS</td>
</tr>
<tr>
<td>Se x Ve x trt</td>
<td>NS</td>
</tr>
</tbody>
</table>

$^\S$MDA, PUA, GPx and GSH are malondialdehyde, plasma uric acid, glutathione peroxidase and glutathione, respectively, and n is the number of birds analyzed.

$^1$Where CTL and TRT represent poults fed normal and furazolidone-containing diets, respectively.

$^\parallel$One unit was the amount of enzyme necessary to catalyze the oxidation (by $H_2O_2$) of 1.0 µmol of reduced glutathione to oxidized glutathione/minute at 25°C, pH 7.0.

$^{ab}$Measurements in the same row with similar alphabetic superscript are not significantly different ($P<0.05$).

$^{1,2}$Values in the same column with similar numeric superscript are not significantly different ($P<0.05$).
Table 5.6. LVIDd and LVISd of poults fed diets with and without furazolidone and supplemented with selenium (Se) and vitamin E (VE) at 2 weeks-of-age

<table>
<thead>
<tr>
<th>Dietary supplements (Se mg/kg)</th>
<th>(VE IU/kg)</th>
<th>LVIDd (cm)</th>
<th>LVISd(cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CTL†</td>
<td>TRT</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
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<td></td>
<td>(n = 19)</td>
<td>(n = 7)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>50</td>
<td>0.54±0.05b2</td>
<td>0.85±0.09a3</td>
</tr>
<tr>
<td></td>
<td>(n = 19)</td>
<td>(n = 10)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0.63±0.05b2</td>
<td>0.76±0.07a4</td>
</tr>
<tr>
<td></td>
<td>(n = 20)</td>
<td>(n = 11)</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>0</td>
<td>0.59±0.04a1</td>
<td>0.64±0.07a5</td>
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<tr>
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<td>(n = 19)</td>
<td>(n = 12)</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0.58±0.05a1</td>
<td>0.58±0.08a5</td>
</tr>
<tr>
<td></td>
<td>(n = 23)</td>
<td>(n = 14)</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>50</td>
<td>0.55±0.05b1</td>
<td>1.04±0.07a1</td>
</tr>
<tr>
<td></td>
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<td>(n = 8)</td>
<td></td>
</tr>
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<td>0.88±0.07a3</td>
</tr>
<tr>
<td></td>
<td>(n = 23)</td>
<td>(n = 14)</td>
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</tr>
<tr>
<td>0.5</td>
<td>50</td>
<td>0.55±0.05b1</td>
<td>0.94±0.09a2</td>
</tr>
<tr>
<td></td>
<td>(n = 22)</td>
<td>(n = 13)</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>100</td>
<td>0.55±0.05b1</td>
<td>0.65±0.09a5</td>
</tr>
<tr>
<td></td>
<td>(n = 20)</td>
<td>(n = 11)</td>
<td></td>
</tr>
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Interaction effects

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</tbody>
</table>

§LVIDd and LVISd are left ventricular internal diastolic and systolic measurements as determined by echocardiography.
†Where CTL and TRT represent poults fed normal and furazolidone-containing diets, respectively.
a,bMeasurements in the same row with similar alphabetic superscript are not significantly different (P<0.05).
1,2Values in the same column with similar numeric superscript are not significantly different (P<0.05).
Table 5.7. LVIDd and LVISd of poults fed diets with and without furazolidone and supplemented with selenium (Se) and vitamin E (VE) at 4 weeks-of-age

<table>
<thead>
<tr>
<th>Dietary supplements (Se mg/kg)</th>
<th>LVIDd (cm)</th>
<th>LVISd (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL †</td>
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</tr>
<tr>
<td></td>
<td>‡</td>
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</tr>
<tr>
<td>0</td>
<td>0.86±0.11b</td>
<td>1.07±0.16b</td>
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<td>(n = 19)</td>
<td>(n = 7)</td>
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<td>0</td>
<td>0.83±0.09b</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(n = 17)</td>
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</tr>
<tr>
<td>0</td>
<td>0.97±0.13b</td>
<td>1.37±0.15b</td>
</tr>
<tr>
<td></td>
<td>(n = 9)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>0.3</td>
<td>0.86±0.09b</td>
<td>1.17±0.14b</td>
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<tr>
<td></td>
<td>(n = 17)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.90±0.10a</td>
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</tr>
<tr>
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<td>(n = 16)</td>
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</tr>
<tr>
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<td>0.91±0.12a</td>
<td>1.59±0.23b</td>
</tr>
<tr>
<td></td>
<td>(n = 12)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>0.3</td>
<td>0.85±0.10a</td>
<td>1.33±0.20b</td>
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<td>(n = 17)</td>
<td>(n = 5)</td>
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<tr>
<td>0.5</td>
<td>0.95±0.09a</td>
<td>1.66±0.18b</td>
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<td>0.95±0.10a</td>
<td>1.44±0.20b</td>
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<td>(n = 15)</td>
<td>(n = 6)</td>
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Interaction effects: Probabilities (P-values)

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<td>NS</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

§LVIDd and LVISd are left ventricular internal diastolic and systolic measurements as determined by echocardiography.
†Where CTL and TRT represent poults fed normal and furazolidone-containing diets, respectively.
abMeasurements in the same row with similar alphabetic superscript are not significantly different (P<0.05).
1,2Values in the same column with similar numeric superscript are not significantly different (P<0.05).
### Table 5.8. Percent (%) shortening of 2 and 4 weeks-old poults

<table>
<thead>
<tr>
<th>Dietary supplements (Se mg/kg) (VE IU/kg)</th>
<th>Week 2</th>
<th></th>
<th>Week 4</th>
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<td>CTL</td>
<td>TRT</td>
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<td>14</td>
<td>41</td>
<td>9</td>
</tr>
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<td>-10</td>
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<td>40</td>
<td>12</td>
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<td>5</td>
<td>53</td>
<td>42</td>
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<td>0.3</td>
<td>-7</td>
<td>-96</td>
<td>39</td>
<td>11</td>
</tr>
<tr>
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<td>-7</td>
<td>-2</td>
<td>33</td>
<td>8</td>
</tr>
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<td>-7</td>
<td>17</td>
<td>43</td>
<td>10</td>
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<tr>
<td>0.5</td>
<td>-14</td>
<td>26</td>
<td>38</td>
<td>9</td>
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</tbody>
</table>

†Where CTL and TRT represent poults fed normal and furazolidone-containing diets supplemented with different combinations of vitamin E (VE) and selenium (Se), respectively.
Table 5.9. Pearson correlation coefficients of LVIDd, LVISd, weight, GPx, PUA, GSH and MDA of 2 week-old-poults fed diets supplemented with selenium (Se) and vitamin E (VE)

<table>
<thead>
<tr>
<th></th>
<th>LVIDd</th>
<th>LVISd</th>
<th>Weight</th>
<th>GPx</th>
<th>PUA</th>
<th>GSH</th>
<th>MDA</th>
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<tr>
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<td>-0.2446</td>
<td>-0.0432</td>
<td>-0.0541</td>
<td>0.3372</td>
<td>0.1167</td>
</tr>
<tr>
<td></td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.4955</td>
<td>0.3928</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVISd</td>
<td>0.8556</td>
<td>1.0000</td>
<td>-0.0857</td>
<td>-0.0440</td>
<td>-0.0401</td>
<td>0.1436</td>
<td>0.0966</td>
</tr>
<tr>
<td></td>
<td>&lt;.0001</td>
<td></td>
<td>0.1766</td>
<td>0.4882</td>
<td>0.5270</td>
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<td>0.1275</td>
</tr>
<tr>
<td>Weight</td>
<td>-0.2446</td>
<td>-0.0857</td>
<td>1.0000</td>
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</tr>
<tr>
<td>GPx</td>
<td>-0.0432</td>
<td>-0.0440</td>
<td>-0.0940</td>
<td>1.0000</td>
<td>-0.0146</td>
<td>0.1292</td>
<td>-0.0245</td>
</tr>
<tr>
<td></td>
<td>0.4955</td>
<td>0.4882</td>
<td>0.1374</td>
<td></td>
<td>0.8179</td>
<td>0.0407</td>
<td>0.6991</td>
</tr>
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<td>PUA</td>
<td>-0.0541</td>
<td>-0.0401</td>
<td>0.0511</td>
<td>-0.0146</td>
<td>1.0000</td>
<td>0.0399</td>
<td>0.1350</td>
</tr>
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<td>0.3928</td>
<td>0.5270</td>
<td>0.4198</td>
<td>0.8179</td>
<td></td>
<td>0.5292</td>
<td>0.0325</td>
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<tr>
<td>GSH</td>
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<td>0.1436</td>
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<td>0.1292</td>
<td>0.0399</td>
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<td>0.2657</td>
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<td>0.0231</td>
<td>&lt;.0001</td>
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<tr>
<td>MDA</td>
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<td>0.0966</td>
<td>-0.0339</td>
<td>-0.0245</td>
<td>0.1350</td>
<td>0.2657</td>
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<td>0.5927</td>
<td>0.6991</td>
<td>0.0325</td>
<td>&lt;.0001</td>
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</tr>
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</table>

\(^8\)LVIDd, LVISd, MDA, PUA, GPx and GSH represents left ventricular internal-diastolic dimension, left ventricular internal-systolic dimension malondialdehyde, plasma uric acid, glutathione peroxidase and glutathione, respectively.
Table 5.10. Pearson correlation coefficients of LVIDd, LVISd, weight, GPx, PUA, GSH and MDA of 4 week-old-poults fed diets supplemented with selenium (Se) and vitamin E (VE)

<table>
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<tr>
<th></th>
<th>LVIDd</th>
<th>LVISd</th>
<th>Weight</th>
<th>GPx</th>
<th>PUA</th>
<th>GSH</th>
<th>MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVIDd</td>
<td>1.00000</td>
<td>0.93847</td>
<td>-0.49408</td>
<td>-0.25017</td>
<td>-0.00330</td>
<td>-0.10293</td>
<td>0.04500</td>
</tr>
<tr>
<td>LVISd</td>
<td>0.93847</td>
<td>1.00000</td>
<td>-0.57498</td>
<td>-0.26484</td>
<td>-0.01384</td>
<td>-0.16048</td>
<td>0.08638</td>
</tr>
<tr>
<td>Weight</td>
<td>-0.49408</td>
<td>-0.57498</td>
<td>1.00000</td>
<td>0.31369</td>
<td>-0.02616</td>
<td>0.24640</td>
<td>-0.06174</td>
</tr>
<tr>
<td>GPx</td>
<td>-0.25017</td>
<td>-0.26484</td>
<td>0.31369</td>
<td>1.00000</td>
<td>-0.09029</td>
<td>0.22314</td>
<td>-0.00930</td>
</tr>
<tr>
<td>PUA</td>
<td>-0.00330</td>
<td>-0.01384</td>
<td>-0.02616</td>
<td>-0.09029</td>
<td>1.00000</td>
<td>-0.06788</td>
<td>0.10237</td>
</tr>
<tr>
<td>GSH</td>
<td>-0.10293</td>
<td>-0.16048</td>
<td>0.24640</td>
<td>0.22314</td>
<td>-0.06788</td>
<td>1.00000</td>
<td>-0.14759</td>
</tr>
<tr>
<td>MDA</td>
<td>0.04500</td>
<td>0.08638</td>
<td>-0.06174</td>
<td>-0.00930</td>
<td>0.10237</td>
<td>-0.14759</td>
<td>1.00000</td>
</tr>
</tbody>
</table>

LVIDd, LVISd, MDA, PUA, GPx and GSH represents left ventricular internal-diastolic dimension, left ventricular internal-systolic dimension malondialdehyde, plasma uric acid, glutathione peroxidase and glutathione, respectively.

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Table 5.11. Necropsy measurements of 2 and 4 weeks-old poults

<table>
<thead>
<tr>
<th>Dietary supplements (Se mg/kg) (VE IU/kg)</th>
<th>WHO(^{\dagger}) (g) Week 2</th>
<th>APEX-T(mm) Week 2</th>
<th>WHO(^{\dagger}) (g) Week 4</th>
<th>APEX-T(mm) Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL</td>
<td>TRT</td>
<td>CTL</td>
<td>TRT</td>
</tr>
<tr>
<td>0</td>
<td>1.73±1.99(^{b1})</td>
<td>2.20±0.32(^{a1})</td>
<td>17.02±1.19(^{b1})</td>
<td>20.01±1.99(^{a1})</td>
</tr>
<tr>
<td>0</td>
<td>2.26±0.32(^{a1})</td>
<td>1.93±0.32(^{b1})</td>
<td>18.58±1.98(^{a1})</td>
<td>15.58±1.98(^{b1})</td>
</tr>
<tr>
<td>0</td>
<td>2.13±0.32(^{a1})</td>
<td>2.10±0.32(^{b1})</td>
<td>19.54±1.98(^{a1})</td>
<td>18.36±1.98(^{a1})</td>
</tr>
<tr>
<td>0.3</td>
<td>2.10±0.32(^{a1})</td>
<td>1.70±0.32(^{b1})</td>
<td>19.87±1.98(^{a1})</td>
<td>12.75±1.98(^{b1})</td>
</tr>
<tr>
<td>0.5</td>
<td>2.03±0.32(^{a1})</td>
<td>1.36±0.32(^{b1})</td>
<td>18.90±1.98(^{a1})</td>
<td>14.67±1.98(^{b1})</td>
</tr>
<tr>
<td>0.3</td>
<td>2.46±0.32(^{a1})</td>
<td>1.93±0.32(^{b1})</td>
<td>18.05±1.98(^{a1})</td>
<td>13.70±1.98(^{b1})</td>
</tr>
<tr>
<td>0.3</td>
<td>2.16±0.32(^{a1})</td>
<td>1.43±0.32(^{b1})</td>
<td>19.03±1.98(^{a1})</td>
<td>15.21±1.98(^{b1})</td>
</tr>
<tr>
<td>0.5</td>
<td>2.06±0.32(^{a1})</td>
<td>1.68±0.23(^{b1})</td>
<td>16.69±1.98(^{a1})</td>
<td>18.03±1.40(^{a1})</td>
</tr>
<tr>
<td>0.5</td>
<td>1.93±0.32(^{a1})</td>
<td>1.93±0.32(^{b1})</td>
<td>17.02±1.98(^{a1})</td>
<td>19.2±1.98(^{b1})</td>
</tr>
</tbody>
</table>

§ WHO and APEX-T represents weight of whole heart, measure from apex to thorax of the heart respectively.
\(^{\dagger}\) Where CTL and TRT represent poults fed normal and furazolidone-containing diets supplemented with different combinations of vitamin E (VE) and selenium (Se), respectively.

\(^{a,b}\) Measurements in the same row with similar alphabetic superscript are not significantly different (\(P<0.05\)).
\(^{1,2}\) Values in the same column with similar numeric superscript are not significantly different (\(P<0.05\)).
CHAPTER 6

Summary of Dissertation

This dissertation research investigated the hypothesis that toxin-induced dilated cardiomyopathy is affected by both genetic background and oxidant/antioxidant status of the turkey. It extended earlier research in which turkey varieties were shown to be differentially affected by toxic levels of Fz. The rationale for the dissertation project was that feeding antioxidants as supplements may modulate the toxicity. The dissertation also addressed the role of genetics on the toxic effect of a once-commonly used drug furazolidone and the potential ameliorative effects on furazolidone toxicity of vitamin E and selenium.

Specific Findings:

1. Comparison of reciprocal crosses fed control and Fz-containing diets showed 25 to 50% reduced left ventricular dilatation for Bourbon Red x Narragansett crossbred poult fed diets containing Fz. The percent heterosis for LVIDd and LVISd was most significant for Bourbon Red x Narragansett cross. Thus, Reciprocal crosses respond differently to Fz-induced DCM and heterosis may significantly influence a turkey’s response to toxic levels of Fz.

2. Analysis of OS levels between poult fed control and Fz-containing diets using various biomarkers showed no differences in oxidative stress levels. Thus, DCM caused by Fz maybe independent of oxidative stress.

3. Assessment of the influence of feeding different combinations of dietary selenium and vitamin E on control and poult fed Fz-containing diets showed that none of the specific
combinations of vitamin E and selenium evaluated had a mitigatory or enhancing effect on dilated cardiomyopathy.

**Future Work:**

This study investigated the effect of OS and feeding different concentrations and combinations of supplemental selenium and vitamin E on the incidence and severity of Fz-induced dilated cardiomyopathy in commercial turkeys. Future studies should focus on the following:

1. Do heritage turkey varieties differ in their response when fed diets containing different concentrations of furazolidone?
2. Do oxidative stress levels vary in heritage turkey varieties previously reported to be more and less susceptible to Fz-induced dilated cardiomyopathy?
3. Does dietary supplementation with antioxidants in Fz-induced dilated cardiomyopathy have a variable effect based on genetic background?
4. How can antioxidants be utilized in the context of genetics to reduce the incidence of dilated cardiomyopathy in commercial turkeys?
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