An Assessment of the Relationship among Oxidative Stress, Adaptive Immunity and Genetic Variations in the Chicken, *Gallus gallus*

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ABSTRACT

Oxidative stress (OS) has been associated with aging and age-related diseases in humans, as well as with the decline in economic trait performance in poultry and other domesticated animals. However, the potential effects of OS on the poultry immune system are not well understood. In addition, the impact of bird genetic variation on redox balance remains to be elucidated. Thus, the central hypothesis of this dissertation is: The bird's adaptive immunocompetence is impacted by their OS level, which is not only influenced by environmental factors, but also related to genetic phenotype of either mitochondrial DNA (mtDNA) or nuclear DNA (nDNA). In the first phase of this study, White Leghorn chickens were provided ethanol at different concentrations in drinking water to induce OS. Biomarkers including malondialdehyde (MDA), glutathione (GSH), and plasma uric acid (PUA) were measured to assess OS before and after ethanol treatment. The adaptive immune response during an OS event was measured by plasma IgG and IgM levels, major lymphoid organ weights, CD4+/CD8+ cell ratio, and histopathological analysis of the immune organs. Results showed that when OS was induced by 10% ethanol, chicken adaptive immune responses decreased; however, when birds were exposed to 2% ethanol, there was an enhancement in antioxidant defense and immune response; These results would suggest a negative correlation between OS level and chicken adaptive immune response. In the second phase of the study, subsets of chickens were selected based on their high (H)- or low (L)-OS to assess for variations in their genetic phenotypes. Using MDA levels, 36 chickens were chosen to scan a

2734-bp region of mtDNA, but no definitive SNP was detected. In another experiment, 40 chickens were conversely selected according to three biomarkers for OS. Although no variation was found at eight SNP loci tested across the mitochondrial genome, mtDNA damage measured by 8-hydroxy-2'-deoxy-guanosine was shown to increase with time, and at higher levels in the high OS birds ($p < 0.05$). Thses results suggest that long-term high OS levels in chickens may increase the somatic mutation of their mtDNA. In the final phase of this dissertation, the effect of nDNA on OS, measured via a genome-wide association study was performed with 18 H and 18 L chickens using the latest chicken 60k SNP microarray for genotyping. Among 56,483 SNPs successfully genotyped, 13 SNPs across five independent loci were associated with OS at significance level of $p \leq 0.001$, and another 144 SNPs were also associated with OS ($p \leq 0.01$). These results indicate new loci and related genes for their genetic influence upon redox balance. In general, experiments carried out on White Leghorn chickens here have shown that adaptive immune response is tightly related to changes of OS. Further, genetic variance in nDNA is associated with the risk of high OS or the ability to better resist it.

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Chapter I

Introduction

The complete reduction of O_2 requires four electrons and forms two molecules of H_2O. In cells of aerobic organisms, partially reduced intermediates such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) are inevitably produced during normal metabolism processes. This group of reactive chemicals is referred as free radicals, or reactive oxygen species (ROS, Finkel and Holbrook, 2000). Although they perform essential functions within certain boundaries, ROS can cause serious oxidative damage to cell components. Therefore, an antioxidant defense system continuously scavenges excessive ROS to maintain a redox balance in the cell. However, the balance could be disrupted by external or internal factors and lead to oxidative stress (OS): the status favors ROS accumulation and oxidative damage (Davies, 2000). Because of the non-specific oxidative damage to cell components like proteins, DNA, and lipids, and their roles in triggering signaling pathways of apoptosis, OS is widely associated with aging and age-related diseases, like cancer, cardiovascular and neurodegenerative abnormalities in humans (Valko et al., 2007).

In chickens, OS is often observed under various stressful conditions, like acute heat (Mujahid et al., 2007), poor hygienic conditions (Star et al., 2008), toxic element exposure (Erdogan et al., 2005), and stress hormone administration, which mimics the stress response under conditions of transport, electric shocks, and fasting (Eid at al., 2003). At the same time, declines of many important economic traits were also observed along with OS in chickens. For example, decreased weight gain and feed efficiency were associated with induced OS (Iqbal et al., 2004). Egg production (Eid et al., 2008) and semen quality (Eid et al., 2006) were also reported to be related with increased OS. In addition, association of OS and heart failure of

fast-growing commercial broilers (Nain et al., 2008) and rancidity of raw chicken meat (Gao et al., 2010) were also proposed. Despite the decline of various traits reported, adaptive immunity, which is also an important trait in chickens, has not attracted enough attention relative to oxidative damage.

Immune cells are likely to be affected by OS. A high percentage of polyunsaturated fatty acids in plasma membranes of immune cells suggested a susceptibility of oxidative lipid damage (Meydani et al., 1995). Moreover, immune cells are frequently exposed to large amount of ROS generated by the immune system itself. In the process of phagocytosis, phagocytes produce lots of hypochloride (OCl-), $O_2^{\cdot -}$ and $\cdot OH$ to kill bacteria (Halliwell, 2006). This non-specific killing serves as the first line of defense against infectious microorganisms. However, overproduction of ROS causes oxidative damage to phagocytes and other immune cells. For example, deRojas-Walker et al. (1995) identified oxidation and deamination products in the DNA of activated macrophages exposed to E. coli lipopolysaccharide. Programmed cell death of T cells, B cells (Buttke and Sandstrom, 1995) and neutrophils (Scheel-Toellner at al., 2004) were also reported upon exposure to stimuli which elicit ROS production and inflammation. However, opposite observations that ROS appear to activate immune cell function were also reported in some studies. For example, though H_2O_2 exposure reduced Th1 cell activity, it enhanced Th2 cytokine secretion on the other hand (Frossi et al., 2008). OS at inflammation sites was also proposed to recruit monocytes and stimulate T cell differentiation (Elahi and Matata, 2008). Remaining inconclusive, the role of OS in the immune system surely deserves more study.

In spite of the influence on cell function and wide impacts on physiological disorders and diseases, genetic causes of OS remain unclear. Related studies indicated OS as a complex

quantitative trait affected greatly by environment and multiple genes (Finkel and Holbrook, 2000). On one hand, genes influencing ROS production could be candidate genes for risk of high OS. For instance, mouse mitochondrial DNA variant 12027T>C, which led to amino acid changes in complex I of mitochondrial respiratory chain, increased $O_2^{\cdot -}$ production of mitochondria, the main source of intracellular ROS (Marchbanks et al., 2003). Manipulation of nuclear genes like *clk-1* (Liu et al., 2005), and *isp-1* (Crofts, 2004) has also been implicated in increased or decreased mitochondrial ROS production. On the other hand, genes controlling antioxidant defense might be valuable in genetic study of OS resistance. *Sod1,* for example, encodes the enzyme CuZn-superoxide dismutase, an important $O_2^{\cdot -}$ scavenger both inside and outside of mitochondrion. A recent study reported that *Sod1*$^{-/-}$ mice had tissue-wide lipid peroxidation and increased oxidative damage to DNA and protein (Muller et al., 2006). Similar results could also be found in knockout mice at some other genes (Muller et al., 2007). It appears that genome-wide studies, which are more suitable for complex traits, are needed to study the network of genes related to OS. Although microarrays have been used in many studies of gene expression in response to OS (Scandalios, 2002), genome-wide studies of DNA variation for risk of high OS or better resistance of OS have not been reported yet.

Considering the potential role of OS in immunocompetence and its great influence on the chicken production industry, the relationship between OS and adaptive immune function of chickens was studied here. In addition, to contribute to our understanding of genetic determinants of risk of OS or the ability to better cope with it, another objective of this study was to evaluate the effect of genetic variation on redox balance in chickens.

The specific aims of this study include:

1. Determine the suitable time and dose for using ethanol as the artificial inducer of OS in chickens.
2. Quantify changes of both OS and adaptive immune response of chickens under ethanol consumption, and identify possible association between these changes.
3. Find possible variation in mtDNA of chicken selected for high (H) or low (L) OS, and analyze the possible association between mtDNA variation and OS in chicken.
4. Test genome-wide SNPs of chickens selected for H or L OS, and identify possible association between nDNA SNPs and OS in chickens.
5. Analyze genes related to mtDNA or nDNA variations which may associate with the risk of high OS or better resistance of it in chickens.

Chapter II

Review of Literature

Reactive Oxygen Species and Oxidative Stress

As early as the mid-1950s, free radicals-induced damage was proposed to be critical in the determination of life span by Denham Harman, and referred to as "Free Radical Theory of Aging" (Wickens, 2001). This theory proposes that the aging of organisms is based on the accumulation of free radical damage in cells over time. "Free radical" was first referred to as a highly reactive chemical having an unpaired electron, like superoxide anion ($O_2^{\cdot -}$) and hydroxyl radical ($^{\cdot}OH$); later, the group was expanded to include chemicals with paired electrons, but equally reactive and easily converted into radicals, like hydrogen peroxide (H_2O_2), and was given the name as reactive oxygen species (ROS, Finkel and Holbrook, 2000). Nowadays, excessive ROS accumulation, which causes OS, is believed to be responsible for much cellular damage and physiological abnormalities, and has been implicated in age-related diseases, such as Alzheimer's disease, Parkinson's disease, and cancer (Valko et al., 2007).

ROS generation

In the cells of aerobic species, a variety of toxic chemical species, including $O_2^{\cdot -}$, H_2O_2, and $^{\cdot}OH$, are inevitably produced during normal metabolic processes. The main source of ROS *in vivo* is aerobic respiration in mitochondria. Other sources-like peroxisomal β-oxidation of fatty acids, cytochrome P450 metabolism of xenobiotic compounds, and stimulation of phagocytosis- also produce ROS.

ROS produced in mitochondria

The mitochondrion is the major site of intracellular ROS production (Figure 2.1). As estimated, around 1-2% of the oxygen consumed by aerobic organisms during physiological respiration in mitochondria is converted to $O_2^{\cdot -}$ (Finkel and Holbrook, 2000). This ROS formation is due to the electron-leakage of the respiratory chain or electron transport chain. Under normal condition, electrons originating from substrates like NADH and succinate are first passed from complex I and II to ubiquinone, also known as coenzyme Q. Then, electrons are transferred to complex III, and then donated to cytochrome c, and to the last transporter - complex IV, where the electrons are finally passed to oxygen and lead to water generation ($O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$). However, there are always some electrons leaking from previous complexes before arriving at complex IV, and reactions of these leaked electrons with oxygen result in the generation of $O_2^{\cdot -}$, the primary precursor of most ROS. $O_2^{\cdot -}$ is unstable, and is quickly reduced to H_2O_2, which is relatively stable and diffuses out of mitochondria. Further, in the presence of reduced transition metals, H_2O_2 can be further reduced to highly toxic $\cdot OH$. There is evidence showing that complex I is the major site of electron leakage and $O_2^{\cdot -}$ generation, while complex III is also regarded as an important site for $O_2^{\cdot -}$ generation (Orrenius et al., 2007). Some believe that these electron escapes are related to direct semiquinone oxidation by oxygen. Others believe the Fe-S centers of these complexes are responsible for the leakage (Kowaltowski et al., 2009).

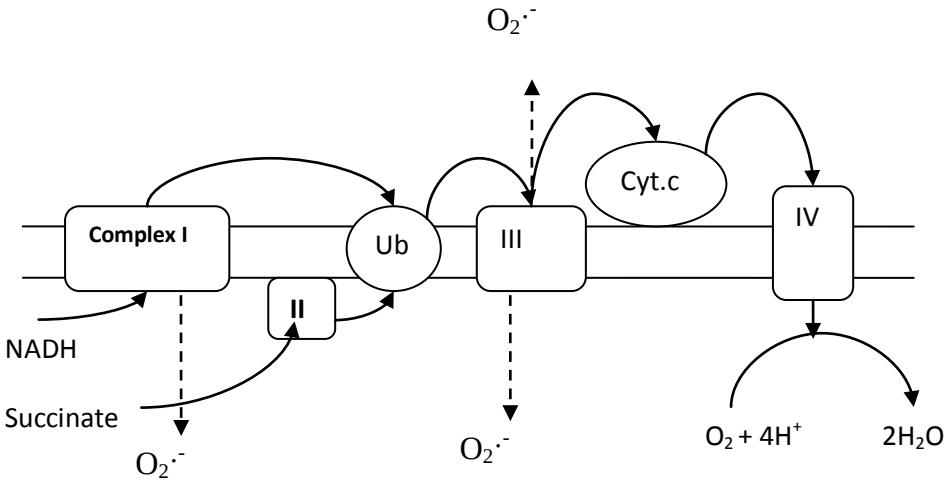

Figure 2.1. Superoxide anion generation from electron transport chain of mitochondria (figure is derived from Orrenius et al., 2007).

In addition to the respiratory chain, ROS are also produced by other components within mitochondria, like monoamine oxidase (MAO) and $p66^{shc}$ (Di Lisa et al., 2009). MAO is a flavoprotein located in the outer membrane of mitochondria. MAO catalyzes oxidative deamination of several amines and leads to a considerable production of H_2O_2. Another mitochondrial source of ROS is related to protein $p66^{shc}$, a 66-KDa isoform of adaptor protein located in both mitochondria and cytoplasm. Because of a redox active sequence in their amino terminal portion, $p66^{shc}$ localized in mitochondria can reduce oxygen to H_2O_2, along with oxidization of cytochrome c.

ROS produced in cytoplasm

Cytosolic enzyme systems also contribute to ROS production. For example, peroxisome generates H_2O_2 as a by-product of fatty acid β-oxidation (Hernandez-Garcia et al., 2010). Microsomal cytochrome P-450 could also directly reduce O_2 to $O_2\cdot^-$, or alternatively form a redox cycling with substrates and oxygen, which detoxify acute doses of toxins and

produce ROS (Beckman and Ames, 1998). In addition, nicotine adenine dinucleotide phosphate (NADPH) oxidases, which were first found in activated neutrophils in 1973, produce superoxide anion ($NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\cdot -}$, Halliwell, 2006). This superoxide production was later proven to kill several microorganisms during phagocytosis, and is essential for phagocytes, like neutrophils and macrophages. Other NADPH oxidase family members in nonphagocytes and the xanthine oxidoreductase system also contribute to cytoplasmic ROS production (Valco et al., 2007).

Antioxidant Defense

To detoxify constantly generated ROS, animals evolved a sophisticated antioxidant defense system to counteract or remove ROS rapidly. There are three levels of defense strategies: prevention, interception, and repair (Sies, 1993). The diversity of this system matches that of oxidants, and the defense mechanism composed of both enzymatic and non-enzymatic branches.

Enzymatic antioxidants

The three major classes of enzymatic antioxidants include superoxide dismutase (SOD) glutathione peroxidase (GPx), and catalase (CAT, Davies, 2000). There are several types of SOD: MnSOD enzyme contains an active manganese site and exists in the mitochondria, while CuZnSOD contains copper and zinc and is present largely in the cytosol. When superoxide, the first intermediate of O_2 reduction is generated, SOD catalyzes the dismutation reaction ($2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$) by about four orders of magnitude at pH 7.4 to accelerate $O_2^{\cdot -}$ removal. Then, the formed H_2O_2 is reduced by enzymes CAT or GPx to water ($2H_2O_2 \rightarrow 2H_2O + O_2$). Except for these three enzymes, there are two less studied, but probably just as important enzymatic antioxidants: the peroxiredoxins and the recently discovered sulfiredoxin. Other

enzymes which have antioxidant properties include paraoxonase, glutathione-S transferases, and aldehyde dehydrogenases (Muller et al., 2007).

Non-enzymatic antioxidants

Non-enzymatic antioxidants could either be water-soluble, like Vitamin C, glutathione (GSH), uric acid, or lipid-soluble, like carotenes, vitamin E, coenzyme Q and others. Generally speaking, water-soluble antioxidants react with oxidants in the cell cytoplasm and blood. For example, glutathione is long known as a major soluble antioxidant in cells because it is highly abundant in cytoplasm, nuclei, and mitochondria (Valco et al., 2007). The conversion between the reduced form (GSH) and the oxidized from (GSSG) removes ROS ($2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$). On the other hand, lipid-soluble antioxidants scavenge ROS in membranes and protect cell membranes from lipid peroxidation. For example, vitamin E, or known as α-tocopherol, reacts with peroxyl radical ($LOO^{\cdot}$) and prevents it from attacking fatty acid side chains in membranes ($\alpha TH + LOO^{\cdot} \rightarrow \alpha T^{\cdot} + LOOH$). In turn, the resulting α-tocopherol radical ($\alpha T^{\cdot}$) is converted back by vitamin C, or ascorbate (Halliwell, 1994). In chickens, vitamin E and selenium are usually used as an antioxidant supplementation to enhance resistance to OS and reduce oxidative damage (Avanzo et al., 2001).

Another interesting antioxidant is uric acid, which is a main nitrogen waste product in most animals, but works as antioxidant in humans and birds (Lin et al., 2008). Lacking urate oxidase, human beings and avian species have a high level of uric acid in the body compared with other animals, but it is much more than a waste. The presence of allantoin, the product of direct oxidation of uric acid by free radicals proves well its antioxidant property (Simoyi et al, 2003). Therefore, a better antioxidant defense system making use of the large amount of uric

acid was proposed as an explanation for the long life span of humans and birds (Costantini, 2008).

Oxidative Stress

Under normal physiological conditions, ROS are produced at a low level, while antioxidants are adequate to cope with them and the oxidative damage they cause is quickly repaired. However, this balance is constantly challenged by intrinsic and extrinsic adverse forces or stressors which increase ROS production or decrease defense ability. The disturbance of redox balance in favor of ROS production leads to a dangerous condition known as oxidative stress (Finkel and Holbrook, 2000). Multiple external stimuli that increase ROS generation or decrease antioxidant levels will be discussed here, while genetic factors will be discussed later.

Increased ROS generation induce oxidative stress

Mitochondria, which are the main source of intracellular ROS production, are susceptible to many stimuli. For example, increased dietary caloric intake, which increases the respiratory rate, has recently been emphasized in increasing ROS production in mitochondria (Wallace, 2005). Similarly, high oxygen tension also contributes to more ROS generation by accelerating the electron flow in the respiratory chain. Mitochondria incubated at higher-than-normal oxygen concentrations generate more ROS than those at normal oxygen levels (Kowaltowski et al., 2009). Moreover, mitochondria incubated in low oxygen environment and then supplemented with oxygen also generate large amounts of ROS after reoxygenation. In addition, chemicals like rotenone, which inhibits electron transfer from complex I to coenzyme Q, can increase ROS generation by stopping normal electron flow (Orrenius, 2007). Another

situation resulting in more ROS production is high concentration of Ca^{2+}. After binding to the mitochondrial membrane, Ca^{2+}-stimulated ROS generation seems to occur mainly at coenzyme Q (Kowaltowski et al., 2009). Moreover, protein $p66^{shc}$, which is mainly located in the cytoplasm, can move into mitochondria under stimulation. This translation will result in increased ROS generation since $p66^{shc}$ can form $O_2^{\cdot -}$ by shuttling electrons from cytochrome c to molecular oxygen (Giorgio et al., 2005).

Cytosolic sources of ROS can also be greatly stimulated by different factors. Growth factors and cytokines such as tumor necrosis factors (TNF-α) and interferon (IFN-γ), can trigger ROS production in non-phagocytic cells. The mechanism proposed was that growth factors and cytokines enhance NADPH oxidase activity (Valco et al., 2007)

Shortage of antioxidants induces oxidative stress

Besides the stimulated ROS production, decreased antioxidant levels could also result in OS. Costantini (2008) discussed OS as a cost of reproduction in birds. Shortage of antioxidants is easily found in laying birds, since more nutrients and antioxidants are needed for egg production in addition to self-maintenance at this period. Similarly, Spears and Weiss (2008) emphasized that OS of dairy cows around parturition probably is due to the lack of antioxidants and trace elements because the plasma concentration of vitamin E dramatically decreases in the periparturient period without additional supplementation.

Oxidative Stress in Chicken

In chickens, OS has been reported to be caused by a multitude of stressors. Heat, cold, pathogenic microbes, toxic element exposure, and pre-slaughter management can all induce excessive ROS production or reduce antioxidant activity. These stressors disrupt homeostasis by the action of stress hormone or other mechanisms.

Stress Hormone-Induced Oxidative Stress

Both broiler and layer chickens are inevitably confronted by various stressors like catching, crating, transporting, fasting, and immune challenges. Being markedly stimulated under stress, the endocrine system participates in the organism's stress response and influences the oxidative status as well. Glucocorticoids, which are the final effectors of the hypothalamic-pituitary-adrenal (HPA) axis, were reported in mammals to induce lipid peroxidation, decreasing antioxidant capacity in multiple organs (Lin et al., 2004). Similarly, corticosterone, as the major adrenal glucocorticoid hormone in birds, was reported to elevate plasma, muscle and liver lipid peroxidation in broiler chicken by dietary corticosterone treatment (Eid et al., 2003; Lin et al., 2004). This induced lipid damage could be alleviated by adding an antioxidant supplement such as tea polyphenols. Additionally, synthetic glucocorticoid injection, like dexamethasone, could also increase lipid peroxidation, and reduce antioxidant activity of GPx in the semen of cockerels (Eid et al., 2006), in muscle and plasma of broiler chicken (Gao et al., 2010), and in liver, plasma and egg yolk of laying hens (Eid et al., 2008). As expected, antioxidant supplementation with vitamin E in these studies could reduce the elevated OS, and prevent the adverse effect of stress hormone on semen, meat quality of broiler chicken, as well as the performance of laying hens. However, the exact mechanism of stress hormone-induced OS has yet to be studied.

Heat-Induced Oxidative Stress

Heat is thought to be a major stressor of poultry because birds have feathers, relatively high metabolic rate, high deep-body temperature, and lack sweat glands (Panda et al., 2008). Heat stress causes great economic losses in terms of decreased growth performance and meat quality (Feng et al., 2008), high mortality (Bartlett and Smith, 2003), and immunosuppression (Tan et al., 2010). Studies of hyperthermic rats and mammalian cells indicated ROS formation and OS in various physiological and metabolic changes under heat exposure (Lin et al., 2006). Similarly, OS was widely observed as part of stress response upon heat exposure in chickens. In broiler chickens, for example, Lin et al. (2006) reported lipid peroxidation was induced in plasma and liver tissue of 5-week-old broiler chickens by acute heat exposure (32 ºC, 6 h). Feng et al. (2008) reported heat treatment increased mitochondrial H_2O_2 production and lipid peroxidation in muscle both in vitro and in vivo study. Yang et al. (2010) reported significant increase of ROS production by heat stress and a quick decline after removal of the heat. Tan et al. (2010) reported oxidative damage in both lipid and protein in liver of chicken under heat stress. In laying hens, ROS formation was significantly augmented by heat exposure (32 ºC, 21 d) in 60-wk-old laying hens (Lin et al., 2008). Lipid peroxidation measured by malondialdehyde (MDA) was significant higher in four 22-wk-old layer lines under 32 ºC exposure for 23 days, while plasma uric acid (PUA) was much lower than control hens (Star et al., 2008). In Japanese quails, MDA level was significantly increased in serum, liver, heart, and kidney under heat stress (34 ºC, 32 d), and supplementation with vitamin C and E could greatly alleviate this increase (Sahin et al., 2003). Although unconfirmed, Mujahid et al. (2006; 2007) suggested that chronic or acute heat induces OS possibly via downregulation of stress-related protein, because they found correlated change of avian uncoupling protein expression. As

another opinion, Tan et al. (2010) suggested that the depressed activity of the mitochondrial respiratory enzymes led to the impaired mitochondrial function and ROS formation under heat.

Toxic Substances-Induced Oxidative Stress

Toxic compounds accumulate in soil, atmosphere, feed and water as the result of industrial practices and pollution. These toxic substances can inhibit growth, damage a wide range of organs, and can disrupt the redox balance of chickens. For example, cadmium, as one of the most toxic substances to which chickens are exposed via contaminated drinking water or feed, was reported to decrease the blood SOD activity and increase MDA level in broiler chicken (Erdogan et al., 2005). However, the mechanism of how cadmium disturbs oxidative status is unknown yet. Another example is T-2 toxin, which is usually found in cereal grains contaminated by *Fusarium* fungi, the most prevalent toxin-producing fungi in northern temperate regions of America, Europe, and Asia. Frankic et al. (2006) and Rezar et al. (2007) reported that T-2 toxin in chicken feed increased DNA damage in spleen leukocytes. Similar studies in mice (Atroshi et al., 1997; Vila et al., 2002) reported that T-2 toxin stimulated lipid peroxidation, causing damage to cell membranes and DNA, and suggested that it mostly worked through oxidative pathways. The last but not the least, TCDD, or dioxin, which was used as herbicides, is a highly toxic substance for domestic chickens because it can induce oxidative damage at small doses. Hilscherova et al. (2003) reported significant lipid peroxidation and oxidative DNA damage in hatchling chicks injected with TCDD at the dose of 150 pg/g egg. Lim et al., 2007 reported that 2 pg/g TCDD had already suppressed liver antioxidant enzyme activity, including GPx, GRx, SOD, and CAT in hatchling chicken. It was suggested that TCDD induced OS because a key transcriptional factor, AhR, has a high affinity

for TCDD-like compounds, and could iniciate gene transcriptional changes that favor ROS production (Lim et al., 2007).

Immune Response-induced Oxidative Stress

The chicken's immune response whether induced by natural infection by microbes or challenge by vaccine, could also induce OS. Keles et al. (2010) reported that natural Marek's Disease infection increased DNA, lipid and protein damage in the peripheral plasma of White-Lohmann hens compared to control hens, while the total antioxidant activity and GSH level also decreased. Costantini and Dell'Omo (2006) also showed that immune challenge like phytohaemagglutinin skin test, which stimulates a T-cell-mediated immune response, stimulates the plasma reactive oxygen metabolite levels, and reduced the total plasma antioxidant as well. These observations are not surprising because various inflammatory cells, like macrophages and neutrophils, generate considerable ROS to kill invading microorganisms when activated. These ROS produced during the phagocytosis process primarily target invaders; however, excessive ROS can cause injury to host cells and organs. For example, deRojas-Walker et al. (1995) identified oxidation and deamination products in the DNA of activated macrophages exposed to *E. coli* lipopolysaccharide.

Oxidative Stress and Immunocompetence

Oxidative stress is widely believed to contribute to the development of diverse age-related diseases and to the aging process in humans. Similarly, whatever the inducer, it has been suggested to impact various economic traits of chickens, like feed efficiency, meat quality, and semen quality. However, the potential effect of OS on immunocompetence is not well understood. Experiments on immune cells seem to show incompatible effects of OS, while

increasing evidence in animals, like chickens, cows, rats and mice, suggested that elevated OS impairs immune function.

General Influence of Oxidative Stress

Oxidative damage on cellular components and biomarkers

Intracellular reactive oxygen species, generated either from mitochondria or elsewhere, can cause oxidative damage to cellular macromolecules, like nucleic acids, phospholipids, and proteins.

Lipid peroxidation is referred to as common oxidative degradation of lipids. For instance, polyunsaturated fatty acid residues of phospholipids are most susceptible to lipid peroxidation by cellular ROS, especially ·OH, by having double bonds (Orrenius et al., 2007). Evidence also shows that this lipid peroxidation can be catalyzed by Fe^{2+} overload. Malondialdehyde, a product of the oxidative degradation of polyunsaturated fatty acids, is usually assayed as a biomarker for lipid peroxidation. In humans, Bloomer (2007) assessed lipid peroxidation level in young cigarette smokers using both MDA and oxidized low-density lipoprotein (oxLDL) as indicators. Plasma MDA was also used as a biomarker for cadmium-induced oxidative damage in broiler chickens (Erdogan et al., 2005).

Similarly, DNA, especially mitochondrial DNA (mtDNA), could be easily damaged upon exposure to ROS. The damage includes the modification of purine, pyrimidine bases, and deoxyribose backbone, the breaks of single or double strands, and cross-links of nucleic acids to other molecules. Oxidative DNA damage accumulates with time and, if unrepaired, may lead to permanent cellular dysfunction. For instance, damaged mtDNA could impair energy metabolism in post-mitotic and/or high energy-demanding tissues, including the heart, skeletal muscle, and brain (Kujoth et al., 2007). Oxidative DNA damage can be measured based on the

level of 8-hydroxy-2'-deoxy-guanosine (8-OHdG), a widely used biomarker, by many approaches, such as immunohistochemical stain, high performance liquid chromatography (HPLC), and quantitative ELISA. For example, Wang et al. (2008) measured 8-OHdG levels in retinas of mice, and showed increased oxidative damage in aged rodent retinas compared to young mice, and that the oxidative DNA damage was primarily found in mtDNA. Another earlier study (Barja and Herrero, 2000) measured 8-OHdG in several species as an indicator of oxidative DNA damage using HPLC. They reported mtDNA damage in heart and brain was inversely correlated with maximum life span of mammalian species.

Oxidative protein damage involves the oxidation of sulfhydryl groups and amino acids close to metal-binding sites, reactions with aldehydes, protein-protein cross-linking, and protein fragmentation (Orrenius et al., 2007). Oxidative damage can dramatically alter the structure of a protein, induce unfolding of the protein, and result in severe dysfunction of enzymatic activity, signal transduction, and channel-forming properties, etc. The protein carbonyls, formed from direct oxidation of amino acids, are usually measured as a marker for protein oxidative damage (Sohal et al., 1993). Alternative parameters for oxidative protein damage also exist, like advanced oxidation protein products and protein thiol levels, as used by Cakatay et al. (2003), who studied damage in skeletal muscle of rats.

Influence of oxidative stress on signaling control

Reactive oxygen species are also important signaling molecules in the regulation of cellular processes like proliferation and apoptosis (Finkel and Holbrook, 2000). Although the events and the process of the activation of signaling pathway in response to ROS are not well understood, many transcriptional factors are activated by ROS in a dose-dependent manner. For example, low levels of intracellular ROS increase the expression of nuclear factor kappa B

(NF-κB), a key transcriptional factor for expression of many inflammatory mediators; under conditions of high OS, however, excess ROS inhibit NF-κB, leading to cell death (Halliwell, 2006). Similarly, exposure to hydrogen peroxide has been reported to block NF-κB activation, resulting in functionally suppressed memory T cells (Malmberg et al., 2001). In addition, ROS have been shown to trigger apoptosis of neutrophils, which were originally protected by some cytokines at inflammation sites (Scheel-Toellner et al., 2004).

Influence of Oxidative Stress in Chicken

Gao et al. (2010) recently reported that increased oxidative stability in skeletal muscle can reduce the rancidity of raw chicken meat and to maintain flavor, indicating the influence of OS on meat quality. Oxidative stress was also associated with low feed efficiency in a broiler line (Iqbal et al., 2004, 2005). In addition, Eid et al. (2006) reported induced OS in cockerels impacts semen quality measured as sperm count, and sperm activity. Moreover, OS has been associated with heart failure in fast-growing broilers. Nain et al. (2008) reported that heart lipid peroxidation was significantly higher in broilers with congestive heart failure, and fast-growing broilers were at high risk of heart failure, compared to leghorn chickens and slow-growing broilers, which are at low risk of heart failure.

Oxidative Stress and Immunosuppresion in Chicken

Oxidative stress was proposed to be responsible for many immunosuppression events in animals as well because of reported deleterious effects on the immune system under deficiency of selenium and vitamin E (Finch and Turner, 1996). A broad class of antioxidants-including vitamin E, vitamin C, carotenoids, flavanoids, zinc, and selenium-have therefore, been evaluated for their ability to reduce OS-induced immunosuppression (Chew and Park, 2004).

In chicken, ROS-induced damage of immune cells has only been implicated in heat-induced OS.

Many studies have shown that heat not only impairs production, but also depresses immune response. Zulkifli et al. (2000) observed decreased antibody titers for Newcastle disease virus (NDV) in female broiler chickens challenged by heat. Mashaly et al. (2004) reported that total white blood cell counts and antibody production against sheep red blood cells (SRBC) significantly decreased in commercial laying hens subjected to heat stress. Acute heat in both broilers and layers has also been reported to increase lipid peroxidation, a measure of OS (Lin et al., 2006, 2008). Due to the damage that OS causes to cells, OS has been proposed to be a significant cause of heat-induced immunosuppression. This hypothesis is supported by experiments in which antioxidant supplementation restored some immune function. For example, Puthpongsiriporn et al. (2001) showed that both vitamins E and C in the diet reduced lipid peroxidation in yolk and plasma and enhanced in vitro lymphocyte proliferation in hens under heat stress. Panda et al. (2008) also reported that vitamins E and C supplementation increased antibody titers against SRBC and NDV, and altered lymphocyte proliferation ratio in White Leghorn layers subjected to heat stress. Two Chinese herbs with similar effects to antioxidants have also been reported to protect cells from damage by lipid peroxidation and to improve immune response when given to hens under heat stress (Ma et al., 2005).

Influence of Oxidative Stress on Immune System of Dairy Cows

Another example of animal studies of OS in immune system concerns the periparturient period of dairy cows, three weeks before and after parturition. During this period, there is

considerable increase in energy demand for fetal growth and lactation. The higher energy demand raises ROS production, thereby exposing cows to high OS (Castillo et al., 2005). The elevated OS level during the periparturient period has been associated with increased susceptibility of dairy cows to a variety of infectious diseases including mastitis (Sordillo and Aitken, 2009). Because supplementation of antioxidants has been shown to ameliorate mastitis, OS have been further implicated in the incidence and severity of this disease (Spears and Weiss, 2008). Politis et al. (2004) observed that vitamin E supplementation during the periparturient period slowed the decrease of neutrophil function that may be caused by OS. Hogan et al. (1990, 1992) reported that selenium and/or vitamin E supplementation, whether by oral or parenteral administration, increased the intracellular killing of bacteria by blood neutrophils insolated from dairy cows in the periparturient period. In subsequent study from this group, Weiss et al. (1997) showed that proportions of clinical mastitis affected quarters in cows receiving low, intermediate, and high vitamin E diets were 25.0, 16.7 and 2.6%, respectively, during the first seven days of lactation. Similarly, β-carotene, another dietary antioxidant, increases blood lymphocyte proliferation as well as phagocytic activity and intracellular killing ability of blood neutrophils (Michal et al., 1994).

Influence of Oxidative Stress on Immune System of Model Animals

In addition to the observations from domestic animal studies, the effects of OS on immune responses have been investigated in rats and mice. Oxidative stress was associated with fluoride-induced immunotoxicity in rats, because a significant diminution of CAT and GPx, and an elevation of MDA in spleen, which indicate the oxidative damage, may explain a reduction of splenocyte counts, and lowered cellular and humoral immunity in rats of the treatment group (Das and Maiti, 2006). Oxidative stress was also indicated in lead-induced

suppression of serum levels of IgA, IgM, and IgG of rats, since the MDA level increased in peripheral blood mononuclear cells, and subsequent antioxidant supplementation with N-acetylcysteine (NAC) significantly reversed the decrease of serum immunoglobulin levels (Ercal et al., 2000). Similar studies also suggested OS increased methanol- (Parthasarathy et al., 2006) and endotoxin- (Victor and Fuente, 2003) related immunotoxicity. Besides chemical-induced OS, environmental factors like noise and hyperbaric exposure were studied in rats for their impact on redox balance and immune function. Srikumar et al. (2006) found that noise also induced OS in plasma and thymus of male rats, and increased OS might suppress the cell-mediated immune response, which was restored when animals were fed Triphala, an herb with antioxidant properties. Xu et al. (2007) reported that the exposure of rats to oxygen in a hyperbaric chamber increased plasma OS and decreased immune function, including decreased peripheral lymphocytes, decreased CD4+ T cell subsets in peripheral blood and spleen, decreased IL-2 excretion, and proliferation of splenic lymphocytes to mitogen ConA stimulation. Additionally, antioxidant-pretreated rats in the same hyperbaric chamber showed attenuated immunosuppression.

Influence of Oxidative Stress on Immune Cells

Immune cells are particularly sensitive to redox status, not only because of the higher percentage of polyunsaturated fatty acids in their plasma membranes, but also because of their frequent exposure to ROS generated by the immune system itself (Meydani et al., 1995). Phagocytes produce $O_2^{\cdot -}$, H_2O_2, $^{\cdot}OH$, and hypochlorous acid (HOCl) to kill bacteria in the process of phagocytosis, which serves as the first line of host defense against microbial infection (Halliwell, 2006). However, this non-specific killing mechanism can cause oxidative damage to phagocytes themselves. For example, deRojas-Walker et al. (1995) identified

oxidation and deamination products in the DNA of activated macrophages exposed to *E. coli* lipopolysaccharide. Chapman et al. (2002) reported that 94% of total HOCl produced during respiratory burst reacts with neutrophil components, while the rest reacts with bacteria. The accumulation of ROS at sites of inflammation may initiate neutrophil apoptosis through a ligand-independent death receptor signaling pathway (Scheel-Toellner at al., 2004).

Besides the phagocytes that generate them, ROS also attack other cells of the immune system. Buttke and Sandstrom (1994) summarized early studies observing programmed cell death of mature peripheral T cells and B cells upon exposure to stimuli that elicit ROS production, and this apoptosis was inhibited by agents with antioxidant activity. Malmberg et al. (2001) described an immunosuppression effect of H_2O_2 on human T cells; exposed to a micromolar level of H_2O_2, T cells produce much less major Th1 cytokines, including IFN-γ, IL-2, TNF-α, and Th2 cytokine measured as IL-10. This loss of both Th1 and Th2 cytokines was attributed to the action of H_2O_2 by blocking NF-κB expression, and indicated suppression in both cellular and humoral immune response. More influence of ROS on T cell signaling and activation were summarized recently (Larbi et al., 2007).

However, opposite observations in which ROS promote activities of immune cells were also reported. Frossi et al. (2008) showed that H_2O_2 exposure reduced Th1 cell activity, but enhanced Th2 cytokine secretion, and the effect lasted as long as a week. In addition, altered ROS production which leads to OS in sites of inflammation was proposed as a mechanism for recruitment of monocytes and stimulation of T cell differentiation (Elahi and Matata, 2008). Though not fully understood, the polarized effect of ROS on lymphocytes may depend on the severity of OS on the site. For instance, depending on the level, ROS was reported either to

activate phagocyte cytokine production at a low level, or to decrease phagocyte activity at a high level (Halliwell, 2006).

Ethanol-Induced Oxidative Stress

As early as the 1960s, oxidative damage in ethanol toxicity was proposed by Di Luzio (1963). Since then, more and more studies showed that both acute or chronic ethanol exposure induced oxidative damage in lipid, protein, or nucleic acids in liver (Colantoni et al., 2000), pituitary gland (Ren et al., 2005), plasma (Husain et al., 2005), parotid (Campos et al., 2005), and white blood cells (Fedeli et al., 2007) of rats. Such damage may be due to the increased ROS production or to decreased antioxidant defense, since increased O_2^-, H_2O_2, oxidized glutathione, and decreased activity of superoxide dismutase, glutathione reductase, catalase, or glutathione peroxidase were reported in different cells or tissues of hamster (Colton et al., 1998), rat (Schlorff et al., 1999; Husain et al., 2005; Maneesh et al., 2005), and human beings (Gotz et al., 2001). Among some of these studies, the effects of ethanol were also shown to be dose-dependent, i.e., that higher ethanol dosage induced higher OS (Schlorff et al., 1999; Colantoni et al., 2000; Husain et al., 2005). The latest studies showed that ethanol-induced OS are tightly associated with its metabolism.

Ethanol can be metabolized in many organs or tissues like liver, stomach, small intestine, and brain, but it is mainly metabolized in liver (Fernandez-Checa et al., 1997). In liver cells (Figure 2.2), the main pathway involves metabolism of ethanol in the cytosol with alcohol dehydrogenase (ADH) producing acetaldehyde, and then in mitochondria, acetaldehyde is further metabolized by aldehyde dehydrogenase (ALDH) to form acetate. In tissues like brain (Figure 2.2), where ADH activity is low, the enzyme cytochrome P450

(CYP2E1) locating on endoplasmic reticulum contributes to ethanol metabolism, and may play an important role at elevated ethanol consumption (Zakhari, 2006).

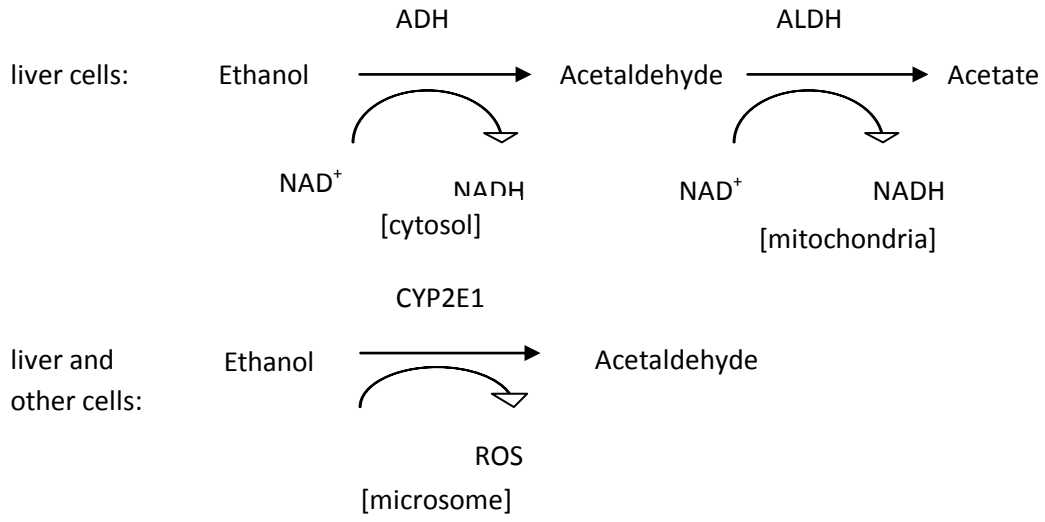

Figure 2.2 Ethanol metabolisms in cells

Through these pathways of ethanol metabolism, ROS are generated at several steps of the process. Firstly, the metabolism of ethanol in liver results in a significant increase of the NADH/NAD+ ratio in both cytoplasm and mitochondria since lots of NADH is generated in both steps. The increased NADH production and its shuttling to mitochondria enhances electron leakage from respiratory chain, which increases ROS production (Das and Vasudevan, 2007). Then, CYP2E1-dependent monoxygenase activity increases by 10-20-fold in ethanol-treated rodents, while elevation of CYP2E1 form of cytochrome P450 enzyme in the liver and other tissues after ethanol use has been demonstrated to generate $O_2^{\cdot -}$, H_2O_2 and in the presence of iron, produces $^{\cdot}OH$ (Albano, 2006). Additionally, ethanol-activated Kupffer cells, specialized macrophages in liver, also contribute to increased ROS production through NADPH oxidation (Zakhari 2006).

Genetic Influence on Oxidative Stress

MtDNA Variation and ROS Production

Mitochondria are the most important generators of intracellular ROS because molecular oxygen is prone to leakage from the electron transport chain (ETC), resulting in $O_2^{\cdot -}$ formation. Since mitochondrial DNA (mtDNA) encodes proteins needed to assemble four of the five complexes in the ETC, it is not surprising that mtDNA variants also control ROS production by influencing OXPHOS efficiency. Marchbanks et al. (2003) reported that a common mtDNA variant 12027T>C, which lead to a amino acid change in ND4 subunit of complex I of the mitochondrial respiratory chain, was significantly associated with increased $O_2^{\cdot -}$ production and schizophrenia in humans. Additionally, Moreno-Loshuertos et al. (2006) reported some common mtDNA variants in mice responsible for different ROS production in mitochondria. Cell lines which have identical nuclear background but carry a $tRNA^{Arg}$ (arginine) variant produce higher intracellular H_2O_2. Although the precise molecular mechanism by which this single nucleotide polymorphism leads to the differential performance of the mitochondrial ETC and ROS production is unclear, the variant probably affects the rate of mitochondrial protein synthesis and the translation fidelity. In this case, complex I, which is an important site of ROS production, is most likely to be affected by this variant, as it contains seven mtDNA-encoded polypeptides. Other studies have shown that mice with this mtDNA variant differ in learning capabilities, sensory development (Roubertoux et al., 2003), and are prone to develop age-related hearing loss (Noben-Trauth et al., 2003).

Nuclear Genes that Impact ROS Production and Redox Balance

Electron transport chain-related genes

While complexes I and III are widely accepted as the major site of ROS production, nuclear DNA-encoded ubiquinone, the electron carrier from complexes I and II to complex III, may play a role in $O_2^{\cdot -}$ production (Orrenius et al., 2007). A mutants line of yeast *Saccharomyces cerevisiae* leading to the lack of ubiquinone was shown by Davidson et al. (2001) to have lower mitochondrial ROS production. Other mutations in the gene *clk-1*, which is required in ubiquinone biosynthesis, were also reported to reduce ROS sensitivity and DNA damage in *Caenorhabditis elegans* (Wong et al., 1995) and mice (Liu et al., 2005). It was suggested that decreased ubiquinone supply reduced the rate of metabolism, and then slowed the accumulation of ROS during respiration. Interestingly, Liu et al. (2005) showed that although total knock out of mouse *clk-1* led to death, heterozygous mice displayed a longer mean life span, indicating that better resistance to OS might increase longevity.

Besides ubiquinone-related genes, other complex-related genes were also reported to impact ROS production. Ishii et al. (1998) reported that *C. elegans* mutants of the gene *mev-1*, which encodes a subunit of succinate dehydrogenase cytochrome b (complex II), increased sensitivity to oxygen and OS. Similarly, Hartman et al. (2001) showed that point mutations of *gas-1*, encoding a subunit of complex I, increased superoxide production in *C. elegans.* Mutation in the Rieske iron-sulfur protein of complex III, encoded by *isp-1*, was also reported to decrease mitochondrial superoxide production (Crofts et al., 2004).

Energy use-related genes

In *C. elegans*, a series of genes related to energy use was reported to increase or decrease ROS production (Finkel and Holbrook, 2000). For instance, the *age-1*, *daf-2* and *daf-16* genes, which are involved in an insulin-like signaling pathway, were shown to regulate redox balance

and longevity; while mutants of *age-1*and *daf-2* showed increased resistance to OS and elevation in CuZnSOD and catalase activities, the *daf-16* mutation displayed decreased resistance to oxidtiave tress (Martin et al., 1996).

The gene p66^{shc}

Another example of a nuclear determinant of ROS production is the gene *shc,* which encodes three proteins: p52^{shc}, p46^{shc} and p66^{shc}, with different molecular masses. The p66^{shc} protein, which is a 66-KDa isoform, is a cytoplasmic signal transducer. First reported by Migliaccio et al. (1999), p66$^{shc-/-}$ cells do not show a normal stress response. Further, ablation of p66^{shc} enhances cellular resistance to paraquat, a common OS inducer, and reduces apoptotic death induced by H_2O_2 or ultraviolet light. Giorgio et al. (2005) found that normally, about 20% of p66^{shc} localizes in mitochondria, where they bind to cytochrome c and shuttle electrons from it to molecular oxygen, resulting in ROS formation. Under conditions of stress, more cytosolic p66^{shc} will translocate to mitochondria, leading to more ROS production (Orsini et al., 2004). This translocation also influences other mitochondrial functions, including Ca^{2+} import and organelle morphology, which may induce the pro-apoptotic activity of p66^{shc} upon OS (Pinton and Rizzuto, 2008).

The gene mth

Among genes selected for extended life span in *Drosophila* strains, some also displayed increased resistance to OS. One of them is *mth*, which encodes a *Drosophila* G-protein-coupled receptor, which is responsible for transducing signal from extracellular stimuli into intracellular secondary message (Finkel and Holbrook, 2000). The heterozygous mutation of

this gene not only enhances resistance to heat, but also increases the resistances to paraquat, the intracellular ROS generator (Lin et al., 1998).

Antioxidant Defense-Related Genes Impact Redox Balance

As the other determinant of intracellular redox balance in mammalian cells, antioxidants are diverse biochemicals that work together to scavenge ROS. In knockout mice, some antioxidants like GPx (Ho et al., 1997) and catalase (Ho et al., 2004) have been shown to be non-essential. These investigations determined that other antioxidants, including CuZnSOD, encoded by the gene *Sod1*, are important scavengers of free radicals. Muller et al. (2006) reported that *Sod1*$^{-/-}$ mice showed tissue-wide lipid peroxidation and increased oxidative damage to DNA and protein. The increased oxidative damage may also explain the earlier observation that *Sod1*$^{-/-}$ female mice showed low fertility, and that both males and females exhibited accelerated age-related hearing loss and macular degeneration (Ho et al., 1998; McFadden et al., 1999). Moreover, these results seem consistent with those of a previous study that overexpression of *Sod1* in *Drosophila* decreased oxidative damage (Reveillaud et al., 1991).

The gene *Sod2*, encoding MnSOD, is another example of a key antioxidant gene. While MnSOD is the main scavenger for $O_2{\cdot}^-$ in mitochondria, genetic shut-down of this antioxidant enzyme caused death of mice in their first month of life and a four-fold increase in oxidative damage to DNA (Melov et al., 1999). In *Drosophila*, overexpression of *Sod2* and catalse decreased extramitochondrial H_2O_2 release, and increased the resistance to intensive oxygen exposure (Bayne et al., 2005).

Using the approach of overexpression or knockout, additional genes, including *MsrA* which encodes methionine sulfoxide reductase (Moskovitz et al., 2001) and *Prxd* which encodes peroxiredoxin (Neumann et al., 2003), have been implicated in free radical scavenge and in maintaining redox balance in mammals. A similar role of *MsrA* was also reported in *Drosophila* (Ruan et al., 2002). In addition, knockout of apolipoprotein D (ApoD) gene increased oxidative damage in *Drosophila*, while ApoD was also suggested to play an important role in protection from OS (Sanchez et al., 2006).

Attempts at Genome-Wide Analysis

The advent of microarray techniques enabled further genome-wide detection of ROS-responsive genes, which are obviously complex gene sets. Using *Drosophila melanogaster* as a model, the global gene expression profile was studied in the aging process, as well as the response to paraquat treatment. Zou et al. (2000) first found the expression of 132 ESTs representing 127 genes changed significantly with aging, and then detected 246 ESTs representing 236 genes valuable in OS response. Since the aging process, may be largely caused by oxidative damage accumulation, some of those aging-related genes might play roles in redox balance regulation and OS resistance. For those genes responses to the ROS inducer, some exhibited unique changes, while 47 ESTs, representing 42 genes, were inferred to regulate the aging process as well, indicating their possible role in regulating OS. In another study, mice having caloric restriction exhibited 120 genes increased in their expression, compared with that in age-matched controls (Lee et al., 2000). Those up-regulated genes might attenuate stress response and oxidative damage in the aging process, because the lean diet has become more and more accepted as contributing to less mitochondrial ROS production and thereby a longer life span (Wallace, 2005). More studies in genome-wide expression profile

under stress stimulation were reported in various species, like *E. coli*, yeast, and plant cells in response to H_2O_2 stimulation (Scandalios, 2002).

Chapter III

Ethanol Induced Changes in Oxidative Stress and Adaptive Immunity of Chickens

Abstract

Oxidative stress (OS) is often observed in chickens under various stressful conditions. To test whether OS impairs the immune system, I evaluated differences in adaptive immune response of chickens exposed to ethanol, which was used at different levels to induce OS. Using biomarkers for OS including malondialdehyde (MDA), glutathione (GSH), and plasma uric acid (PUA), metrics of OS were quantified at 0, 8, 14 days post-ethanol exposure for male chickens in study 1, and at 0, 7, and 21 days post-ethanol consumption for female chickens in study 2. The dosages of ethanol were 0, 2, 6, and 10% for study 1, and 0, 2, and 10% in study 2. Total plasma IgG and IgM were determined by ELISA before and after sheep red blood cell (SRBC) challenge in both studies, and major lymphoid organ weights were measured. Peripheral blood CD4+ and CD8+ T lymphocytes, counted by flow cytometry, and histopathological analysis of major lymphoid organs were carried out in study 2. The results of study 1 indicated polarized effects of ethanol on both OS and adaptive immunity: birds consume water containing 2% ethanol had higher plasma levels of antioxidants ($p < 0.001$), when those given 6 and 10% ethanol had decreased antioxidant but increased oxidative damage ($p < 0.001$). Additionally, birds provided 2% ethanol showed enhanced IgG levels and higher lymphoid organ weights ($p < 0.01$), while those on 10% ethanol had lower IgG ($p < 0.05$) and lymphoid organ weights ($p < 0.001$). There were significant correlations between lymphoid organ weights and levels of biomarkers for OS. In study 2, ethanol treatment did not induce many significant differences in OS in different chicken groups; there were also limited

differences in immunological parameters. While birds on 10% ethanol had decreased PUA ($p < 0.001$), they had the lowest lymphoid organ weights ($p < 0.001$). Birds on 2% ethanol showed higher levels of plasma GSH, and higher CD4+ cell count and CD4+/CD8+ ratio. There were no significant differences in antibody and histopathological analyses among the birds on different ethanol levels. Collectively, these results suggest that OS induced by ethanol appear to be correlated with changes in adaptive immune response which may contribute to regulation of immunocompetence in chicken.

Key words: Chicken, Ethanol, Oxidative Stress, Adaptive Immunity

Introduction

Oxidative stress (OS) is defined as the physiological imbalance in which reactive oxygen species (ROS) production exceeds the amounts of antioxidants that defend cells against ROS damage. Under normal physiological conditions, the production of reactive oxygen species (ROS), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), is balanced by that of antioxidants like superoxide dismutase, vitamins C, and E, and glutathione (GSH, Davies, 2000). However, cellular redox balance could be disrupted by external or internal factors, which leads to OS. In chickens, OS has been reported to arise from heat (Lin et al., 2006; Feng et al., 2008), pathogenic microbe stimulation (Keles et al., 2010), and toxic element exposure (Erdogan et al., 2005; Rezar et a., 2007). Additionally, other stressors like cold, transport, and pre-slaughter management that impact corticosterone secretion could also stimulate OS in chickens (Eid et al., 2003; Lin et al., 2004).

Because it causes physiological disorders and cell damage, OS has been widely associated with aging and age-related disease in humans (Valco et al., 2007). The deleterious effect of OS is believed to be due to ROS-induced oxidative damage to cell components like proteins, DNA, and lipids, and the triggering of specific signaling pathways of apoptosis (Finkel and Holbrook, 2000). Considering these effects, OS has also been associated with the decline of performance in various economic traits of chickens, including feed efficiency (Iqbal et al., 2004), meat quality (Gao et al., 2010), risk of heart failure (Nain et al., 2008), and other traits. However, impacts of OS on the immune system of chickens are not well understood.

Like other cells, immune cells are also susceptible to toxic effects of OS. For example, the human memory T cells are functionally suppressed after exposure to minute levels of H_2O_2 (Malmberg et al., 2001). Also, oxidative DNA damage has been detected in activated

macrophages (deRojas-Walker et al. 1995), which produce great amount of ROS under exposure to *E. coli* lipopolysaccharide, but over-production of ROS leads macrophages to damage themselves. Neutrophils go through apoptosis at sites of inflammation which had accumulation of ROS (Scheel-Toellner et al., 2004). Evidence of the influence of ROS on T cell and B cell activation and apoptosis were summarized by Buttke and Sandstrom (1994) and Larbi et al. (2007).

The possible damage of immune cells from ROS exposure suggested that OS might also impact immune function. Indeed, emerging evidence from both model and farm animals supported this hypothesis. In rats, OS induced by hyperbaria (Xu et al., 2007), noise (Srikumar et al. (2006), or toxin (Ercal et al., 2000) has been associated with impairments of both innate and adaptive immune responses. Moreover, antioxidant treatment in these studies prevented or alleviated related immunosuppression events. Similarly, OS induced by higher energy demand in the periparturient period increases susceptibility to a variety of infectious diseases including mastitis in dairy cows (Sordillo and Aitken, 2008). In chickens, OS has mostly been implicated in heat-induced decrease in lymphocyte proliferation and antibody titers, while supplementation with antioxidants including vitamins E and C (Puthpongsiriporn et al., 2001; Panda et al., 2008) and certain herbs (Ma et al., 2005) improved immune function after impairment by heat stress.

On the other hand, studies have shown that both acute and chronic ethanol exposure induced oxidative damage in multiple tissues and cells, including the liver (Colantoni et al., 2000), pituitary gland (Ren et al., 2005), plasma (Husain et al., 2005), testes (Maneesh et al., 2005), and white blood cells (Fedeli et al., 2007). Ethanol could therefore be used to induce OS, providing an experimental tool for assessing the relationship among OS and immune function.

Furthermore, compared to other stressors like heat and cold, the mechanism by which ethanol induces OS is relatively well understood. Initially, mitochondrial ROS production is stimulated by the increased NADH production from the metabolism of ethanol in liver cells, and CYP2E1-dependent ethanol oxidation in brain and other tissues then could contribute to ROS generation; additionally, when activated by ethanol, Kupffer cells, a specialized macrophages in liver, produce more ROS (Zakhari 2006; Das and Vasudevan, 2007). This background provided the rationale for using ethanol to test the hypothesis that OS contributes to the immunosuppressive events in chickens under different stress condition. Using different levels of ethanol in the drinking water, parameters characterizing adaptive immune response as well as biomarkers for OS were measured in male or female White Leghorn chickens.

Materials and Methods

To test the hypothesis described above, two experimental studies were carried out. One involved males and the other females. Experimental techniques were generally common to both experiments. Specific differences are detailed below.

Animals and Ethanol Administration

In the first study, day-old male commercial White Leghorn chickens (n = 96, Brickland Hatchery, Blackstone, VA) were raised to three weeks of age on a floor pen according to standard procedure (Table 3.1). On day 21, birds were randomly distributed to 16 cages (6 birds/cage) followed by nine days of acclimation. Beginning on day 30, birds were divided into four groups of four cages each and provided *ad libitum* access to water containing 0, 2, 6, or 10% (v/v) of ethanol (absolute, 99.5%, reagent ACS, Acros Organics) as the only water source for two weeks.

In the second study, day-old female commercial White Leghorn chickens (n = 600, same source) were divided into six pens of 100 each and conventionally raised on the floor for 33 days. They were then divided into three groups of two pens each and provided water *ad libitum* with 0, 2, or 10% (v/v) ethanol as the only water source for three weeks. All procedures were carried out in accordance with the approval of the Virginia Tech Institutional Animal Care and Use Committee (#09-070-APSC).

Sampling and Antigenic Challenge

To assess biomarkers for OS and antibody levels before and after ethanol use, blood samples were obtained by brachial venipuncture on one day before and 8 days, 14 days post ethanol treatments from study 1, and on one day before and 7 days, 21 days after ethanol

exposure from study 2. Plasma was obtained immediately after blood collection and snap-frozen on dry ice before storage at -20^{o}C. Six days before the third blood collection of both studies, chickens were injected with 0.1ml of 25% sheep red blood cells (SRBC, Cleveland Scientific, Cleveland, OH) via brachial venipuncture to induce a primary immune response. Birds were also weighed on days 0, 8, and 15 in the first study, and upon sacrifice on 15 days post-ethanol use, liver and major lymphoid organs, including thymus, bursa of Fabricius, and spleen, were immediately removed and weighed. Similarly, on days 0, 7, 21 in study 2, body weight and major lymphoid organs were weighed. The lymphoid organ weights were adjusted by body weights and expressed as g/kg body weight (BW).

Biomarkers for Oxidative Stress

Malondialdehyde (MDA). Plasma MDA level, used as a biomarker of lipid peroxidation, was determined spectrophotometrically using a commercially available colorimetric kit (Northwest Life Sciences, Vancouver, WA) based on the approach described by Jentzsch et al. (1996). Minor modifications were made here by using 96-well microplates and reducing the amount of reagents to 20% of recommendation. Briefly, 50 µL calibrator or plasma sample were mixed with 2 µL of butylated hydroxytoluene, 50 µL phosphoric acid (1 mol/L) and 50 µL reconstituted 2-thiobarbituric acid reagents. Following incubation at 60 ^{o}C for 60 min, the solutions were centrifuged at 10,000×g for 60 sec. Absorbance of calibrators and samples was determined at 532 nm, and background readings at 570 nm were subtracted.

Glutathione (GSH). Total blood GSH level was measured using a commercially available kit (Northwest Life Sciences, Vancouver, WA) based on the method of Teitze (1969). Some changes were made regarding sample dilution, reading wavelength and intervals. Briefly, 100 µL of heparinized whole blood from each bird were first deproteinated using 200 µL cold 5%

metaphosphoric acid, and diluted 40-fold prior to use. Then 50 µL calibrator or diluted samples were mixed with 50 µL 2-nitrobenzoic acid and 50 µL glutathione reductase. After 5 min-incubation at room temperature, 50 µL NADPH were added to each well, and total GSH was quantified immediately by a kinetic analysis of 10 absorbance records at 412 nm at 30-second intervals.

Plasma Uric Acid (PUA). PUA was quantified by an enzymatic method using a colorimetric commercial kit according to the procedure suggested by the manufacturer (BioAssay Systems, Hayward, CA). Briefly, 2,4,6-tripyridyl-s-triazine was added to plasma samples to form a blue-colored complex with iron in the presence of uric acid. The concentrations of PUA were then estimated using calibration curves at 590 nm.

Measurements for Adaptive Immunity

IgG and IgM Analysis. Total plasma IgG and IgM were determined as described by Sakai et al. (2006) using quantitative ELISA Kits (Bethyl Laboratories, Montgomery, TX), with some modifications involving sample dilutions, washing times and HRP dilutions. Briefly, plasma samples were diluted 213,000-fold for the IgG assay, and 3,000-fold for the IgM assay. Then, 96-well microplates were coated with 100 µL of 0.01 mg/ml capture antibodies followed by a blocking procedure. After washing twice, 100 µL samples or standards were added and incubated for 1 hour at room temperature. The plates were then washed three times and incubated with horseradish peroxidase (HRP)-conjugated goat anti-chicken antibody for 1 hour. After three washes and addition of a substrate (3,3',5,5'-tetramethyl benzidine) and H_2SO_4 to stop the reaction, optical density was measured at 450 nm and the concentrations of IgG and IgM were calculated based on standard curves. Here, the HRP conjugate was diluted 25,000-fold for the IgG assay, and 15,000-fold for the IgM assay.

Flow Cytometry Analysis of CD4+/CD8+ cells. Approximately 5 mL of peripheral blood were collected from individual birds (n = 6-18) in study 2 at each designated time point (Days 0, 5, 9, 14 post-ethanol use). Peripheral blood lymphocytes were isolated immediately by density centrifugation followed by a gentle "swirl" technique for isolating the buffy coat layer as described by Gogal et al. (1997). After two washes with phosphate-buffered saline (PBS), isolated lymphocytes were enumerated and adjusted to 5×10^6 cell/mL, and then stained with conjugated monoclonal antibodies for 30 min at 4^oC. Mouse anti-chicken fluorescein isothiocyanate (FITC)-conjugated CD4 and phycoerythrin (PE)-conjugated CD8 antibodies (SouthernBiotech, Birmingham, AL) were used at a final concentration of 2.5 µg/mL. Stained lymphocytes were then washed and resuspended in 100 µL PBS and analyzed in an EPICS-XL flow cytometer (Coulter, Hileah, FL). From each sample, 5000 events were collected and analyzed using the Immuno-4 software program (Coulter, Hileah, FL).

Histopathological Analysis. Major lymphoid organs, including thymus, bursa of Fabricius, and spleen, were harvested from two chickens of each group before treatment (day 0), and four chickens of each group after treatment (days 7 and 21) in study 2. Tissue samples were fixed in 10% neutral buffered formalin, and processed through graded ethanol, a xylene substitute, and embedded in paraffin. After cutting to 5 µm thickness and staining with hematoxylin and eosin (H&E), sections were evaluated microscopically for any histological damage. Image acquisition was carried out using a digital camera (Nikon, Japan), and the histopathology analysis was performed in a blinded fashion.

Statistical Analyses

All data are presented as the least squares means ± standard error (SEM). Analyses of variance were performed using the General Linear Model procedure of SAS (version 9.1, 2007,

SAS Institute Inc., Cary, NC). The model included effects of ethanol and cages/pens with a critical value of $p < 0.05$. Pairwise comparisons of least-squares means for ethanol effect were performed using the Tukey test. Correlations among biomarkers for OS and immunological parameters were analyzed using the CORR procedure of SAS with a level of significance set at $p < 0.05$.

Results – Study 1

Oxidative Stress

Beginning at similar levels, plasma MDA level increased in all groups as the experiment went on (Table 3.2). However, the extent of increase differed. After eight days of ethanol consumption, increases of MDA level were 42, 7, 43, and 110% for birds exposed to water, 2%, 6% and 10% of ethanol respectively. The MDA level of the 10% ethanol group was significantly higher than those of other groups in pair-wise comparisons ($p < 0.001$, compared to control and 2% group; $p < 0.01$, compared to 6% group). Six days later, the increment of MDA levels expanded to 110, 69, 75, and 158% (compared to day 0) for the four groups, respectively, but differences among the groups were not significant ($p > 0.05$). Conversely, total plasma GSH as a indicator of antioxidant decreased sharply in all groups after eight days of treatment, but the GSH level of 2% ethanol groups remained significantly higher than those of the control ($p < 0.001$) and 10% groups ($p < 0.001$) at day 8, and higher than that of the control group ($p < 0.05$) at day 14. Another antioxidant indicator, PUA concentration of the four groups began at similar levels on day 0, then decreased differentially (-15, -8, -19, and -29%) at day 8, and slightly rebounded at day14 (+10, +18, -8, and -13% compared to day 0). Pair-wise comparisons displayed higher PUA in the 2% group compared to the 10% group at day 8 ($p < 0.05$); and higher PUA in the control and 2% groups compared to the 6% ($p < 0.01$) and 10% groups ($p < 0.001$) at day 14.

Adaptive Immunity

Plasma IgG and IgM Levels. After eight days of ethanol exposure in drinking water, total plasma IgG (Table 3.3) level increased in birds exposed to water, 2% and 6% ethanol (+51, +86, and +46%, respectively), but decreased in birds exposed to 10% ethanol (-6%). After two

weeks of ethanol exposure and antigen challenge, total plasma IgG of the four groups increased 125%, 254%, 90%, and 15% respectively, compared to day 0. The 2% ethanol group had higher IgG level than the control group ($p = 0.41$), 6% ($p < 0.05$) and 10% groups ($p < 0.001$), while the 0% group was also significantly higher than 10% group ($p < 0.05$). No significant differences were observed in total plasma IgM levels among groups of all the time points measured .

Body and Major Lymphoid Organ Weights. As shown in Table 3.4, although all groups began at similar weights, the average body weight of birds on 10% ethanol was significantly less than those on 0, 2 and 6% ethanol ($p < 0.01$, day 8; $p < 0.001$, day 15). Relative weights of the major lymphoid organs revealed clear polarized effect of two weeks of ethanol use. Thymuses from the 2% group were 35.4% heavier than those of the control group ($p < 0.01$), whereas thymuses from the 10% ethanol group were 39.6% lighter ($p < 0.001$) than those of the control birds. Although the adjusted spleen weights were not significantly different among the 0, 2, and 6% groups, those of the 10% group were much lower ($p < 0.001$). Like the thymus, bursas from the 2% ethanol group were the heaviest, while those in the 10% group were the lightest ($p < 0.001$).

Correlation Analysis

Following two weeks of ethanol treatment, there were significant correlations between biomarkers for OS and major lymphoid organ weights (Fig. 3.1). For example, plasma MDA on day 8 was strongly negatively correlated with major lymphoid organ weights on day 15 ($p < 0.05$), while PUA on day 8 positively correlated with these organ weights ($p < 0.05$). Similarly, GSH was positively correlated with thymus and bursa weight after two weeks of ethanol consumption ($p < 0.05$). The general correlations between biomarkers for OS and

immunological parameters of all time points are shown in Table 3.5, although significance was not shown for other time points.

Results – Study 2

Oxidative Stress

For some unknown reason, birds in the 2% and 10% ethanol groups displayed significantly higher lipid peroxidation before ethanol treatment than those in the control group ($p < 0.001$, Table 3.2). After 7 days of ethanol supply in their drinking water, MDA level did not change in the 2% and 10% group, but increased in the control group, which then was about the same as in the other groups. Fourteen days later, all groups decreased about 35% in plasma MDA level, and there was no group difference. For total GSH, birds on 2% ($p < 0.01$) and 10% ($p= 0.06$) ethanol use showed lower levels than control birds on day 0, and was still lower on day 7 ($p < 0.05$ for the 10% group). On day 21, group differences disappeared. Similarly, PUA level differed a lot at the very beginning ($p < 0.001$), when the control group had the lowest level, and then increased 65% and became the highest level on day 7. At the same time, the 10% group fell to the lowest PUA level and remained lowest 21 days post-ethanol treatment ($p < 0.001$).

Adaptive Immunity

Plasma IgG and IgM Levels. From one day before to day 7 of ethanol treatment, total plasma IgG level increased in all groups to different extents: 60%, 121% and 56% for control, 2% and 10% ethanol groups, respectively (Table 3.3). However, there were no significant group differences. On day 21, the IgG levels in chicken without SRBC challenge did not change much in all groups, while in challenged chickens, it increased 176%, 259%, and 143% for

control, 2% and 10% ethanol groups, respectively (compared to day 0). The group difference was not significant. Similarly, no significant group difference in total plasma IgM was detected among groups, although there were increases as time went on and after SRBC chanllenge.

Body and Major Lymphoid Organ Weights. At beginning, average body weight of birds in control and 2% ethanol group were similar, and that of the 10% group was higher ($p < 0.001$, Table 3.4). There was no difference in the relative lymphoid organ weights among groups on day 0. After one week of ethanol treatment, birds on 2% and 10% ethanol use gained less body weight than the control, and relative lymphoid organ weights were lightest in the 10% group. Then, group differences increased on day 21, as both body and lymphoid organ weight of birds under 10% ethanol use were significant lower than those of control birds ($p < 0.001$).

Blood T Lymphocytes Ratio. Ethanol consumption caused polarized changes in peripheral blood T lymphocyte subsets. Although percentage of CD4+ lymphocytes all began around 37%, the proportion (Fig. 3.2A; Table 3.6) in the 10% ethanol group was consistently lower than those in the other two groups, while it remained relatively high in the 2% group after treatment. Especially nine days post-ethanol consumption, the proportions of CD4+ subsets of the 2% group were 28.5% and 43.5% ($p < 0.05$) higher than those given water and 10% ethanol, respectively. The CD8+ T lymphocyte population (Fig. 3.2B), although was not significantly different among ethanol treatment groups, decreased slightly with time. Meanwhile, the CD4+/CD8+ ratios (Fig. 3.2C) of all groups showed a slight increase, with the ratios of the 10% ethanol group starting the highest on day 0 and ending the lowest by day 14. However, the group differences did not reach a significant level.

Histopathological Analysis. There were no obvious differences in gross pathology or histopathology of major lymphoid organs from chicks on different levels of ethanol exposure

after 3 weeks (Fig. 3.3A-I). The H&E-stained organ sections from treated chicks appeared similar to those of the control chickens at all three sampling time-points; results only from day 21 are shown.

Discussion

Study 1

I investigated the effects of moderate and excessive ethanol consumption on OS and adaptive immune response of chickens. Generally, results suggest that the ethanol-induced changes in biomarkers for OS and for adaptive immunity in chickens paralleled one another.

In study 1, the dose-dependent elevation of OS in chicks treated by 6% and 10% ethanol, indicated by increased MDA and reduced GSH and PUA, is consistent with previous studies. Husain et al., (2005) reported dose-dependent increase of plasma MDA in rats under high ethanol intake for 12 weeks. Significant reductions in enzymatic antioxidant levels were also shown in a dose-dependent manner in rat plasma following acute oral ethanol consumption (Schlorff et al., 1999). A difference here is that the MDA level of chicken was significantly higher only in the high ethanol group on day 8. The ethanol dosage, 10% compared to 20-30% in the former study (Husain et al., 2005), may explain the quick disappearance of group differences in lipid peroxidation. Alternatively, the action of PUA in chicken may be another explanation, since PUA was suggested to be a highly effective ROS scavenger in avian and primate species (Simoyi et al., 2003). Moreover, the significant decrease of PUA detected on day 14 suggested a lasting OS in chicken at 6% and 10% ethanol dose.

At the same time, the adaptive immune responses appear impaired in birds of 6% and 10% ethanol dose in study 1. The SRBC-induced IgG level was significantly lower in birds of the 6% and 10% ethanol groups, showing an impaired humoral immune response. Although there was no difference in IgM levels among groups, further study is needed to determine whether group difference in IgM level appeared prior to the time evaluated here, since a peak

of IgM is supposed to appear and disappear soon before IgG production (Tizard, 2009). Then, the dose-dependent decrease of relative lymphoid organ weights in the high ethanol group might indicate an impaired development of both T and B lymphocytes and further weak host immunocompetence, because the thymus and bursa are the sources of T and B cell production, respectively, and the biggest secondary lymphoid organ, spleen, serves as the site for antigen-dependent lymphocyte differentiation (Tizard, 2009). My results are also consistent with the reports of Jerrells et al. (1990) and Padgett et al. (2000), in which the thymus and spleen cells were significantly decreased due to severe ethanol exposure.

The decreased antibody production and lymphoid organ weights may be due to a slow immune cell proliferation or cell damage, and the concurrence of ethanol-induced OS suggesting OS might play a role. According to the deleterious effect of ROS (Finkel and Holbrook, 2000; Halliwell, 2006), influence of ethanol-induced OS is in two aspects: non-specific oxidative damage to cell components like proteins, DNA, and lipids; and controlling cellular signaling pathways. For example, ethanol-induced oxidative damage to blood lymphocytes has been detected before in mice (Sibley et al., 1995; Fedeli et al., 2003). In a later study reported by Fedeli et al. (2007), the ethanol-induced DNA damage of white blood cells was reduced by taking ethyl pyruvate, a ROS scavenger. Actually, immune cells are more sensitive to oxidative damage because of the high percentage of polyunsaturated fatty acids in their plasma membranes (Meydani et al., 1995). This evidence indicated that immunosuppression events observed here might due to oxidative damage to macromolecules of immune cells. Similarly, Ercal et al. (2000) suggested that toxin-induced oxidative damage in peripheral blood mononuclear cells contributed to the significant reduction in rat serum antibody levels in their study. As another possibility, ethanol-induced OS here could also

impacted proliferation and triggering of cell death in the immune system through a signaling pathway. Malmberg et al. (2001) suggested that H_2O_2 exposure suppressed human memory T cell function by blocking NF-κB expression, and Hampton and Orrenius (1998) also reported that excess ROS could down-regulate this key transcriptional factor and lead to cell death. In my study, decreased lymphoid organ weights could be a result of ROS-induced cell loss. Similarly, cell loss in the immune system was associated with hyperbaria-induced OS (Xu et al., 2007) and fluoride-induced OS (Das et al., 2006). To verify these hypotheses, lymphoid organs need an examination of cell damage; cytokine production could be monitored; and *in vitro* proliferation could also be assayed.

On the other hand, there were increased antioxidant level, as indicated by GSH and PUA, of birds in the 2% ethanol dose in study 1. A low level of ethanol use appears to enhance antioxidant defense and decrease OS in birds. Though moderate consumption of red wine for two weeks increased antioxidant status and reduced OS in humans (Micallef et al., 2007; Tsang et al., 2005), data on the positive effects of moderate ethanol use, without antioxidant ingredient, on oxidative status are lacking. However, there were some clues in a previous study that rats at low ethanol dose for 12 weeks showed slightly increased in the activities of antioxidant enzymes and ratio of reduced to oxidized glutathione (GSH/GSSG) compared to controls (Husain et al., 2005).

Correspondingly, increased immunocompetence was shown in birds at 2% ethanol consumption when their antioxidant level was increased. There was higher total plasma IgG following SRBC challenge in chicken on 2% ethanol, and increased weights of thymus and bursa in birds on 2% ethanol. These data appear to accord with the beneficial effects of decreased OS reported by Hogan et al. (1992) that injection of vitamin E enhanced the

neutrophil killing response to bacteria in dairy cows, and by Puthpongsiriporn et al. (2002) that vitamin E supplementation, which might decrease heat-induced OS, increased lymphocyte proliferation in chicken. Actually, low levels of intracellular ROS was reported to increase NF-κB expression and cell proliferation, while high levels affect these marksers adversely (Hampton and Orrenius, 1998). To test whether the beneficial effects on lymphoid organs and the antibody prodction in chicken given low dose of ethanol are due to low-OS activated proliferation, lymphocytes could be isolated from those organs and be recorded on their *in vitro* proliferation.

Collectively, our results from study 1 supported the hypothesis that OS involved in the ethanol induced changes in chicken adaptive immunity. Then, to test whether the biphasic effect of ethanol also works in female chickens and whether there is again a negative correlation between OS and adaptive immune response, study 2 was carried out.

Study 2

As expected, GSH and PUA decreased significant in a dose-dependent manner in one or two of the time points measured after ethanol consumption, indicating the increased need for ROS removal after high ethanol dose. However, different from study 1, there was no group difference in plasma MDA level at any time-point measured after ethanol administration, indicating that ethanol-induced OS in study 2 was not high enough to cause significant lipid peroxidation. The less-significant OS induced by ethanol here compared to that of study 1 may be due different antioxidant ability of male and female, as supported by Hartman et al. (2006). It may be also due to the different housing method used in study 2. Raised in floor pen compared to cages, chicken had more space and movement, and this might accelerate ethanol detoxification and reduce oxidative damage. On the other hand, there are huge group

differences in biomarkers before ethanol use, which indicated the existence of other possible stressors. This interference could reduce the ethanol effect on OS among treatment groups, and it may also explain the absence of a beneficial effect of ethanol on redox balance in study 2.

Interestingly, like the indistinctive OS in chickens at 10% ethanol dose and the absence of significant improvement in oxidative status in chicken of 2% ethanol use, the group difference in immune response in study 2 is not as significant as that of study 1. Based on total plasma IgG and IgM levels, humoral immune responses were similar among groups. Only a slightly higher IgG level was observed in the 2% group on day 21. For cellular immunity, the CD4+ helper T cell population appeared higher in birds at 2% ethanol dose on day 9, and there was a trend of diminished circulating CD4+ lymphocyte ratio and reduced CD4+/CD8+ ratios in birds on 10% ethanol dose, although this decrease could be more significant if the birds were challenged (e.g., by a pathogenic agent). For the major lymphoid organ weights, significant decreases were detected again in the 10% ethanol group on day 21, but the increase in the 2% group was not significant. Further, histopathological analysis did not show visual lesions or obvious cell loss in those major lymphoid organs of chickens exposed to 10% ethanol dose. It is possible that cell damage was not severe enough to exhibit histological lesions, which means a more accurate assay are needed here; or OS only slowed lymphocyte proliferation and organ development, but did not damage cell components, in which case *in vitro* proliferation may be needed. Collectively, the change of the immune system still correlated to ethanol-induced OS of female chicken, and the simultaneous reduced regulatory effect on both OS and the immune system again supported their association.

Summary

In summary, observations of study 1 showed a polarized effect of ethanol on both oxidative status and adaptive immunity: when high ethanol intake (6% and 10%) appeared to increase OS and suppress immune function dose-dependently in male chicken, 2% ethanol appeared to lower OS and to enhance adaptive immune response. Correlation analysis suggested that OS plays a role in biphasic immunoregulatory effects of ethanol. In study 2, the biphasic ethanol effects on OS were less significant and group differences in immunological parameters were also smaller. Based on these studies, we suggest that OS plays an important role in immunoregulation, and ROS may mediate immune response through oxidative damage of cellular components or/and their regulatory function on cell proliferation. To test this hypothesis, a more accurate examination of cellular damage, like immunohistochemistry staining, could be carried out; flow cytometry could be used to assay apoptosis of lymphoid cells; the production of cytokines, like IL-2, IL-4, TNF-2, and *in vitro* proliferatioin could also be good measurements.

Table 3.1 Experimental design for both studies.

	Study 1	Study 2
Chicken	Day-old White Leghorn	Day-old White Leghorn
Gender	Male	Female
Number	96	600
Housing	16 cages	6 pens
Ethanol treatment	0%, 2%, 6%, 10%	0%, 2%, 10%
Age of first treatment	30 days of age	34 days of age
Treatment duration	2 weeks	3 weeks
Biomarkers measured[a]	MDA, PUA, GSH	MDA, PUA, GSH
Immunological parameters measured	IgG, IgM,	IgG, IgM
	relative lymphoid organ weight	relative lymphoid organ weight
		CD4+, CD8+ ratio
		histopathology

[a]Malondialdehyde (MDA) indicates lipid peroxidation, and total glutathione (GSH), and plasma uric acid (PUA) were measured as antioxidants.

Table 3.2 Mean levels of oxidative stress biomarkers measured in chicken plasma before and after different doses of ethanol use

	Ethanol concentration in the water (v/v)							
	Study 1					Study 2		
	0%	2%	6%	10%		0%	2%	10%
MDA[a] *(µmol/L)*					*MDA (µmol/L)*			
Day 0[b]	0.66 ± 0.07[c]	0.91 ± 0.08	0.77 ± 0.07	0.72 ± 0.07	Day 0 [b]	0.50 ± 0.03	0.74 ± 0.03***	0.72 ± 0.03***
Day 8	0.94 ± 0.08	0.97 ± 0.08	1.10 ± 0.08	1.51 ± 0.08***	Day 7	0.77 ± 0.03	0.74 ± 0.03	0.71 ± 0.03
Day 14	1.4 ± 0.22	1.54 ± 0.22	1.34 ± 0.21	1.85 ± 0.24	Day 21	0.5 ± 0.02	0.49 ± 0.02	0.46 ± 0.02
GSH(µmol/L)					*GSH(µmol/L)*			
Day 0	382.2 ± 43.3	307.7 ± 47.9	328 ± 46.1	298.3 ± 47.0	Day 0	1643 ± 29	1497 ± 29**	1552.1 ± 28
Day 8	105.6 ± 12.6	179.2 ± 12.6***	139.2 ± 12.3	84.4 ± 13.8	Day 7	1622 ± 23	1579.5 ± 22	1543 ± 23*
Day 14	129.1 ± 18.2	203.7 ± 17.4*	173.8 ± 16.3	174.7 ± 18.9	Day 21	1512 ± 31	1535 ± 30	1535 ± 31
PUA(µmol/L)					*PUA(µmol/L)*			
Day 0	670.4 ± 33.6	643.1 ± 34.3	645.8 ± 33.6	659.2 ± 34.6	Day 0	260 ± 7	324.7 ± 7.1***	353.1 ± 7***
Day 8	571.2 ±32.1	594.6 ± 33.2	524.8 ± 31.4	469.3 ± 34.2	Day 7	429.9 ± 8.9	393.7 ± 8.7**	329.7 ± 8.8***
Day 14	739.4 ± 27.6	756.7 ± 27.9	594.9 ± 26.1**	575.6 ± 30.3***	Day 21	366.6 ± 7.3	356.2 ± 7.3	312.9 ± 7.6***

[a]Malondialdehyde (MDA) indicates lipid peroxidation, and total glutathione (GSH), and plasma uric acid (PUA) were measured as antioxidants.

[b]In study 1, Day 0 represents 29 days of age and one day before ethanol induction, day 8 and day 14 represents 8 days and 14 days after ethanol consumption. In study 2, Day 0 represents 33 days of age and one day before ethanol induction, day 7 and day 21 represents 7 days and 21 days after ethanol induction.

[c]The data are expressed as least-squares mean ± SEM. n = 20 to 24 per group for study 1, n = 81 to 153 per group for study 2. Significant difference compared to control: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 3.3 Mean levels of total IgG and IgM measured in chicken plasma before and after oral administration of different doses of ethanol

	Ethanol concentration in the water (v/v)							
	Study 1					Study 2		
	0%	2%	6%	10%		0%	2%	10%
IgG (mg/ml)					*IgG (mg/ml)*			
Day 0[a]	4.24 ± 0.56[b]	3.27 ± 0.57	4.12 ± 0.55	5.17 ± 0.55	Day 0	2.52 ± 0.22	2.36 ± 0.22	2.9 ± 0.21
Day 8	6.4 ± 0.69	6.07 ± 0.71	6 ± 0.69	3.94 ± 0.71	Day 7	4.03 ± 0.43	5.2 ± 0.41	4.5 ± 0.42
Day 14 (SRBC)	9.57 ± 0.89	11.56 ± 0.93	7.85 ± 0.87	5.93 ± 0.87*	Day 21	4.07 ± 0.55	4.35 ± 0.58	4.16 ± 0.57
					Day 21 (SRBC)	6.96 ± 0.52	8.47 ± 0.51	7.06 ± 0.53
IgM(mg/ml)					*IgM(mg/ml)*			
Day 0	0.13 ± 0.02	0.11 ± 0.02	0.17 ± 0.02	0.14 ± 0.02	Day 0	0.17 ± 0.01	0.15 ± 0.01	0.17 ± 0.01
Day 8	0.17 ± 0.02	0.15 ± 0.02	0.13 ± 0.02	0.17 ± 0.01	Day 7	0.26 ± 0.03	0.32 ± 0.03	0.23 ± 0.03
Day 14 (SRBC)	0.13 ± 0.01	0.14 ± 0.02	0.16 ± 0.02	0.15 ± 0.01	Day 21	0.36 ± 0.03	0.36 ± 0.03	0.33 ± 0.03
					Day 21 (SRBC)	0.63 ± 0.03	0.62 ± 0.03	0.68 ± 0.03

[a] In study 1, Day 0 represents 29 days of age and one day before ethanol induction, day 8 represents 8 days after ethanol consumption, day 14 represents 14 days after ethanol induction, and 6 days after 0.1ml 25% sheep red blood cell (SRBC) challenge. In study 2, Day 0 represents 33 days of age and one day before ethanol induction, day 7 represents 7 days after ethanol consumption, and day 21 (SRBC) stands for 21 days after ethanol induction and 6 days after 0.1ml 25% sheep red blood cell (SRBC) challenge.

[b]The data are expressed as least-squares mean ± SEM. n = 16 to 22 per group in study 1, n = 21 to 49 per group in study 2. Significant difference compared to control: $^{*}p < 0.05$.

Table 3.4 Body weight and relative major lymphoid organ weight of chicks before and after oral administration of different doses of ethanol

Ethanol concentration in the water (v/v)								
	Study 1					Study 2		
	0%	2%	6%	10%		0%	2%	10%
Body weight (g)					*Body weight (g)*			
Day 0[a]	299.8 ± 6.5[b]	298.8 ± 6.5	300.5 ± 6.5	288.3 ± 6.5	Day 0[a]	370.1 ± 2	373.7 ± 2	380.1 ± 2***
Day 8	426.7 ± 10	420.4 ± 10	415.4 ± 10	366.7 ± 10***	Day 7	560.7 ± 30	497.9 ± 30.1	506.1 ± 29.5
Day 15	601.2 ± 10.5	589.8 ± 10.5	572.3 ± 10.5	506.4 ± 11***	Day 21	696.5 ± 5.7	702.8 ± 5.6	628.9 ± 5.5***
Thymus (g/kg body weight)					*Thymus (g/kg body weight)*			
					Day 0	2.53 ± 0.1	2.63 ± 0.11	2.45 ± 0.1
Day 15	4.78 ± 0.31	6.51 ± 0.32**	5.28 ± 0.31	2.91 ± 0.33***	Day 7	3.33 ± 0.14	3.4 ± 0.15	3.16 ± 0.14
					Day 21	4.9 ± 0.14	4.75 ± 0.13	3.32 ± 0.13***
Spleen (g/kg body weight)					*Spleen (g/kg body weight)*			
					Day 0	1.05 ± 0.03	1.06 ± 0.03	0.98 ± 0.03
Day 15	2.69 ± 0.15	2.58 ± 0.15	2.33 ± 0.15	1.48 ± 0.15***	Day 7	1.41 ± 0.05	1.44 ± 0.05	1.2 ± 0.05**
					Day 21	2.12 ± 0.04	2.05 ± 0.04**	1.27 ± 0.04***
Bursa (g/kg body weight)					*Bursa (g/kg body weight)*			
					Day 0	2.36 ± 0.08	2.39 ± 0.08	2.25 ± 0.08
Day 15	6.88 ± 0.27	7.42 ± 0.27	6.11 ± 0.27	3.45 ± 0.28***	Day 7	2.93 ± 0.11	3.02 ± 0.11	2.58 ± 0.11
					Day 21	3.68 ± 0.09	3.71 ± 0.09	2.5 ± 0.09***

[a] Day 0 represents one day before ethanol induction, and 29 days of age in study 1, 33 days of age in study 2, and day 7, 8, 15, and 21 represents 7, 8, 15, and 21 days after ethanol consumption.

[b]The data are expressed as least-squares mean ± SEM. n = 22 to 24 per group in study 1; n = 112 to 200 per group for body weight, and n = 34 to 86 per group for organ weight in study 2. Significant difference compared to control: ** $p < 0.01$, *** $p < 0.001$.

Table 3.5 Pearson correlation coefficients of oxidative stress and Adaptive Immunity before, and after ethanol oral administration[a].

	Day 0[#]	Day 8	Day 15			
	IgG	IgG	IgG	thymus	spleen	bursa
Day 0						
MDA	-0.01	-0.03	-0.03	0.12	0.03	-0.04
GSH	0.01	-0.13	-0.02	0.03	0.05	-0.07
PUA	0.20	0.22	-0.11	-0.06	0.05	-0.04
Day 8						
MDA	0.18	-0.14	-0.10	-0.35**	-0.28*	-0.36***
GSH	-0.32*	-0.14	0.04	0.31**	0.13	0.29*
PUA	-0.19	0.12	0.10	0.24*	0.23*	0.26*
Day 14						
MDA	0.08	0.03	-0.03	-0.01	-0.09	-0.19
GSH	-0.04	0.06	-0.15	0.05	-0.10	-0.06
PUA	-0.16	0.13	0.19	0.09	0.15	0.24*

[a] Oxidative stress was measured by three biomarkers: malondialdehyde (MDA) indicates lipid peroxidation, and total glutathione (GSH), and plasma uric acid (PUA) were measured as antioxidants.

Day 0 represents one day before ethanol induction; day 8, 14, 15 represent 8, 14, 15 days after ethanol consumption.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 3.6 Peripheral blood T lymphocytes ratio before and after oral administration of different doses of ethanol

	Ethanol concentration in water (v/v)		
	0%	2%	10%
CD4+ (% lymphocytes)			
Day 0[a]	34.46[b]	38.75	37.3
Day 5	39.66 ± 4.5	36.24 ± 4.5	32.35 ± 4.5
Day 9	31.54 ± 2.7	40.52 ± 2.7	28.24 ± 2.7
Day 14	43.89 ± 2.9	46.13 ± 2.9	42.33 ±2.9
CD8+ (% lymphocytes)			
Day 0	16.29	13.53	11.91
Day 5	14.51 ± 1.28	11.85 ± 1.28	14.71 ± 1.28
Day 9	13.71 ± 1.51	13.56 ± 1.51	12.37 ± 1.51
Day 14	12.59 ± 1.44	9.8 ± 1.44	13.11 ± 1.44
CD4+/CD8+			
Day 0	2.1	2.87	3.16
Day 5	2.73 ± 0.45	3.12 ± 0.45	2.31 ± 0.45
Day 9	2.46 ±0.33	3.12 ± 0.33	2.61 ± 0.33
Day 14	4 ± 0.74	5.03 ± 0.74	3.36 ± 0.74

[a]Day 0 represents 28 days of age and six days before ethanol induction, and day 5, 9, 14 represent 5, 9, 14 days after ethanol consumption.

[b]The data are expressed as least-squares mean ± SEM, n = 4 to 6 per group for day 5, 9, and 14. Data for day 0 are shown as least-squares mean, n = 2 per group.

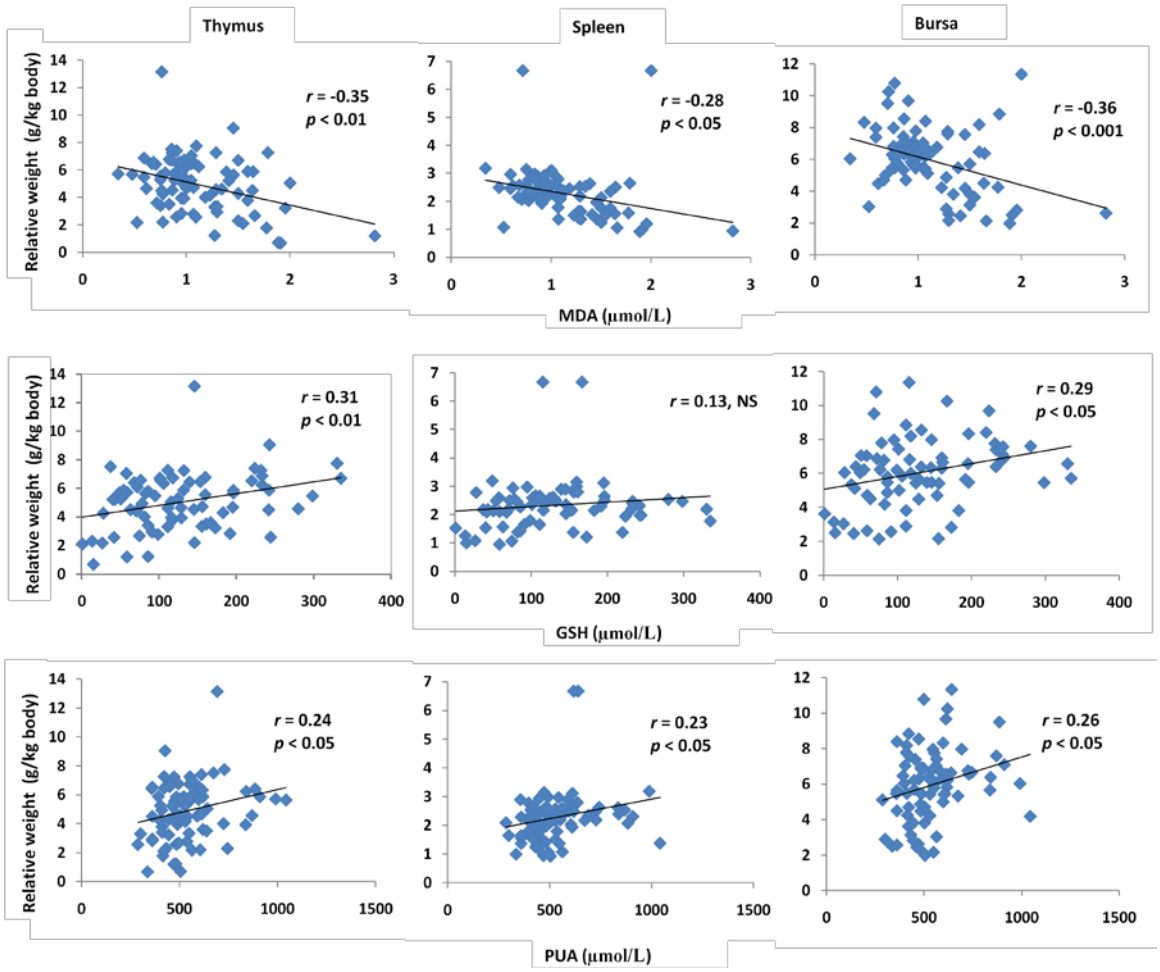

Figure 3.1 General correlations between oxidative stress biomarkers on day 8 and major lymphoid organ weights on day 15. Oxidative stress biomarkers are measured as concentration of malondialdehyde, MDA, glutathione, GSH, and plasma uric acid, PUA, while major lymphoid organ weights were measured as thymus, spleen, bursa, g/kg body weight. Coefficients and significance level of Pearson's parametric correlations for each pair are shown in each figure. NS = non-significant. Day 8 and 15 represent 8 days and 15 days after ethanol consumption.

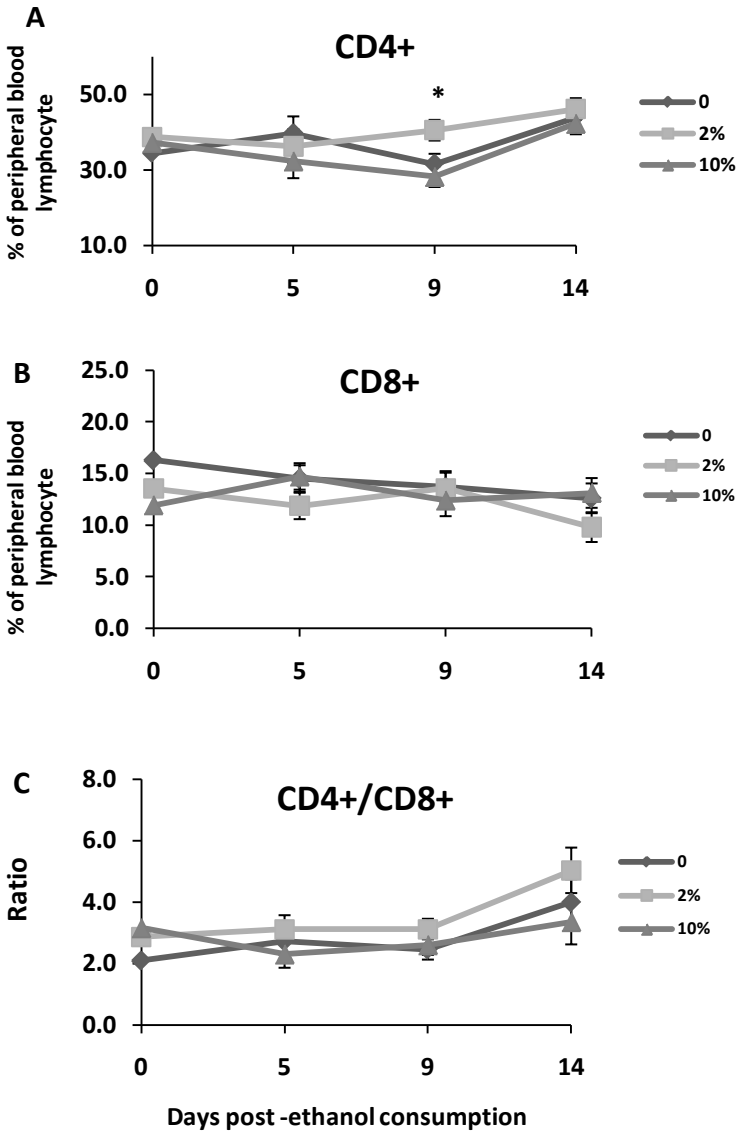

Figure 3.2 Peripheral blood T lymphocyte ratios. **(A)** CD4+ and **(B)** CD8+ cell percentage and **(C)** CD4+/CD8+ ratio were measured before and after oral administration of different doses of ethanol (0, 2, and 10%, v/v). Time points 0, 5, 9, and 14 represent corresponding days post-ethanol consumption, and also stands for 28, 39, 43, and 48 days of age, respectively. The data are expressed as least-squares mean ± SEM. *n* = 2 per group for Day 0, n = 4 per group for Day 5, and n = 6 per group for days 9 and 14. * $p < 0.05$, compares the difference between 2% and 10% group.

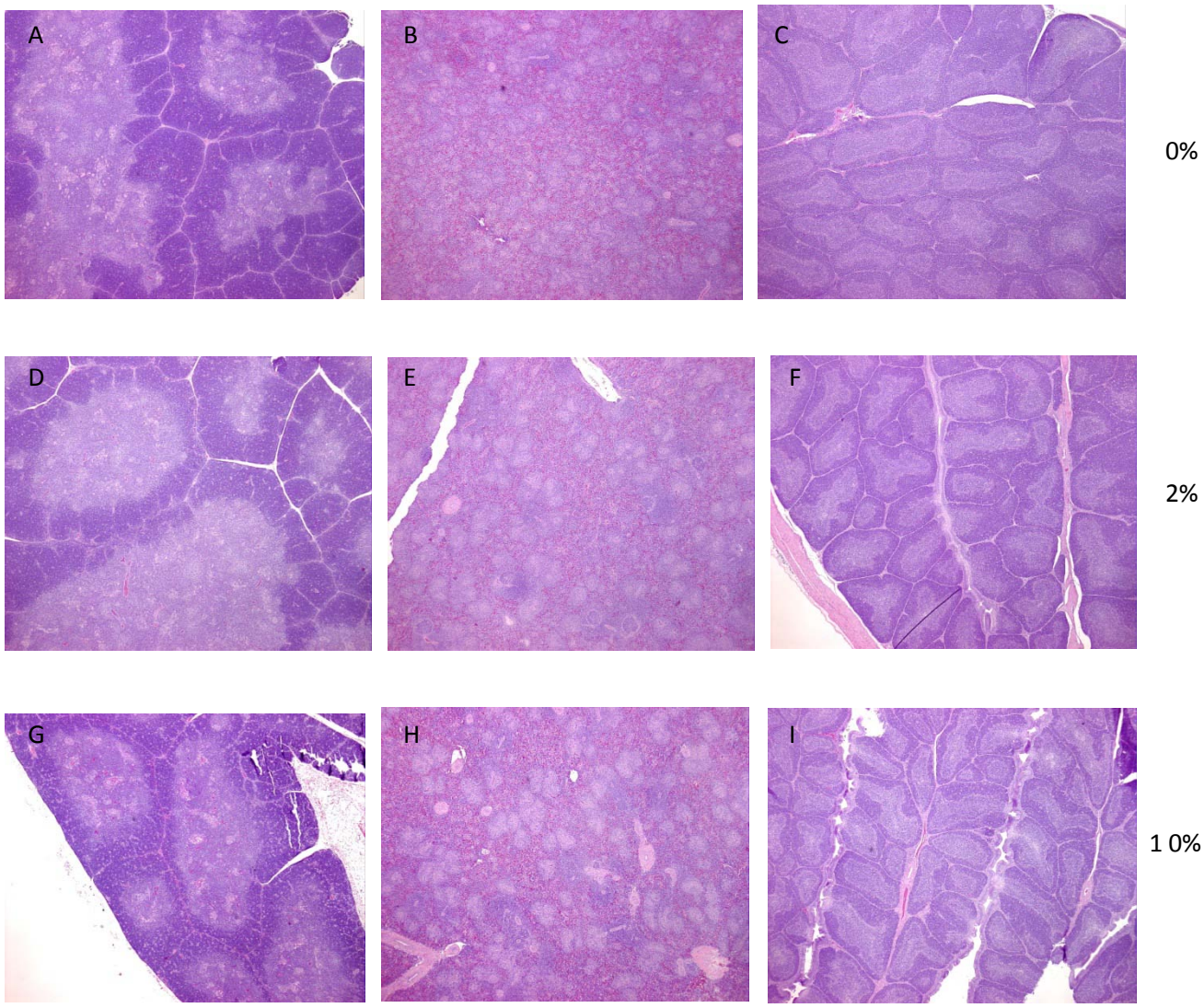

Figure 3.3. Histopathological analysis of major lymphoid organ of chicks under different doses of oral ethanol (0, 2, and 10%, v/v) administration. Hematoxylin and eosin (H&E)-stained longitudinal-sectioned thymus (A, D, G), spleen (B, E, H), and bursa (C, F, I) were collected 21 days post-ethanol consumption, at 55 days of age. Original magnification was 4×.

Chapter IV

Mitochondrial DNA Variation and Damage in Chickens Divergently Selected for High or Low Oxidative Stress

Abstract

Mitochondrial DNA (mtDNA) variations are increasingly linked to complex disorders and the risk of age-related diseases. Without obvious influence on respiratory function, these mtDNA haplotypes are often related to oxidative stress (OS). Although yet inconclusive, these observations indicate a relationship between common mtDNA variation and intracellular reactive oxygen species (ROS) generation. This study was carried out to test whether any mtDNA variations relate to different OS levels in chickens. Firstly, based on an OS marker, 36 chickens out of 96 were selected divergently to do a PCR-based sequencing on a 2734-bp region of mtDNA. Then, another 40 chickens were selected out of 600 for the highest or lowest OS based on three biomarkers, and tested for eight mtDNA SNP loci using a microarray chip. And last, mtDNA damage as determined by 8-hydroxy-2'-deoxy-guanosine (8-OHdG) was measured in 12 chickens by quantitative ELISA. Insignificant differences for environmental factors, like pen, ethanol, and period, the diversely selected chickens showed significant differences in their OS levels, which indicate genetic influence. However, no variation was found in the tested region or SNP loci in those chickens, except for a putative SNP, 6827A>C. The mtDNA damage, however, increased with time ($p < 0.05$), and was significantly higher in chickens with high OS ($p < 0.05$). Based on these observations, whole-mtDNA sequencing may still be needed to verify whether mtDNA variation contributes to different OS levels in chickens, while the increased mtDNA damage with age and plasma oxidative level may induce further mutation in birds over the long-term.

Key words: mtDNA variation, SNP, oxidative stress, 8-OHdG

Introduction

Mitochondria are the major places of energy production in eukaryotes (Chinnery and Schon, 2003). Independent of the nucleus, with its own genome, mtDNA is a small circular dsDNA existing at thousands of copies per cell. Although only 16-17kb in size in most animals, mtDNA is very important because all the 22 tRNAs, 2 rRNAs, and 13 polypeptides it encodes are essential for the assembly of enzyme complexes of mitochondrial respiratory chain or electron transport chain (ETC, Taylor and Turnbull et al., 2005). Mitochondrial DNA exhibits high sequence variability in both coding and non-coding regions, and is frequently used in population and phylogenetic analysis (Pereira, 2000). As a developing area, mtDNA variations were also associated with complex phenotypes or diseases in both human and other animals. For instance, a common mtDNA variation was related to resistance to Marek's disease in chickens (Li et al., 1998). Haplogroup J of mtDNA in humans had higher probability of developing two common mtDNA mutations that lead to a common mitochondrial disease, LHON (Leber's hereditary optic neuropathy, Torroni et al., 1997). Mitochondrial DNA polymorphisms or haplotypes were also related to the risk of Parkinson's disease (Van der Walt et al., 2003), Alzheimer's disease (Van der Walt et al., 2004), sperm motility problem (Ruiz-Pesini et al., 2000), diabetes (Poulton et al., 2002), cardiomyopathy (Khogali et al., 2001), and longevity (Tanaka et al., 1998; Niemi et al., 2003)

Apart from mtDNA mutations, which often causes defect to the respiratory chain and lead to energy deficiency, common mtDNA variations seem not to affect respiratory function in humans (Carelli et al., 2002) and mice cells (Battersby et al., 2001). Other mechanisms may exist to explain complex phenomenon related to mtDNA haplotypes. OS, induced by more reactive oxygen species (ROS) generation or less antioxidant defense, is often observed in

diseases like cancer, diabetes, and neurodegenerative disorders in humans (Valco et al., 2007; Kirches, 2009), suggesting that mtDNA variation may contribute to the risk of these late-onset diseases through OS. Actually the ETC, encoded by both nuclear DNA and mtDNA, is the major generator of intracellular ROS (Finkel and Holbrook, 2000), and changes of electron transportation could increase mitochondrial ROS production (Wallace, 2005). However, direct evidence of the relationship between mtDNA variants and mitochondrial ROS production is limited. Marchbanks et al. (2003) reported a heteroplasmic mtDNA variant 12027T>C causing amino acid changes in the ND4 subunit of NADH-ubiquinone reductase, was significantly associated with increased superoxide production and schizophrenia in humans. Moreno-Loshuertos et al. (2006) reported mice cell lines with identical nuclear background but different mtDNA variants showed increased intracellular hydrogen peroxide. Although it remains inconclusive, if common mtDNA variations could affect mitochondria ROS generation, it will help us better understand many mitochondrial diseases.

Therefore, to test whether mtDNA variation is responsible for the different OS levels in chickens, White Leghorn chickens, the typical egg-type chickens, were divergently selected for oxidative level and tested for possible mtDNA variation. As long as 16775 bp, chicken mtDNA harbors lots of variations. Guan et al. (2007) reported that 113 single-nucleotide polymorphisms (SNPs) have been identified in the chicken, *Gallus gallus*. Here, I scanned a 2734-bp region compact with tRNAs for variations, then tested eight SNP loci throughout the mitochondrial genome. The biomarkers used here included malondialdehyde (MDA), glutathione (GSH) and plasma uric acid (PUA). As one of the predominant forms of oxidative DNA lesion, 8-OHdG has been widely used as a biomarker for DNA damage (Valavanidis et

al., 2009). Hence, mtDNA damage was also measured as 8-hydroxy-2'-deoxyguanosine (8-OHdG) to test whether its concentration is consistent with the OS level and possible variation.

Material and Methods

Animal selection

As described in Chapter III, 600 day-old female commercial White Leghorn chickens (Brickland Hatchery, Blackstone, VA) were divided into six flour pens of 100 each and conventionally raised. From 34 days of age, they were provided water *ad libitum* with 0, 2, or 10% (v/v) ethanol as the only water source for three weeks. Plasma samples were obtained one day before, 7 and 21 days post-ethanol treatments for assaying biomarkers. Blood cells separated from plasma were stored at -20^{o}C for observation of genetic variation.

For the resequencing-based mtDNA variation analysis, plasma MDA levels were measured in 96 birds (n=16/pen) randomly selected from the sample pool on day 7 post-ethanol treatment, according to the procedure described in Chapter III. A total of 36 chickens (n=6/pen, including 3 highest and 3 lowest chickens) from six pens were selected for the mtDNA variation test based on their MDA levels (Table 4.1).

For the microarray-based SNP assay, 40 birds (18 highest, 18 lowest and 4 median ones) were divergently selected for OS level out of 829 records of three period, based on three biomarkers levels (MDA, PUA, and GSH). Details on divergent selection are documented in Chapter V. The LS-means of biomarkers in different classes and the significance level are shown in Table 4.2.

For the mtDNA damage analysis, 12 of the 40 birds selected were used. Six of them are from day 0, the other six from day 21. Also, half are high OS, as the others are low.

Mitochondrial DNA variation analysis

PCR-based Resequencing. Total DNA was isolated from 36 selected birds following the recommended protocol of the DNeasy Tissue/Blood kit (Qiagen, Valencia, CA). A PCR-based resequencing method (Smith et al., 2001) was used to scan a part of a 2734-bp region of the chicken mtDNA genome (GenBank) for variations. As shown in Table 4.3 and Figure 4.1, seven genes are located in this region, including five tRNA genes and parts of two protein-encoding genes. Five overlapping pairs of primers were designed to cover this whole region using Primer 3 software (Rozen and Skaletsky, 2000) as shown in Table 4.4. Following primer synthesis, PCR was performed using a standard protocol, and amplicons were purified and sequenced according to the procedure described in Guan et al. (2007). The PCR conditions were: initial denaturation at 95 ^{o}C for 5 min; 35 cycles of 95 ^{o}C for 1 min , 55 ^{o}C for 45 s, 72 ^{o}C for 2 min; then a final extension at 72 ^{o}C for 5 min. PCR was performed in a total of 25 μl using 50 ng of genomic DNA,0.25 μl FailSafe enzyme mix (Epicentre, WI), 12.5 μl 2 × PREMIX G (supplied by the manufacturer), 25 pmol of each primer, and 11 μl water.

Microarray based- SNP analysis. Total DNA was isolated from 40 selected birds following the recommended protocol of the DNeasy Tissue/Blood kit (Qiagen, Valencia, CA). After adjusting the concentration to 50 ng/μl, DNA samples were sent to DNA LandMarks Inc. (Saint-Jean-Sur-Richelieu, Quebec, Canada) for genome-wide SNP genotyping, among which eight mtDNA SNPs are our focus here (Table 4.5, Figure 4.1). Other details of genotyping will be documented in Chapter V.

Mitochondrial DNA damage assay

mtDNA Isolation. The mtDNA was isolated using a salt precipitation method (Li et al., 2003). It is based on the principle that most of the linear DNA and protein will precipitate in saturated sodium acetate solution, while mtDNA which is circular, will remain in supernatant.

Briefly, 150 µl blood precipitate (about 10^7 blood cells) were first washed in 5 ml cold PBS, then in 5.5 ml Solution I (Tris-HCl, 10 mmol/L; NaCl, 10 mmol/L; $MgCl_2$, 5 mmol/L; pH 7.5), and 5.5 ml Solution II (Tris-HCl, 10 mmol/L; NaCl, 400 mmol/L; EDTA, 2 mmol/L; pH 8.0). Then, blood cells were resuspended in 930 µl Solution II, 70 µl 20 mg/ml Enzyme K, 120 µl 10% SDS, and incubated at 50 ^oC for 4 hours. After incubation, 300 µl saturated sodium acetate was added to tubes, and gentle mixing was applied before centrifuging tubes at 15,000 rpm, 4 ^oC for 15 min. Next, supernatants were transferred to new tubes, and same volume of mixed phenol:chloroform:Isoamyl alcohol 25:24:1 solution was added to purify mtDNA. The supernatant s were transferred to new vials, and 2 volume of ice-cold 100% ethanol was added to precipitate the mtDNA pellet. Then, isolated mtDNA were washed twice with 70% ethanol, dried, and dissolved in 20 µl double-distilled water and stored in -20 ^oC before use. To check the integrity of isolated mtDNA, products were resolved on 1% agarose gels containing 20% SYBR green (75 v for 55 min, 5 µl sample/ ladder were loaded). The purity and concentration of isolated mtDNA was further determined by a NanoDrop spectrophotometer (NanoDrop Technologies, Montchanin, DE).

PCR amplification. To make sure that mtDNA was free of nuclear DNA contamination, a PCR amplification was carried out on nuclear DNA control and mtDNA samples. A 1200 bp segment of the W chromosome, a female-restricted region, was amplified. The reaction mix contained 50 ng DNA template, 2.5 µM of each primer, and 12.5 µl PCR Master Mix (Promega, Madison, WI) in a total volume of 25 µl. Primers for this amplification of nuclear DNA were 5'- CTGTGATAGAGACCGCTGTGC-3' as forward and 5'-CAACGCTGACACTTCCGATGT-3' as reverse primers (Li et al., 2008). PCR was initiated at 94 ^oC for 2 min; followed by 35 circles of 94 ^oC for 35 s, 55 ^oC for 1 min; 72 ^oC for 1 min,

and a final extension at 72 ^{o}C for 10 min. The amplified products were resolved on 2% agorose gels containing 20% SYBR green (75 v for 55 min, 10 µl sample/ ladder were loaded).

8-OHdG ELISA. Mitochondrial DNA damage was detected using a commercially available 8-OHdG ELISA kit (Cosmo Bio Co., Tokyo, Japan). Widely used as a biomarker for oxidatively damaged DNA, 8-OHdG could be formed by hydroxy radical or singlet oxygen action (Mecocci et al., 1993). According to the manufacture's protocol, competitive *in vitro* ELISA were carried out in each mtDNA sample to quantify this oxidative DNA adduct. All mtDNA samples were adjusted to 10ng/µl before use. Standard 8-OHdG (arranged from 0.5 to 200ng/ml) and sample DNA assays were performed in duplicate.

Statistical Analyses

Analyses of variance were performed using the General Linear Model procedure of SAS (version 9.1, 2007, SAS Institute Inc., Cary, NC). The model for MDA level comparison included effects of ethanol, pens, and high or low (H/L) class. Model for comparison of three biomarkers in selected 40 chickens includes ethanol, pen, period, and H/L/M class. Model for 8-OHdG level comparison included period, ethanol, and H/L class. Pair-wise comparisons of least-squares means were performed using the Tukey test. The critical value was set as $p < 0.05$.

Results and Discussion

The mean level of the three H and L chickens selected from each pen based on their MDA level ranged from 0.289 – 1.096 μM/ml (Table 4.1). There was no significant difference among ethanol treatment groups, but significant differences among pens ($p = 0.002$) and between H and L classes ($p < 0.0001$).

Using blood samples from these selected 36 birds, five pairs of primers amplified expected products (615-642bp, as shown in Table 4.4), and a total of 360 sequences were sequenced and analyzed. As shown in Figure 4.2, a putative SNP was found in three blood samples at 6827bp, which is located in gene cytochrome oxidase subunit I. However, it seems that it was not restricted to the H or L class of OS level. Except for this putative SNP, no variation was identified in this 2734-bp region in the 36 tested chickens.

Least-square means for MDA, GSH and PUA concentrations differed significantly among three classes of chickens divergently selected for OS level (Table 4.2). In addition, the three biomarkers were significantly correlated within birds ($p < 0.01$). At the same time, the environmental factors including ethanol, pen, and period effects were not significant different among those selected chickens. However, the later scans for possible variation in eight SNP loci of mtDNA did not show any differences among chickens.

Although the two mtDNA variation assays did not show genetic differences among chickens selected for different OS level, the mtDNA damage analysis showed interesting results. As shown in Fig. 4.3, mtDNA was successfully isolated from blood samples without nuclear DNA contamination. The 8-OHdG level, which indicated DNA oxidative damage, showed significant differences in mtDNA between chickens of H and L classes ($p = 0.029$,

Fig. 4.4A). Similarly, there was also a significant difference in mtDNA damage between blood samples of day 0 and day 21 (p = 0.018, Fig. 4.4B). However, being limited by the funding, this assay was only carried out in 12 birds, which means further verification may be needed on these results.

In general, chickens showed a great difference in their OS level independent of environmental factors, like pen or ethanol treatment effect in our study, which indicated the role of genetic effects. Similarly, Iqbal et al. (2004, 2005) also reported that the OS level was significantly higher in a broiler line which had low feed efficiency. Then, the tested part of the mitochondrial genes, including a 2734-bp region and eight SNP (Figure 4.1), showed no variation. This similarity of mtDNA sequence according to our test confirmed the identical origin of this batch of chickens. However, the lack of variation in the tested region and SNP loci dose not excluded the possibility of genetic various in the remaining parts of the mitochondrial genome. The study of mitochondrial genome in humans and model animals showed big difference in mtGenome among individuals even within species (Wallace, 2005). For example, the D-loop or control region, which is around 1.1-kb in length and important in mtDNA replication and transcription, is frequently used in population-genetic studies because it has several highly variable segments (Pereira, 2000). Other parts of mtDNA, mostly coding region, could also harbor common variants or adaptive mutations, though less frequently, are used for evolutionary study (Chinnery, 2006). Therefore, it seems remains a good chance to find variations in other part of mtDNA in those chickens.

Further analysis is needed to test whether there is an association between mtDNA variation and oxidative level in chickens. Even a single nucleotide change could influence mitochondrial ROS production. Moreno-Loshuertos et al. (2006) found that a common SNP

on the $tRNA^{Arg}$ (arginine) gene of mouse mtDNA is responsible for the increased ROS production in cell lines within the same nuclear DNA background. Although the precise molecular mechanism by which this single nucleotide polymorphism leads to the differential performance of the mitochondrial ETC and ROS production is unclear, the variant probably affects the rate of mitochondrial protein synthesis and the translation fidelity. In this case, Complex I, which is an important site of ROS production, is most likely to be affected by this variant as it contains more mtDNA-encoded polypeptides (7 out of 13). At the same time, the mitochondrial ETC not only is encoded by the 37 mtDNA genes, but also by nuclear DNA which encodes 1500 genes for mitochondrial biogenesis (Wallace, 2005). In addition, nuclear DNA also codes for the complex antioxidant system, including enzymatic and non-enzymatic components, which are the other determinant of the intracellular OS level (Scandalios, 2002). Therefore, nuclear DNA could be responsible for the genetic influence on OS level of chickens as well.

No matter whether the extremely distributed OS level in chickens is affected by mtDNA or nuclear DNA, the significantly higher mtDNA damage in the high OS group will increase the risk of new mtDNA mutation in those chickens. In fact, lack of active repair and protection from histones, mtDNA is highly susceptible to oxidative damage (Cadenas and Davies, 2000); Yakes et al. (1997) reported that mtDNA damage is more extensive and persists longer than nuclear DNA damage in human cells following OS. Krishnan et al. (2008) indicated that mutations like deletion are most likely occurs during the repair of damaged mtDNA. Moreover, some of these mutations could induce more ROS generation, and worsen OS (Jou at al., 2007; Wu et al., 2010). This vicious cycle will sooner or later defect the ETC, and reduce energy production, causing energy deficiency, and show disorders first in energy-

demanding tissues like nerves, muscles, and pancreas. On the other hand, the greatly increased mtDNA damage in the later period here is consistent with the free radical theory of aging, which proposes that free radical-induced oxidative damage will accumulate with age, and lead to increased mtDNA mutation, cell disorder and impact upon longevity (Finkel and Holbrook, 2000). Thus, long-term monitoring of these chickens may reveal new mutations in mtDNA. Since our conparism of mtDNA oxidative damage among brids is based on limited sample size, more measurements are needed in the future to test our results and hypothesis.

Table 4.1 Means and standard deviations of melondialdehyde level of chickens chosen from each group for high or low oxidative stress.

Pen[a]	Ethanol treatment	Classification[b]	Mean ± SD (μM/ml)
1	10%	L	0.289±0.012
		H	0.780±0.175
2	0%	L	0.298 ± 0.015
		H	0.667±0.015
3	2%	L	0.307±0.085
		H	0.731±0.051
4	0%	L	0.386±0.015
		H	0.877±0.055
5	10%	L	0.412±0.054
		H	1.096±0.303
6	2%	L	0.371±0.086
		H	0.877±0.015

[a] 16 birds/pen were randomly chosen for the MDA assay.

[b] L and H represent chickens divergently selected for low(L) and high(H) OS based on MDA level. N=3 per class.

Table 4.2 Summary of Least-square means of three biomarkers for chickens selected for high or low oxidative stress.

Category[a]	N[b]	LSmeans ± SEM (μM/ml)					
		MDA[c]		GSH		PUA	
H	18	1.00±0.07		1211±38		281.5±18	
L	18	0.33±0.07	p <0.0001	1876±38	p<0.0001	469.9±18	p <0.0001
M	4	0.58±0.16		1790±80		226.5±38	

[a] The birds were selected and categorized into high (H), low (L), and medium (M), based on their ranking of three biomarkers for oxidative stress.

[b] number of birds selected for this category

[c] Malondialdehyde (MDA) indicates lipid peroxidation, and total glutathione (GSH), and plasma uric acid (PUA) were measured as antioxidants. Significance levels shows comparison of LSmeans among categories.

Table 4.3 Mitochondrial genes located in region targeted for PCR-based resequencing [a].

Genes	Begin [b](bp)	End (bp)	Gene length / resequenced length (bp/bp)
NADH dehydrogenase subunit II	5241	6281	1040/996
tRNA-Trp	6280	6355	75/75
tRNA-Ala	6362	6430	68/68
tRNA-Asn	6434	6506	72/72
tRNA-Cys	6508	6573	65/65
tRNA-Tyr	6573	6643	70/70
cytochrome oxidase subunit I [b]	6645	8190	1545/1375

[a] Target region resequenced is 2734 bp in length.

[b] The location is based on the chicken mitochondrial genome, *Gallus gallus* (GenBank: NC_001323).

Table 4.4 Primers and sizes of amplicons for PCR-based resequencing for chicken mtDNA variation analysis.

Primer ID	Primers[a]	Length (bp)[b]	Tm (^{o}C)
Mt1	F(5285): 5'-ggg aac cag cat cac aat ct-3'	623	56
	R(5907): 5'-tga ttt ggg cta ggg ata gg-3'		
Mt2	F(5831): 5'-ccc aca act cac tat tct cac c-3'	622	56
	R(6452): 5'-tcg aag ccc atc tgt cta gg-3'		
Mt3	F(6355): 5'-gcc caa cta aga cca aca gg-3'	615	56
	R(6969): 5'-agg gag gga gga gtc aga ag-3'		
Mt4	F(6862): 5'-cat cat gat cgg tgg ctt c-3'	624	56
	R(7485): 5'-ccg att gac agt atg gct ca-3'		
Mt5	F(7377): 5'-atc ctc atc ctc cca ggt tt-3'	642	56
	R(8018): 5'-gga gcc gat tga gga tag tg-3'		

[a] Primer binding-sites in the chicken mitochondrial genome (GenBank: NC_001323) are presented in parentheses, F and R are forward and reverse primer, respectively.

[b]Length in base pairs (bp) of the expected amplicon based on primer-binding sites.

Table 4.5 Eight SNPs tested in microarray-based mtDNA variation assay.

SNP name[a]	Related gene	Begin[b] (bp)	End (bp)	lengths (bp)
10080_COIII	cytochrome oxidase subunit III	9923	10706	783
10668_COIII	cytochrome oxidase subunit III	9923	10706	783
12187_ND4	NADH dehydrogenase subunit IV	11486	12863	1377
15466_CYTB_MT	cytochrome b	14893	16035	1142
16262_ND6	NADH dehydrogenase subunit VI	16184	16705	521
16370_ND6	NADH dehydrogenase subunit VI	16184	16705	521
5831_ND2_MT	NADH dehydrogenase subunit II	5241	6281	1040
8472_COXII_MT	cytochrome oxidase subunit II	8331	9014	683

[a]These eight SNPs were newly discovered by the USDA genome-wide marker- assisted selection consortium, and the exact position is unpublished.

[b] Gene location is based on the chicken mitochondrial genome (GenBank: NC_001323)

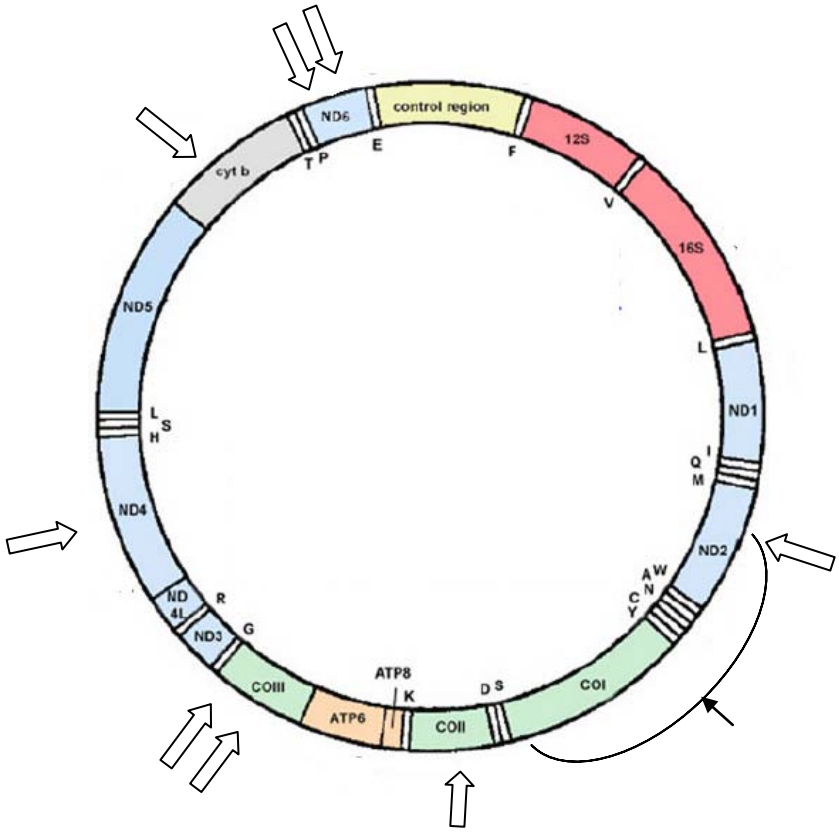

Figure 4.1 Positions of resequenced region and SNPs tested in the mitochondrial genome. Open arrows mark the rough locations of eight SNPs tested. Closed arrow marks the rough location of the 2734-bp resequenced segment. The chicken (*Gallus gallus*) mtGenome is derived from a published figure (Pereira and Baker, 2004).

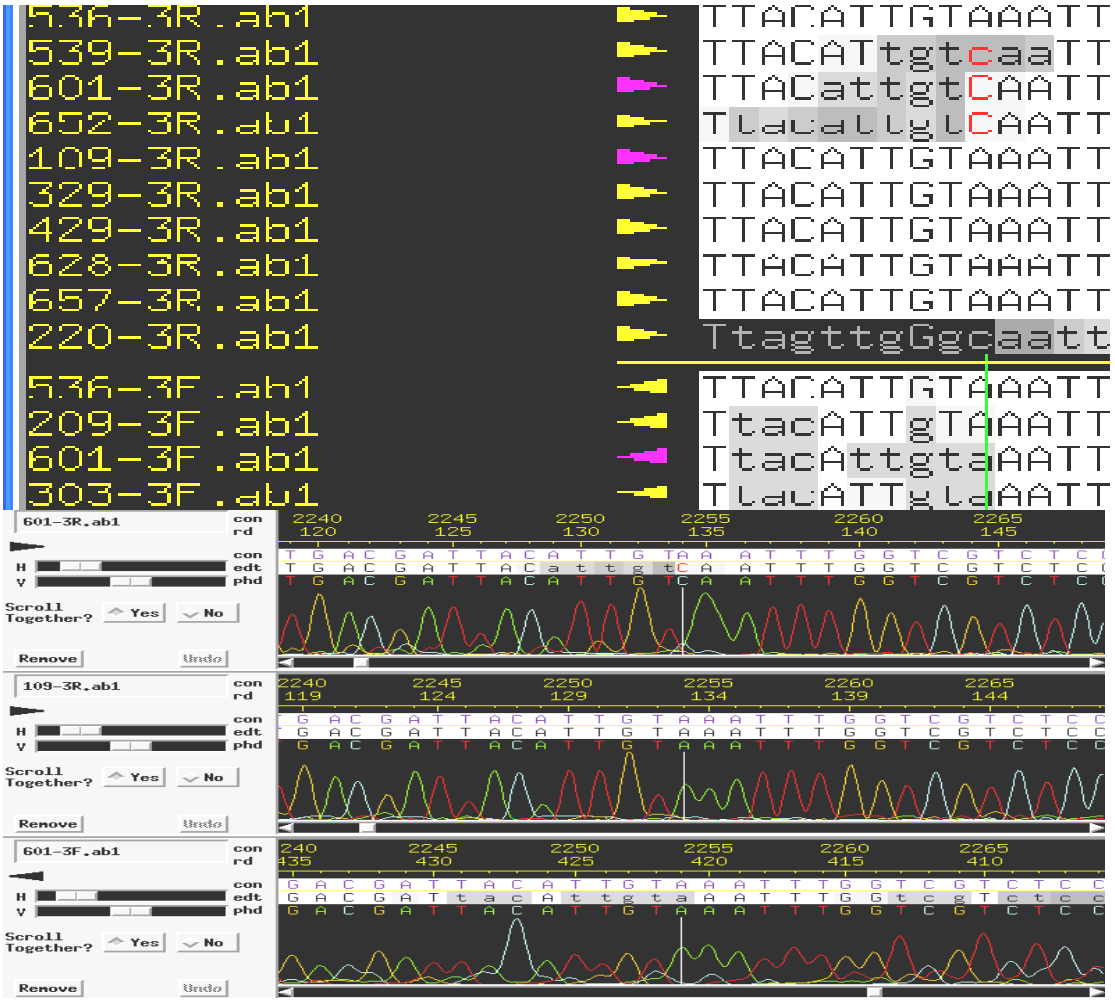

Figure 4.2 Alignment showing putative SNP – position corresponds to 6827bp in 10 reverse and 4 forward primer sequences according to the chicken mtDNA (NC_001323)

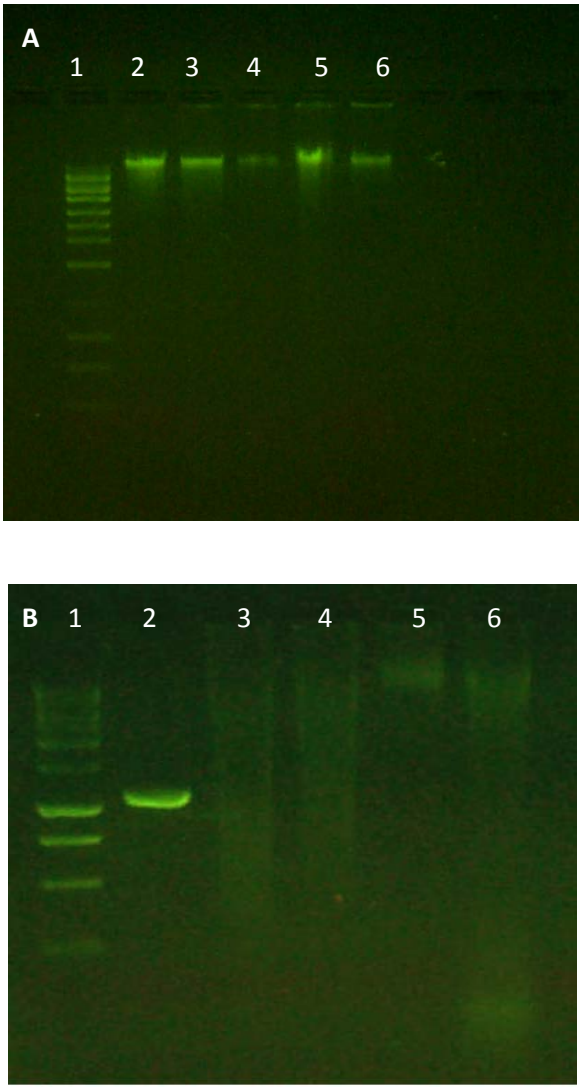

Figure 4.3 Examples of gel pictures for mtDNA isolation. (A) mtDNA isolation: lane 1 represent 24-kb Ladder, lane 2 is nuclear DNA control, 3-6 are separate isolations of mtDNA from four birds. (B) PCR amplification of nuclear DNA region: lane 1 is 1-kb ladder, lane 2 is PCR products of nuclear DNA, 3-6 are PCR products of mtDNA from four birds.

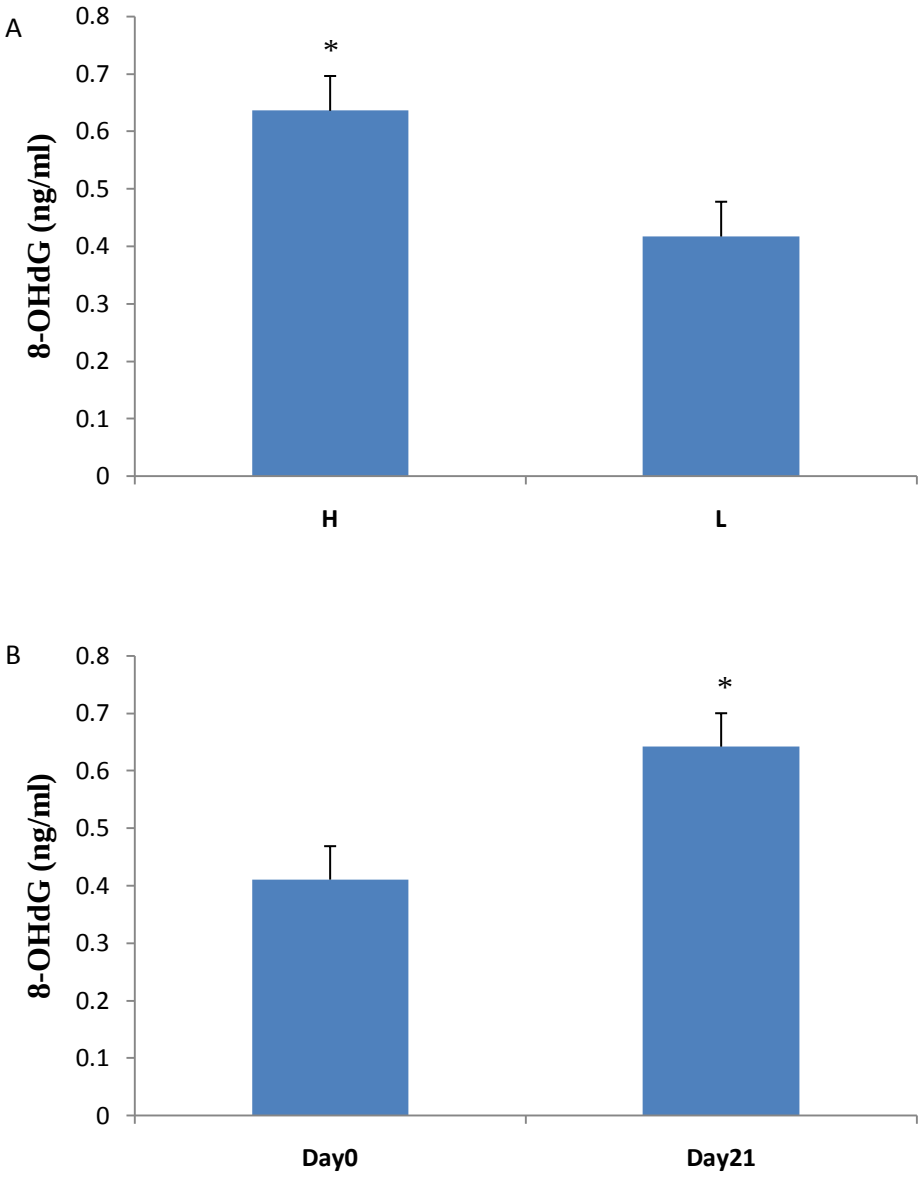

Figure 4.4 8-OHdG accumulations in mtDNA measured by enzyme-linked immunosorbent assay in blood cells. (A) Chickens selected for high (H) and low (L) oxidative stress; (B) Chickens sampled one day before treatment (Day 0) and 21 days after experiment (Day 21). Results are presented as LSmeans ± SEM. n = 6 in each case. *$p < 0.05$.

Chapter V

Genome-wide SNP Analysis for Chickens Divergently Selected for High or Low Oxidative Stress

Abstract

Oxidative stress (OS) has long been associated with aging and age-related diseases in humans. It is also increasingly implicated in economic traits, like meat quality and feed efficiency, in farm animals including chickens. Related genetic studies indicated OS is a complex quantitative trait, which needs genome-wide interpretation. Thus, I used the latest high-density chicken 60k single nucleotide polymorphism (SNP) microarray for a genome-wide association study of OS. A total of 40 chickens were selected for high (H), median, or low (L) OS level based on three biomarkers: malondialdehyde (MDA), glutathione (GSH), and plasma uric acid (PUA). For genotyping, DNA samples of these chickens were assessed for genome-wide SNPs using a microarray. A Fisher's exact test was carried out comparing different genotype frequencies between chickens selected for extreme phenotypes, 18 H and 18 L, of each SNP. Quality assessment showed that all 40 samples had an average call rate above 98%. The association study identified 13 SNP loci out of 56,483 significantly associated with OS ($p \leq 0.001$), and another 144 SNPs were showed significance levels below 0.01. The most significant SNPs predominantly located on chromosomes 1, 4, 6, and 10, and some of them were linked to genes likely to influence redox balance. Identification of new candidate loci and related regions may contribute to our understanding of genetic causes of OS. This study also demonstrated the power of genome-wide SNP association in discovering genetic regulation of complex traits as OS.

Key words: oxidative stress, SNP array, genome-wide association, chicken

Introduction

In the 1950s, Denham Harman first proposed the "free radical theory of aging", in which reactive oxygen species (ROS) - induced damage is a cause of aging (Finkel and Holbrook, 2000). Since then, oxidative stress (OS), defined as the unbalanced redox state that favors ROS production over antioxidant defense, has been widely associated with human diseases like cancer, diabetes, cardiovascular and neurodegenerative disorders (Valco et al., 2007), and with the aging process (Paradies et al., 2010). Considering possible damage to cell components like proteins, DNA, and lipids, and its regulatory function in cell signaling, OS has also been increasingly emphasized in farm animals. In chickens, OS was suggested to impact various economic traits, including meat quality (Gao et al., 2010), feed efficiency (Iqbal et al., 2004; 2005), semen quality (Eid et al., 2006), and heart failure in fast-growing broilers (Nain et al., 2008). In spite of its wide deleterious influence, genetic regulation of OS remains unclear.

As one aspect of OS, ROS are generated from multiple sources in cells. Mitochondria are the major generator of intracellular ROS in aerobic animals, for superoxide ($O_2^{\bullet -}$), the precursor of most ROS, is constantly produced from the respiratory chain as a by-product of the oxidative respiration (Kowaltowski et al., 2009). In addition, other components within mitochondria, including the monoamine oxidase (MAO) and adaptor protein $p66^{shc}$, also generate ROS by oxidative deamination and direct oxidization of cytochrome c, respectively (Di Lisa et al., 2009). Meanwhile, cytosolic enzyme systems also contribute to ROS production. For example, acyl-CoA oxidase initiates fatty acid oxidation in peroxisomes and produces hydrogen peroxide (H_2O_2, Hernandez-Garcia et al., 2010), cytochrome P-450 can directly reduce O_2 to $O_2^{\cdot -}$ in microsomes (Beckman and Ames, 1998), and NADPH oxidase produces large amounts

of $O_2^{\cdot -}$ in the plasma membrane of activated phagocytes (Halliwell, 2006). It is unclear whether genetic regulation is involved in all these ways of ROS generation, but studies here reported that mitochondrial ROS generation could be reduced or increased in animals that have mutations in mitochondrial-related genes, like *clk-1* (Liu et al., 2005), *isp-1*(Crofts et al., 2004), *mev-1* (Ishii et al., 1998), *gas-1* (Hartman et al., 2001), and $p66^{shc}$ (Giorgio et al., 2005).

On the other hand, an even more complex antioxidant network exists in cells to cope with the constantly generated ROS, and this defense system is composed of both enzymatic and non-enzymatic antioxidant branches. The three major enzymatic antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase which remove $O_2^{\bullet -}$ or H_2O_2 both inside and outside mitochondria (Valko et al., 2007). Besides these, many other enzymes also have antioxidant properties, like peroxiredoxins and the recently discovered sulfiredoxin (Muller et al., 2006). Non-enzymatic antioxidant which can be water- or lipid-soluble, include vitamins C and E, uric acid, glutathione, and others (Davies, 2000). Working together, these antioxidants prevent, intercept and repair oxidative damage (Sies, 1993). Genetic studies previously showed genes related to this antioxidant defense system could influence the intracellular oxidative level, although it appears some are more essential than others. For instance, studies conducted on knockout mice showed GPx (Ho et al., 1997) and catalase (Ho et al., 2004) were not essential, while the genes *Sod1* (Muller et al., 2006), *Sod2* (Melov et al., 1999) and *Prxd* (Neumann et al., 2003) that encode CuZnSOD, MnSOD and peroxiredoxin, respectively, have been implicated as having a great role in free radical scavenge.

Multiple intracellular sources of ROS generation and the complex antioxidant system imply a sophisticated genetic influence on OS. However, genome-wide studies of

genetic influence on oxidative status are still limited. Therefore, I applied the latest 60k chicken SNP array to do a genome-wide association study (GWAS) of OS level in chickens. As a promising model for OS, chickens have many favorable traits. First, birds live up to three times longer than mammals of the same size, although they have higher metabolic rate, body temperature, and blood glucose levels, all of which should lead to more tissue damage and disorders (Costantini, 2008). This paradoxical phenomenon indicates that chickens resist OS by either a lower ROS production rate, or a stronger antioxidant defense system. Second, similar to humans, chickens exhibit a high level of plasma uric acid (PUA) with antioxidant properties, which is restricted to avian and primate species (Simoyi et al., 2003). The whole chicken genome is among the first to be uncovered and a large number of SNPs have been discovered. For phenotyping, the biomarkers malondialdehyde (MDA), glutathione (GSH), and PUA, representing either oxidative lipid damage or antioxidant level, were assessed, considering that there is no simple universal method by which the OS could be assessed.

Material and Methods

Animal and Blood Collection

As described in Chapter III, 600 day-old female commercial White Leghorn chickens (Brickland Hatchery, Blackstone, VA) were divided into six flour pens of 100 each and conventionally raised. From 34 days of age, they were provided water *ad libitum* with 0, 2, or 10% (v/v) ethanol as the only water source for three weeks. Plasma samples were obtained one day before, 7 and 21 days post-ethanol treatments for assaying biomarkers. Blood cells separated from plasma on the above time-points were stored at -20^{o}C before the scanning for genetic variation.

MDA, GSH, PUA level

For the microarray-based SNP assay, 40 birds (18 highest, 18 lowest and 4 median ones) in total were divergently selected for OS level, indicated by three biomarker levels (MDA, PUA, and GSH). This selection was based on rankings for these three biomarkers from each pen at each time-point. For day 0, 38 - 43 birds with three biomarker records were used to carry out ranking for each of the six pens; and similarly, 58 - 70 birds with full records on day 7 were used for ranking for six pens separately; and for day 21, records of 30 - 37 birds from each pen were used to conduct rankings. In total, 18 rankings were made from 829 full records. The highest (H) one and lowest (L) one from each ranking, and the median one from four rankings of the first time-point were used to isolate total DNA.

Total DNA isolation and SNP analysis

Total DNA was extracted from peripheral blood of 40 birds according to the procedures described in Chapter IV. The purity and concentration of isolated DNA was

determined by a NanoDrop spectrophotometer (NanDrop Technologies, Montchanin, DE). Genotyping of these DNA samples was performed by DNA Landmarks, Inc. (Saint-Jean-Sur-Richelieu, Quebec, Canada), according to the manufacturer's protocols. The chicken 60k SNPs BeadArray used in this study was fabricated by Illumina Corp. (San Diego, CA), with iSelect Infinium technology (http://www.illumina.com). Probes laid down on this array are oligos, selected to be equally distributed throughout the chicken genome. The quality of these typing assays was evaluated by a number of parameters including call rate.

Statistical Analyses

Analyses of variance were performed using the General Linear Model (GLM) procedure of SAS (version 9.1, 2007, SAS Institute Inc., Cary, NC). The model for biomarker comparison in selected chickens included ethanol, pen, period, and OS ranking. Correlations among biomarker were analyzed using the CORR procedure. Using GLM, the model for the comparison of each biomarker among genotypes excluded environmental effect since they proved insignificant. The statistical significance of the genotype frequency difference for each SNP between high- or low-OS chickens was evaluated by Fisher's exact test. Pair-wise linkage disequilibrium (LD) analysis between SNPs was done using GENEPOP (http://wbiomed.curtin.edu.au/genepop; Raymond and Rousset 1995).

Results

In total, 40 chickens-with 18 high (H), 18 low (L) and 4 medium OS levels- were selected based on three biomarkers. Environmental effects including ethanol, pen, and period were not significant for those selected chickens. Their least-square means for MDA, GSH and PUA differed significantly among H, L and median classes ($p < 0.0001$, Table 4.2). In addition, the three biomarkers were significantly correlated in individual birds ($p < 0.01$).

The results of genotyping of these 40 chickens were reported by DNA Landmarks Inc. (Saint-Jean-Sur-Richelieu, Quebec, Canada). Quality assessment showed that all 40 samples had an average call rate above 98%. The final dataset consisted of 40 chickens with genotype data for 56,483 SNPs in whole genome. Results of the Fisher's exact test for each SNP among 36 birds (18H, 18L) showed that genotype frequencies were significantly different between H and L birds at 13 tested loci ($p \leq 0.001$, Table 5.1, Figure 5.1), while those of another 144 SNPs distributed over the genome were also highly associated ($p < 0.01$, Table 5.4).

As shown in Table 5.1, comparison of genotype frequency unveiled 13 SNPs associated with H or L chickens most significantly, with *p*-values varying from 0.0001 to 0.001. It appeared that alleles like A at rs13818962, T at rs13938103, T at rs14010706, etc. were significantly associated with high OS, while other alleles exist more frequently in L chickens. Then, Table 5.2 showed the comparison of each biomarker among genotypes at the 13 SNPs. It appeared that the three biomarkers had their most significant associations with different SNPs. Malondialdehyde level, indicating lipid peroxidation, differed the most among genotypes at SNP rs13818962 and GGaluGA071419 ($p =$

0.0085). The total GSH level differed mostly at SNP rs14590417 with a p-value < 0.0001, and at four other SNPs (p = 0.0002). The PUA level, meanwhile, showed the most significant difference among genotypes of SNP rs14010706 (p < 0.0001), rs13818962, rs13938103, and GGaluGA071419 (p = 0.0014).

Interestingly, the 13 SNPs with the most significant levels, were located only on four chromosomes: 1, 4, 6, 10, and SNPs were quite near each other on the same chromosomes (Table 5.3, Figure 5.2). On chromosome 1, three SNPs were located within 0.1 Mb; the four SNPs on chromosome 4 also stood around 0.1 Mb apart. Two of the five SNPs on chromosome 10 were located within 0.3 Mb, while the other three, which also stood within 0.1 Mb, were 7 Mb away. Pair-wise LD analysis of these most relevant SNPS showed that high LD existed among SNPs located withine 0.3 Mb on same chromosomes ($P < 10^{-7}$); SNP rs15573469 on chromosome 10 is significantly associated with two SNPs around 7 Mb away (p < 0.01); and significant linkage was also observed among SNPs on different chromosomes, like SNP rs14590417 is linked with all four SNPs on chromosome 4, and two SNPs on chromosome 10 (p < 0.01).

Examination of the chromosomal location of each SNP showed that only SNP rs15573469 was located within gene *wdr72*. When scanning in a 100kb flanking area for adjacent genes, other SNPs stood apart from one to five genes with a distance from 7.2kb to 94kb, except for three SNPs on chromosome 4 for which no genes were found in this area. Together, 11 genes and 4 hypothetical proteins were found near the 13 SNPs.

Analysis of the location of other 144 SNPs with significance levels lower than 0.01 was shown in Table 5.4. Those SNPs were scattered in almost every chromosome published. However, chromosome 1 had the most: 25 significant SNPs; chromosomes 2,

3, 4, 5, 6, and 8 had from 11-20 SNPs; and the other 15 chromosomes have fewer than 5 SNPs at the 0.01 significance levels. The next scan for genes linked to these SNPs showed two synonymous SNPs in coding regions of gene *arnt2* and *tmem8a*, two SNPs in 3'UTR, and another 66 SNPs in introns of 54 genes, and the rest SNPs were located in intergenic area.

Discussion

Consistent with previous studies, 13 most significant SNPs and 144 significant SNPs found here implicated a complex genetic cause of intracellular oxidative status. Differed association levels between SNPs and different biomarkers supported genetic impact for both: ROS production and antioxidant defense. Meanwhile, significant SNPs were relatively compressed on several chromosomes, and tightly associated with each other, suggesting the existence of QTL in related regions.

A functional analysis was carried out on the 11 candidate genes adjacent to 13 SNPs. Although none of them were known as familiar OS genes, the putative functions of six genes showed potential influence on OS. First, gene *stim2* encodes protein stromal interaction molecule 2, an important regulator of Ca2+ concentration in the cytosol (Berna-Erro et al., 2009). Interestingly, STIM2 was not only linked to various cellular disorders but also associated with mitochondrial function for its feedback regulation of Ca2+ concentration (Brandman et al., 2007), and the overload of mitochondrial matrix Ca2+ was known to lead to enhanced ROS generation (Brookes et al., 2004). Then, gene *slc18a2* has been implicated in protection against human neuropsychiatric diseases, like Parkinson's disease (Glatt et al., 2006) and schizophrenia (Talkowski et al., 2008), and neurodegenerative disease in mice (Caudle et al., 2007). Coincidently, these diseases have long been associated with OS (Valco et al., 2007). Similarly, variants of gene *mctp2* have also been associated with schizophrenia (Djurovic et al., 2009). These associations with OS-related diseases may suggest their role in maintaining redox balance. Then, although genes *kiaa1598* and *kiaa1370* are not known for their physiological functions, another gene of the group, *kiaa0415,* was reported in a recent study for an essential function of in DNA double-strand break repair (Slabicki et al., 2010). The ability to

repair damage and maintain genome integrity is an antioxidant defense strategy. Finally, gene *mapk6*, which encodes the enzyme mitogen-activated protein kinase 6, has been reported to respond to paraquat-induced OS (Yuasa et al., 2001) and also associated in pathogenesis of cancer, another disease related to OS (Hoeflich et al., 2006).

Other genes detected in the 100kb flanking region are associated with development and normal physiological function, which appear less associated with OS. The gene *shox* is known as the genetic cause of human idiopathic short stature (Chen et al., 2009), and its expression was shown to affect the length of skeletal elements (Tiecke et al., 2006). The *vax1* gene, conserved in vertebrates, was shown to be essential for development of the optic nerve (Hallonet et al., 1999). In a recent study, it was reported to work with *vax2* and to control neuroepithelial segregation (Mui et al., 2005). The gene *wdr72*, with a significant SNP located in the last intron, encodes a 927-aa protein, which is poorly understand, but a recent study reported that mutations of human *wdr72* is related to unhealthy dental enamel development (Walid et al., 2009). *kcnk18*, which codes for member 18 of the KCNK family, is related to function of nerves and muscles for its role in potassium-selective channels (Goldstein et al., 2001). *Pdzd8* encodes a 1154-aa protein, and belongs to a large protein group with a PDZ domain that functions in cytoskeletal organization, a recent study found that protein PDZD8 promotes retroviral infection in humans by initiation of reverse transcription (Henning et al., 2010).

In spite of these important genes found nearby, the SNPs associated with them are not necessarily linked to their function. On the other hand, SNPs could also regulate genes as far as 250kb away (Chen et al., 2009). Although it is not easy to link the SNPs to the genes whose expression they affect, SNPs are increasingly used as the new generation of DNA markers for many benefits. For example, they are widely distributed within

genomes, and they are relatively easy to evaluate and interpret. In fact, genome-wide association analysis using SNPs has already helped us in understanding many complex human diseases, like type 2 diabetes (Sladek et al., 2007) and multiple sclerosis (Godde et al., 2004). As a developing area in animal genomic studies, high-density SNP microarrays have been constructed for chicken, dog, cattle, horse, pig and sheep (Abasht and Lamont, 2007; Fan et al., 2010), and they are anticipated to play an important role in genome-wide study for economically important traits.

To our knowledge, this is the first genome-wide SNP association study for OS in all species. Although several genome-wide studies have been reported, they were based on expression changes after OS induction in model organisms like *E. Coli*, yeast, plants (Scandalios, 2002), fly (Zou et al., 2000), mouse (Lee at al., 2000) and rat (Zhang et al., 2002) and human cells (Crujeiras et al., 2008). The up- or down-regulated genes in these studies surely reflect antioxidant defenses to some extent, but they also included genes in signaling pathways triggered after OS. In addition, expression levels varied a lot depending on the specific inducer of OS. Conversely, SNPs, with proper coverage and density over the genome, could reflect the genetic makeup and to the greatest extent the genetic cause of OS, and is less changeable. Thus, data from this study will contribute to our knowledge on the genetic background of OS.

However, the power of our results is limited because of the small sample size. As discussed by Hirschhorn and Daly (2005), large sample sizes in GWAS are very important for detecting varants that each has modest effect on a complex trait and for increasing statistical power. On one hand, small sample size risk missing variants which contribute to a target trait with more modest effects. On the other hand, and more importantly, false-positive results will be increased by reduced criteria in declaring

significant association. For example, the *p*-value threshold of 0.001 could bring 56 false-positive associations in a 56,000-SNP study by chance alone, while a few real causal alleles may be among them. Therefore, further verification are needed in future to distinguish real relevant SNPs from false-positives in studies like ours that applied a small sample size in GWAS under a financial pressure.

Table 5.1 Genotype frequency comparisons between high and low chickens on single nucleotide polymorphism (SNPs) at *p*-value ≤ 0.001.

SNP ID[a]	Context[b]	Alleles: A/B[c]	Genotype Frequency[d] /*n*						/ *p*-value[e]
			AA		AB		BB		
			H	L	H	L	H	L	
rs13818962	CTACCATCCTAGAACCCTTCCCAGTC[A/C]AAGGTGGGTTTAATTAAAATTCAGT	A/C	-	-	0.44/16	0.14/5	0.06/2	0.36/13	0.0005
rs13938103	CTTATGTCAACAAAGAACAAATTGTT[C/T]ATAGCTTCTGGTATCTGAAAGGCAC	T/C	0.31/11	0.03/1	0.19/7	0.28/10	-	0.19/7	0.0001
rs14010706	GGATGTTAACAATATTTTGCAGTCAA[C/T]GTGCTTCAGGTAAGTTAGGAATGAG	T/C	0.47/17	0.14/5	0.03/1	0.28/10	-	0.80/3	0.0002
rs14489496	AGTGCAACGTCTTACACATGAACTTA[C/T]TGAAACTTACTTGGTTCACGTGGGC	T/C	0.25/9	0.5/18	0.25/9	-	-	-	0.0010
rs14489711	AAGAAAAGGGATTCAGGATAACTGTT[C/T]GATTTTTCAATGGTCACATCCTCCA	T/C	-	-	0.25/9	-	0.25/9	0.5/18	0.0010
rs14590417	CTCCACAGACAGAGAATAGGGTAACC[A/G]AAGTACAAATATTAACAGTTCTTTC	A/G	0.25/9	0.5/18	0.25/9	-	-	-	0.0010
rs15573469	AAAGTGGAATTCTCACTCCTCTGATG[C/T]AGATTTTATCATGGACTTTAAATGC	T/C	0.03/1	0.23/7	0.52/16	0.13/4	-	0.1/3	0.0005
rs15573695	CACTGACACTTTCCTTGTGGACAGAC[A/G]GACAGCAGTGCTCTGTGCCGACACC	A/G	-	0.13/4	0.47/14	0.13/4	0.03/1	0.23/7	0.0005
rs16434507	ATCTAGTTACTTAATACAAAGTTCAC[A/G]GCATTTGAAAAGAGACTGAGAGCTT	A/G	-	-	0.25/9	-	0.25/9	0.5/18	0.0010
rs16434646	ATGTGCATACTTATAAAGTATATGTC[A/G]CAATGCTAAGTAAAACAATATTAAA	A/G	0.25/9	0.5/18	0.25/9	-	-	-	0.0010
GGaluGA043801	ATTTCCCTAAAATCAGCTGAAGCAA[A/G]CAAATAGAAGTTTAAGACGTTGATC	A/G	-	0.22/8	0.22/8	0.25/9	0.28/10	0.03/1	0.0002
GGaluGA043810	GATGGCTGACTGTTTTAAATAAGAT[A/G]CACGTACCTCTCAGCTACGACAGTG	A/G	-	0.22/8	0.22/8	0.25/9	0.28/10	0.03/1	0.0002
GGaluGA071419	AGCTACAAAGAAGGGTGAAGAGCCC[A/G]GAGTAAACTCATTTGGGCTTTTTAA	A/G	0.06/2	0.36/13	0.44/16	0.14/5	-	-	0.0005

[a] SNP names beginning with "rs" can be traced in GenBank, while those with "GGaluGA" are from an unpublished resource from the USDA genome-wide marker-assisted selection consortium.

[b] Sequence context of SNPs was based on chicken genome (*Gallus gallus* genome Build released in GenBank). SNPs are highlighted by red color.

[c] Alleles having a higher percentage in H birds are shown in red, while those existing more often in L birds are shown in green.

[d] The frequency is that of each genotype: AA, AB, or BB, as *n* is the number of birds. H and L represented the chickens divergently selected for high and low ranking of biomarkers for oxidative stress, respectively.

[e] Significance level are shown as two-tail *p*-value of Fisher's exact test.

Table 5.2 Comparison of biomarkers for oxidative stress among genotypes of 13 SNPs at *p*-value ≤ 0.001.

SNP ID[a]	Genotype/n[b]	MDA[c]		GSH		PUA	
		LSMean ± SEM	F-value/*p*-value	LSMean ± SEM	F-value/*p*-value	LSMean ± SEM	F-value/*p*-value
rs13818962	AA/0	-	7.71/0.0085	-	8.14/0.007	-	11.9/0.0014
	AC/25	0.799±0.08		1451±67		314±22	
	CC/15	0.423±0.11		1763±86		439±29	
rs13938103	TT/14	0.881±0.11	4.79/0.0141	1341±86	6.22/0.005	274±29	7.93/0.0014
	TC/19	0.625±0.09		1638±74		389±25	
	CC/7	0.3±0.16		1831±122		456±41	
rs14010706	TT/26	0.808±0.08	5.08/0.0112	1439±63	6.17/0.005	304±19	13.02/<0.0001
	TC/11	0.391±0.12		1775±97		482±30	
	CC/3	0.331±0.24		1926±187		412±57	
rs14489496	TT/30	0.563±0.08	6.03/0.0188	1684±56	17.5/0.0002	385±22	5.04/0.0306
	TC/10	0.942±0.13		1219±96		287±38	
	CC/0	-		-		-	
rs14489711	TT/0	-	6.03/0.0188	-	17.5/0.0002	-	5.04/0.0306
	TC/10	0.942±0.13		1219±96		287±38	
	CC/30	0.563±0.08		1684±56		385±22	
rs14590417	AA/31	0.575±0.08	5,24/0.0278	1683±53	20.89/<0.0001	379±22	3.18/0.0825
	AG/9	0.944±0.14		1171±99		297±41	
	GG/0	-		-		-	
rs15573469	TT/8	0.446±0.15	1.75/0.1739	1834±109	5.97/0.0021	424±41	3.11/0.0381
	TC/23	0.776±0.09		1391±64		315±24	
	CC/3	0.332±0.25		1791±179		477±67	

rs15573695	AA/4	0.336±0.21	3.29/0.0314	1825±163	4.12/0.0131	480±58	3.01/0.0429
	AG/21	0.849±0.09		1416±71		323±25	
	GG/8	0.446±0.15		1834±115		424±41	
rs16434507	AA/0	-	6.03/0.0188	-	17.5/0.0002	-	5.04/0.0306
	AG/10	0.942±0.13		1219±96		287±38	
	GG/30	0.563±0.08		1684±56		385±22	
rs16434646	AA/30	0.563±0.08	6.03/0.0188	1684±56	17.5/0.0002	385±22	5.04/0.0306
	AG/10	0.942±0.13		1219±96		287±38	
	GG/0	-		-		-	
GGaluGA043801	AA/8	0.3±0.14	5.2/0.0103	1835±116	5.4/0.0088	448±39	6.67/0.0033
	AG/19	0.65±0.09		1599±75		382±25	
	GG/13	0.889±0.11		1358±91		276±31	
GGaluGA043810	AA/8	0.3±0.14	5.2/0.0103	1835±116	5.4/0.0088	448±39	6.67/0.0033
	AG/19	0.65±0.09		1599±75		382±25	
	GG/13	0.889±0.11		1358±91		276±31	
GGaluGA071419	AA/15	0.423±0.11	7.71/0.0085	1763±86	8.14/0.007	439±29	11.87/0.0014
	AG/25	0.799±0.08		1451±67		314±22	
	GG/0	-		-		-	

[a] SNP names begin with “rs” can be traced in GenBank, while those with “GGaluGA” are from an unpublished resource from the USDA genome-wide marker-assisted selection consortium.

[b] Number of chicken in each genotype and 40 chickens were used in total.

[c] Comparison of malondialdehyde (MDA), glutathione (GSH), and plasma uric acid (PUA). All data are presented as the least-squares means ± standard error (SEM).

Table 5.3 Candidate genes located in the flanking areas (± 100kb) of significant SNPs ($p \leq 0.001$)

SNP ID[a]	Chr[b]	Position[c]	Allel		Candidate Gene	Distance (kb)[d]	Gene ID[e]	Annotation	Putative function
			H	L					
GGaluGA043801	1	133761585	G	A	SHOX	+82.1	418669	Short stature homeobox	Development
					LOC769702	+94.2	769702	Hypothetical protein LOC769702	
GGaluGA043810	1	133774723	G	A	SHOX	+68,9	418669	Short stature homeobox	Development
					LOC769702	+81.1	769702	Hypothetical protein LOC769702	
rs13938103	1	133867348	T	C	LOC769702	-9.9	769702	Hypothetical protein LOC769702	
					SHOX	-15.6	418669	Short stature homeobox	Development
rs14489496	4	74934932	C	T	-	-	-	-	-
rs16434507	4	74952642	A	G	-	-	-	-	-
rs16434646	4	75293823	G	A	-	-	-	-	-
rs14489711	4	75434013	T	C	STIM2	+22.1	422799	Stromal interaction molecule 2	Ca2+ concentration
rs14590417	6	30601651	G	A	KCNK18	-7.2	771448	Potassium channel, subfamily K,	K+ current
					SLC18A2	+18.2	423921	Solute carrier family 18(vesicular	Monoamine
					VAX1	-41.8	395582	Ventral anterior homeobox 1	Development
					PDZD8	+40.9	428984	PDZ domain containing 8	
					KIAA1598	-86.5	771423	KIAA1598	
rs15573469	10	9551012	C	T	WDR72	intron	427497	WD repeat domain 72	
					LOC415414	-55.2	415414	similar to Munc13-1	
rs15573695	10	9828805	A	G	MAPK6	+89.3	415419	mitogen-activated protein kinase 6	Extracellular signal
					KIAA1370	+90.7	415416	KIAA1370	
rs14010706	10	16925710	T	C	LOC768396	-14.9	768396	Hypothetical protein LOC768396	
					MCTP2	-57.1	415508	Multiple C2 domains,	Intercellular signal
					IPI00580478.1	+69.2		Ensembl	
rs13818962	10	16996650	A	C	IPI00580478.1	0		Ensembl	
					LOC768396	-85.9	768396	Hypothetical protein LOC768396	
GGaluGA071419	10	17030258	G	A	IPI00580478.1	-25.4		Ensembl	

[a] SNP names begin with “rs” can be traced in GenBank, while those with “GGaluGA” are from an unpublished resource from the USDA genome-wide marker-assisted selection consortium.

[b] “Chr” stands for chromosome.

[c] SNP location was based on chicken genome 2.0.

[d] The smallest distance in kilobase pair (kbp) between candidate gene and SNPs. Negative “-”stands for down-stream, and “+” for up-stream.

[e] Gene ID and annotations were retrieved from the *Gallus gallus* genome Build released in GenBank.

Table 5.4 Single nucleotide polymorphism (SNPs) with significant differences in genotype frequencies between high and low chickens in Fisher's exact test ($p < 0.01$).

SNP ID[a]	Chr[b]	Chr Position	Alleles	position[c]	linked Gene	Gene ID
Gga_rs13624529	1	135859780	C/T	intron	ATP10A	418685
Gga_rs13828802	1	16264366	A/C		-	
Gga_rs13879676	1	67673220	A/G		-	
Gga_rs13893940	1	86873611	C/T	intron	CCDC80	395074
Gga_rs13919572	1	111749939	A/G	intron	DSCAM	418525
Gga_rs13937909	1	133556771	A/C		-	
Gga_rs13941413	1	136740206	A/C	intron	REV1	418703
Gga_rs13952451	1	148741641	G/T		-	
Gga_rs13975251	1	176383356	C/T		-	
Gga_rs13975272	1	176400961	A/C		-	
Gga_rs13975919	1	177082112	A/G	intron	DCLK1	418903
Gga_rs13976073	1	177220175	C/T	intron	DCLK1	418903
Gga_rs14886565	1	133476914	C/T		-	
Gga_rs14886832	1	133881131	A/G		-	
Gga_rs14907328	1	158493187	C/T	intron	MYCBP2	418808
Gga_rs14919554	1	176356551	A/G		-	
Gga_rs15378688	1	111719155	C/T	intron	DSCAM	418525
Gga_rs15378859	1	111820219	C/T	intron	DSCAM	418525
Gga_rs15453071	1	148686592	C/T		-	
Gga_rs15507599	1	177279251	C/T		-	
GGaluGA022937	1	67488278		Intron	RECQL	378910
GGaluGA022991	1	67659600			-	
GGaluGA043655	1	133133873			ASMT	396286
GGaluGA043745	1	133532496			-	
GGaluGA055840	1	176499258			-	
Gga_rs13544185	2	30048846	A/G	intron	ITGB8	420605
Gga_rs13669086	2	37037730	A/G	intron	UBE2E2	776043
Gga_rs14158578	2	30221969	A/C		-	
Gga_rs14195212	2	64642522	G/T		-	
Gga_rs14242536	2	125069362	C/T		-	
Gga_rs14242723	2	125225693	A/G		-	
Gga_rs14251774	2	136022467	A/G	intron	ZFPM2	420269
Gga_rs14262964	2	146189443	A/G	intron	FAM49B	420330
Gga_rs14262989	2	146165441	G/T	intron	FAM49B	420330
Gga_rs15079411	2	30406437	A/G		-	
Gga_rs15084679	2	36978709	A/G	intron	UBE2E2	776043
Gga_rs15950748	2	36891889	G/T	intron	UBE2E2	776043
Gga_rs16136964	2	136180431	A/G	intron	ZFPM2	420269
GGaluGA150681	2	64865123			-	
GGaluGA157336	2	91674059			-	
Gga_rs14309211	3	2450238	C/T	intron	RTN4	378790
Gga_rs14309220	3	2492925	G/T		-	
Gga_rs14400207	3	99391679	A/G		-	
Gga_rs15253393	3	1941129	C/T		-	
Gga_rs15253427	3	1861658	A/G	intron	RCJMB04_3a6	421199
Gga_rs15298130	3	25277967	C/T		-	
Gga_rs16215158	3	2427662	A/G		-	
Gga_rs16215167	3	2467682	C/T	intron	RTN4	378790
Gga_rs16215195	3	2548283	A/G		-	
Gga_rs16215227	3	2682368	A/G	intron	SPTBN1	421216
GGaluGA203504	3	2343152		intron	XPO1	421192

SNP ID[a]	Chr[b]	Chr Position	Alleles	position[c]	linked Gene	Gene ID
Gga_rs14308187	3	2380429	C/T		-	
Gga_rs14308191	3	2378356	A/G		-	
Gga_rs14308276	3	2150549	C/T	intron	LOC421195	421195
Gga_rs14308283	3	2119913	A/G	intron	LOC421195	421195
Gga_rs14308413	3	1900890	C/T		-	
Gga_rs14308443	3	1801669	G/T		-	
Gga_rs14308446	3	1800164	C/T		-	
Gga_rs14432157	4	14300318	C/T		-	
Gga_rs14466944	4	50974445	A/C	intron	MFHAS1	422628
Gga_rs14506283	4	93690713	C/T		-	
Gga_rs14692661	4	93416180	G/T		-	
Gga_rs15538350	4	35629161	A/G		-	
Gga_rs16045173	4	93287577	C/T		-	
Gga_rs16411398	4	53932459	A/G		-	
Gga_rs16448604	4	88829504	A/G	intron	JMJD1A	422917
GGaluGA269219	4	88203732			-	
GGaluGA269514	4	88874031			RMND5A	422920
GGaluGA270890	4	93237409		intron	FBXO41	428805
GGaluGA270900	4	93258513		intron	CCT7	428806
Gga_rs13649677	5	2881449	C/T		-	
Gga_rs13787156	5	3652758	G/T	intron	CCDC34	421609
Gga_rs14353136	5	3986903	C/T	intron	METT5D1	421606
Gga_rs14353262	5	3673492	A/G	intron	LGR4	428605
Gga_rs14508135	5	2783063	C/T	intron	TMEM16E	422973
Gga_rs14538448	5	44468553	A/G		-	
Gga_rs14538484	5	44531480	C/T		-	
Gga_rs14538871	5	45123939	C/T		-	
Gga_rs14743483	5	16865196	A/G	intron	PNPLA2	431066
Gga_rs15649344	5	3229497	A/C		-	
Gga_rs15649770	5	2452703	C/T	intron	LOC770845	770845
Gga_rs15999871	5	3510315	A/G		-	
Gga_rs16456203	5	2288762	C/T	intron	LOC428823	428823
Gga_rs16472804	5	18339765	A/G		-	
GGaluGA272527	5	2658460			-	
GGaluGA277246	5	18048509			-	
GGaluGA285477	5	45179065			-	
Gga_rs13580315	6	28077808	C/T	intron	GPAM	423895
Gga_rs14583785	6	24551545	C/T	intron	C10orf6	423858
Gga_rs14587851	6	28153252	C/T		-	
Gga_rs14747095	6	34879854	C/T	intron	LOC426320	426320
GGaluGA294302	6	4157854			-	
GGaluGA302170	6	24652699		intron	SUFU	374118
GGaluGA302199	6	24705864			-	
GGaluGA303729	6	28034286			-	
GGaluGA306463	6	34763463		intron	LOC768931	768931
GGaluGA306492	6	35014054			-	
GGaluGA307146	6	36566998		intron	SCFD1	423307
Gga_rs14636977	8	5921062	C/T	intron	RGSL1	429067
Gga_rs14638008	8	6963802	C/T		-	
Gga_rs15898713	8	3446004	C/T	intron	CDC73	424356
Gga_rs15903982	8	6986712	C/T		-	
Gga_rs15925489	8	21279369	C/T		-	
Gga_rs16619959	8	3502594	A/G	intron	CDC73	424356
Gga_rs16621320	8	4621832	C/T		-	

SNP ID[a]	Chr[b]	Chr Position	Alleles	position[c]	linked Gene	Gene ID
GGaluGA323225	8	4154247		intron	PACS2	424375
GGaluGA323987	8	5849089			-	
GGaluGA329868	8	22520309		3'UTR	MKNK1	771961
Gga_rs14679801	9	21560431	C/T	intron	EVI1	424997
Gga_rs16676292	9	21135756	A/G	intron	TNIK	424987
GGaluGA334181	9	2736297			-	
GGaluGA342852	9	21543999		intron	EVI1	424997
Gga_rs13677428	10	4643810	C/T	intron	CRABP1	374211
Gga_rs14007557	10	13933500	C/T	intron	MESDC2	415475
Gga_rs14007588	10	13959486	A/G	intron	KIAA1199	415476
Gga_rs14948714	10	14133203	C/T (synonymous)	extron 10	ARNT2	415481
GGaluGA070113	10	14384514		intron	LOC770352	770352
Gga_rs15624861	11	19538676	C/T		-	
GGaluGA079424	11	19548317		intron	NAV2	89797
Gga_rs14063877	13	16191141	A/G	intron	PHF15	416315
Gga_rs14070631	14	2349641	C/T		-	
Gga_rs14076471	14	8562162	C/T	intron	SMG1	416605
Gga_rs14096555	15	12667819	C/T		-	
GGaluGA110657	15	10434475			-	
GGaluGA111841	16	424347	A/G (synonymous)	Exon 10	TMEM8A	58986
GGaluGA116106	17	7836255			-	
Gga_rs14114530	18	8757097	A/G		-	
Gga_rs14116631	19	1682276	A/G	intron	AUTS2	417481
Gga_rs14116644	19	1689497	C/T	intron	AUTS2	417481
Gga_rs14121275	19	5979279	A/G	intron	TAOK1	417583
Gga_rs15171637	20	3930620	A/G		-	
Gga_rs16162993	20	3665091	A/G	intron	SLC32A1	419167
Gga_rs16163006	20	3669811	A/G	intron	SLC32A1	419167
Gga_rs16163378	20	3835715	A/G	3' UTR	DHX35	419172
GGaluGA176766	20	3838846			-	
Gga_rs16078288	22	2910718	A/G		-	
Gga_rs15196832	23	1877417	C/T	intron	C1orf160	419582
Gga_rs15203512	23	4291485	A/G		-	
GGaluGA187066	23	215083		intron	C20orf4	419118
GGaluGA189162	23	4136419			-	
GGaluGA192546	24	3942494		intron	TRIM29	419754
GGaluGA192936	24	4488827		intron	HTR3A	428247
GGaluGA192967	24	4517479		intron	ZBTB16	419759
Gga_rs14303550	27	858508	A/G		-	

[a] SNP names beginning with "rs" can be traced in GenBank, while those with "GGaluGA" are from an unpublished resource from the USDA genome-wide marker-assisted selection consortium.

[b] "Chr" stands for chromosome.

[c] Relative position with genes, gene ID and annotations were retrieved from the *Gallus gallus* genome Build released in GenBank.

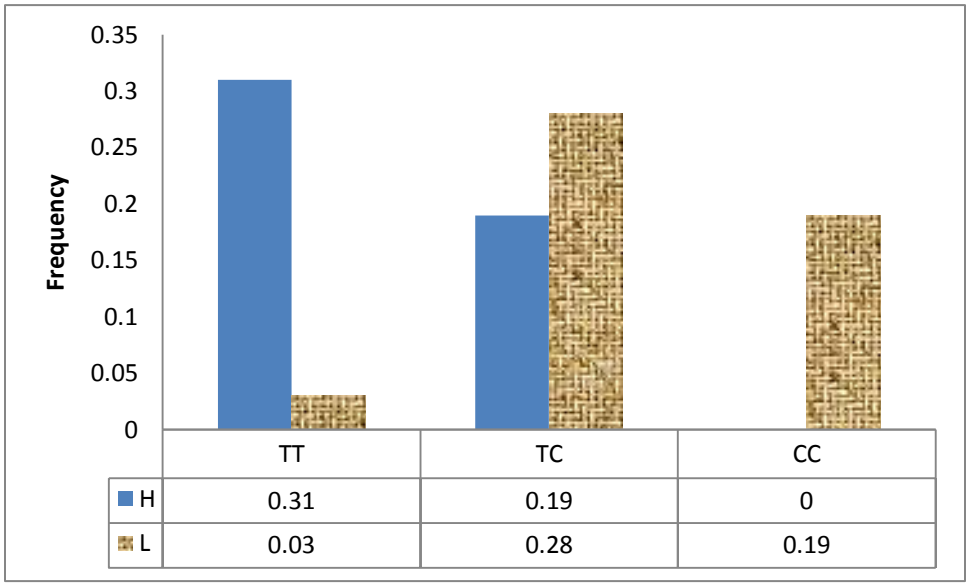

Figure 5.1 Genotype frequencies for the representative SNP (rs13938103) with different phenotypes-high (H) and low (L) ranking of biomarkers of oxidative stress.

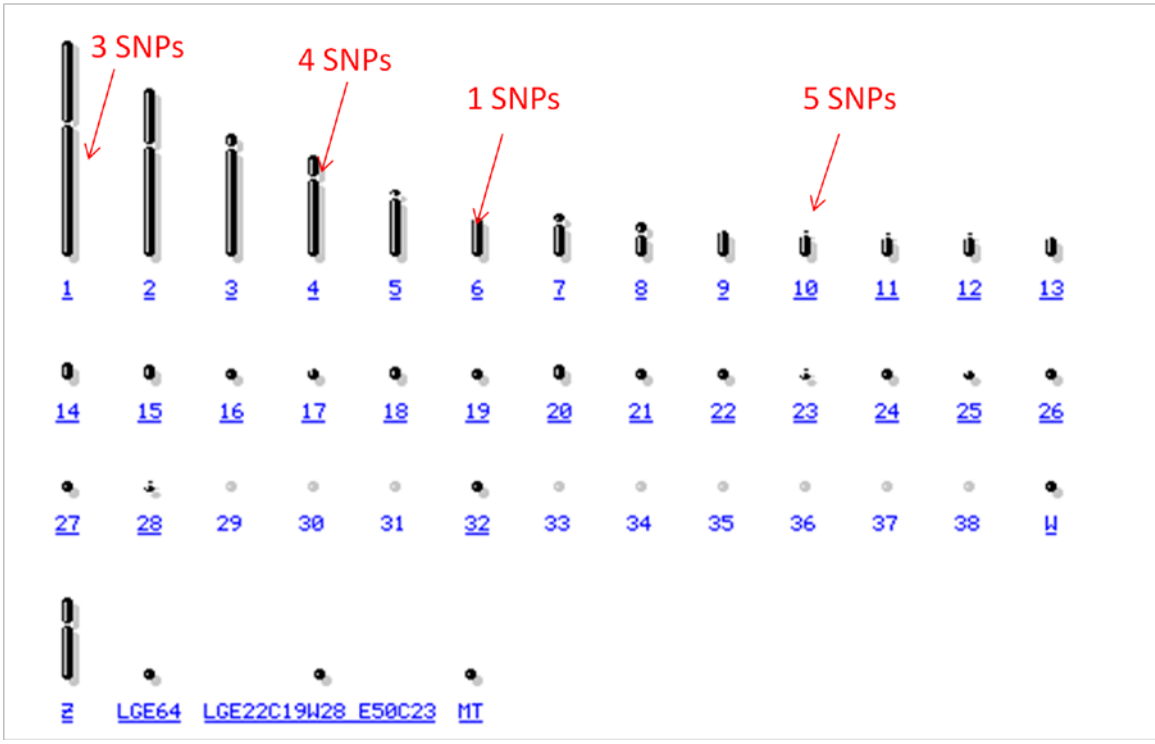

Figure 5.2 Location of the 13 most relevant SNPs ($p \leq 0.001$) on chromosomes shown on *Gallus gallus* genome view, build 2.1 released in GenBank.

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Chapter VI

Conclusion and Future Work

This dissertation project investigated the hypothesis that oxidative stress (OS) is related to adaptive immune responses in chickens, *Gallus gallus*, and the genetic variance has a role in OS level. The rationale of the research was that different dosages of ethanol use is believed to induce different levels of OS, and that immune responses could be measured in chickens of high (H)- or low (L)- OS to test their correlation. Similarly, genetic variation of both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) were assessed in divergently selected chickens for association analysis between genetic background and OS. Referring to the aims, several findings of this study were as follows:

Specific findings:

1. For male White Leghorn chickens, 10% ethanol in drinking water was enough to induce OS in chicken raised in cages within one to two weeks, while 2% ethanol use appeared to stimulate antioxidant defense and to reduce OS in this chickens.
2. A tight association between OS level and adaptive immune response was found in White Leghorn chickens. Results showed that when OS was induced by 10% of ethanol dose, immune response of chickens decreased; when OS was reduced by 2% ethanol dose, some beneficial effects in immune response were detected; when the OS level was not influenced significantly by ethanol induction, the changes of immunological parameters were also reduced.
3. No variation was found at tested regions and SNP loci in mtDNA of chickens selected for H- or L- OS, except for a putative SNP, 6827A>C. The mtDNA

damage, however, increased with time ($p < 0.05$), and was significantly higher in chickens exhibiting high OS ($p < 0.05$).

4. A genome-wide SNP analysis of chickens selected for H- or L- OS showed that 13 new SNPs in probably 5 independent loci were significantly associated with OS ($p \leq 0.001$), and another 144 SNP associated with OS in chickens at a *p*- value less than 0.01. Analysis of genes adjacent to the most significant SNPs suggested several candidate genes for OS, including genes *sitm2, slc18a2, mctp2, kiaa1598, kiaa1370,* and *mapk6*.

Future work:

This study showed a connection between OS and adaptive immune response under ethanol induction, and some candidate SNP loci in nDNA associated with OS. However, if there is a direct effect of OS on immune system is yet unclear, and more genetic studies are needed to confirm my results. Specific investigations that could be carried out in the future include:

1. Evaluate the changes in OS and immune response in chickens receiving ethanol treatment along with antioxidant supplementation.
2. Determine whether different results on OS level and immune response in study 2 are due to different sex or housing.
3. Examine cell damage in lymphoid organs using a more accurate method; Monitor cytokine production and lymphocytes *in vitro* proliferation.
4. Use another OS inducer, like paraquat, to confirm the results.
5. Test the whole mtDNA molecule for variations in more chickens selected for H or L OS.

6. Enlarge the sample population in genome-wide SNP analysis to avoid the possibility of false positive and confirm these new candidate SNP loci.

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