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Inosine ameliorates the effects of hemin induced oxidative stress in broilers

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**Inosine Ameliorates the Effects of
Hemin Induced Oxidative Stress in Broilers**

Christen N. Seaman

**Thesis submitted to the
Davis College of Agriculture, Forestry, and Consumer Sciences
At West Virginia University
In partial fulfillment of the requirements for the degree of**

**Master of Science
In
Animal and Veterinary Sciences**

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Joseph Moritz, Ph.D.
Knox Van Dyke, Ph.D.
Janet Tou, Ph.D.**

Division of Animal and Veterinary Sciences

**Morgantown, West Virginia
2007**

**Keywords: Uric Acid, Oxidative Stress,
Broilers, Hemin, Inosine**

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ABSTRACT

Inosine Ameliorates the Effects of Hemin Induced Oxidative Stress in Broilers

Christen N. Seaman

Previous studies have shown that chronic treatment of broilers with hemin decreases plasma concentrations of uric acid and increases leukocyte oxidative activity (LOA) whereas the inclusion of inosine produces an inverse effect. The objective of these studies was to determine whether or not inosine could serve as a potential therapy for oxidative stress. Oxidative stress was induced in broilers by the administration of hemin. Dietary inclusion of inosine was then begun either before the onset of oxidative stress induced by hemin, or as a post treatment once oxidative stress was established. Four-week-old broilers were individually banded and divided into four treatment groups (Control, Hemin, Inosine, Hemin/Inosine). Throughout the study control birds (n=10) were injected daily intraperitoneal with a buffer solution, while hemin birds (n=10) were injected daily intraperitoneal with a 20mg/kg body weight hemin buffer solution. Leukocyte oxidative activity (LOA) and plasma uric acid (PUA) were measured on day eight. Results showed that hemin birds had higher levels of LOA (P=0.03) and lower PUA (P=0.11). On day 10, control and hemin birds were subdivided into inosine birds (n=5) and hemin/inosine birds (n=5). These birds were given 0.6M/ kg of feed/day of dry inosine. Plasma concentrations of uric acid and LOA were then measured on day 15. Results showed that inosine raised concentrations of PUA (P=0.0001) and lowered LOA (P=0.004) induced by hemin. In a separate study, stereological analyses of inosine vs. control birds revealed no anatomical differences in kidney morphology between treatments (P=0.05). The results of these studies support the view that uric acid reduces oxidative stress by functioning as an antioxidant. Uric acid treatment has the potential to decrease the amount of damage generated by free radicals during times of oxidative stress associated with disease states.

DEDICATION

First and foremost I have to thank God, for he is the fountain from which all blessings flow, and without his strength I would have never made it this far. To the most wonderful parents anyone could ask for I say thank you. My parents, Keith and Colleen Seaman, have stood by my side during all my years of education. They have watched with loving eyes and accepting hearts as I have followed my dreams and aspirations. I would also like to thank Dr. Hillar Klandorf, my committee chair, for his guidance, patience, and constant reassurance during my term as his student, in both undergraduate and graduate work. Many thanks go out to Dr. Knox Van Dyke, Dr. Joe Moritz, and Dr. Janet Tou for their priceless knowledge, support, and use of their facilities. Graduate student, Brian Lewis assisted during several experiments and his helpfulness and unconditional friendship was greatly appreciated. Lab technician Elizabeth Falkenstein and farm workers Fred Roe and Bill Miller contributed during each project and must be thanked for their efforts.

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LITURATURE REVIEW

Inosine Ameliorates the Effects of Hemin Induced Oxidative Stress

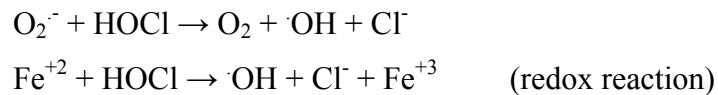
INTRODUCTION

Molecular oxygen is arguably the most important element of the periodic table. Without it there would be no water, no air to breathe and most species on earth would cease to exist. Although oxygen is important to life on earth, it acts as a two edged sword providing numerous advantages on one side, but on the other hand, has the potential to create multiple forms of toxicity (Fridovich, 1983). For example, aerobic organisms such as fungi, plants and animals combine oxygen with food in order to run important metabolic processes for efficient energy production. However, some toxicity naturally arises during these metabolic processes, as highly reactive oxygen species (ROS) are created as harmful by-products (Ames et al., 1993, Sinitsyna et al., 2006). Once these ROS are created, they react within the cell with a force so powerful, that it has the ability to eradicate the life of any organism that creates them. Continued existence now depends upon how well the elaborate antioxidant defense mechanism of the organism can protect itself against the oxidative damage caused by free radicals (Halliwell et al., 1990).

REACTIVE OXYGEN SPECIES

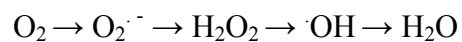
Aerobic cells are constantly battling an oxygen paradox that can ultimately decide whether an organism lives or dies. Oxygen plays an indispensable supporting role on the road of life but also act as a powerful poison. Metabolites of oxygen, known as reactive oxygen species (ROS), lead to oxidative free radicals via enzymatic redox chemistry that threaten numerous systems in the body and can ultimately lead to disease states and even death. Because oxygen is a universal electron acceptor many ROS are generated as by-products of the bodies own metabolism, exposure to ionizing radiation and environmental pollutants (Sinitsyna et al., 2006). Most ROS are defined as “any species that has one or more unpaired electrons,” (Halliwell et al., 1984). The oxidative powers of O₂ are controlled by the parallel spins of its unpaired electrons. In order for

O_2 to accept electrons from another species, the spins of the electrons have to be opposite of each other (Fridovich, 1999). This restriction prevents O_2 from being a reactive enough molecule to cause damage. However, O_2 can become highly reactive when a single electron is added and the spin on the molecule is released (Benzie, 2000). It is because of these unpaired electrons that catabolic processes lead to oxygen free radicals such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), the hydroxyl radical ($\cdot OH$), and the particularly reactive peroxyxynitrite ($OONO^{\cdot-}$).



Numerous sites in the body are responsible for oxygen free radicals and ROS generation, but four main systems are the primary producers: mitochondrial electron transport chain (ETC), peroxisomal fatty acid metabolism, cytochrome P-450 reactions, and phagocytic cells (“respiratory bursts”) (Ames et al., 1993).

One of the cells most significant sources of oxidants comes from the leakage of $O_2^{\cdot-}$, H_2O_2 , and $\cdot OH$ through the membrane wall of the mitochondria (Beckman et al., 1998). Instead of being completely reduced to water, up to 4% of the oxygen consumed in the mitochondria may be incompletely reduced. The mitochondrial ETC consumes oxygen and produces one-electron reductions of O_2 to form $O_2^{\cdot-}$ which then goes on to yield H_2O_2 and $\cdot OH$ (Ames et al., 1993).

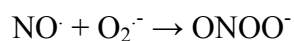


Another source of oxidants is peroxisomes, which are responsible for degrading fatty acids and other molecules. Peroxisomal β -oxidation of these fatty acids leads to the by-product H_2O_2 . Those H_2O_2 molecules that escape degradation by catalase have the ability to cause further oxidative damage to the cell. While H_2O_2 has no unpaired electrons and therefore is not directly referred to as a radical species, it can cross biological membranes with ease where it can then react with various other molecules to generate numerous ROS as well as damage to DNA (Halliwell et al., 1990).

Phagocytic cells are part of the bodies own natural immune defense systems that digest foreign objects such as bacteria or virus-infected cells. This is accomplished by engulfing the foreign invader and then destroying it with an oxidative burst containing a mixture of oxidants and free radicals such as nitric oxide (NO), O_2^- , H_2O_2 , and hypochlorite. From this burst, ROS are released into the body and free to create multiple forms of oxidative damage. Therefore, chronic forms of inflammation result in an increase in oxidant and free radical generation, which can be a major risk factor for diseases like cancer (Ames et al., 1993). Overall, phagocytic cells have an inevitable consequence because they are part of an innate immune system.

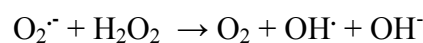
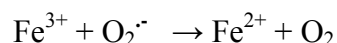
Lastly, cytochrome P-450 enzymes operate as a primary defense mechanism against xenobiotic compounds, toxic chemicals found in plants (Beckman et al., 1998). These enzymes, created by the bodies own natural defenses, help eliminate some of the toxic effects that an organism might encounter from plants and other dietary chemicals. Unfortunately, many of these enzymatic reactions reduce O_2 to O_2^- resulting in oxidant by-products that cause oxidative stress and lead to DNA damage (Ames et al., 1993).

While these four reactant sources of oxidants are familiar, there are numerous other sources which contribute to the balancing act between oxidants and antioxidants. One such reaction that leads to an increase of ROS is the formation of peroxynitrite. This complex reaction occurs very rapidly between the two radicals, superoxide and nitric oxide (Blough et al., 1985).



This in turn leads to a more dangerous oxidant that has the ability to rapidly oxidize DNA, lipids and proteins, which leads to cell damage, and eventually death (Whiteman et al., 2002). Besides radicals combining to form ROS, metal ions like copper and iron also have the ability to encourage the generation of ROS via Fenton chemistry. Iron plays a key function in energy generation, O_2 transport and DNA synthesis all because it acts as a cofactor within the active site of many important enzymes (Kalinowski and Richardson, 2005). Iron is able to switch between ferric and ferrous forms, which allow it to donate as well as accept electrons (Kalinowski and Richardson, 2005). Too much iron can be toxic; a simple mixture of an iron salt and H_2O_2 can set

into motion, a chain of events that will lead to Haber-Weiss reactions (Halliwell et al., 1984, Kalinowski and Richardson, 2005).



Once reactive oxidative species are created, the bodies own natural defense systems come into play in order to minimize the generation and help counteract the damages that are brought on by these highly reactive and unstable species. Over time, constant exposure to these deleterious ROS leads to cellular damage and life threatening diseases. Degenerative diseases of aging such as cancer, cardiovascular disease, arthritis, and inflammatory response are all due to the formational changes that occur to DNA, lipids and proteins (Whiteman et al., 2002). Reactive oxidative species attack nucleic acids, which in turn generates adducts of base and sugar groups, strand breaks in the back bone, and DNA-protein crosslink's (Sinitsyna et al., 2006). With DNA being the back bone of life it is easy to see why any substance that could possibly change its conformations is potentially carcinogenetic and tumor promoting (Halliwell et al., 1984, Leonard et al., 2003). Along with DNA changes, ROS have the ability to oxidize proteins and affect lipid peroxidation. Lipid peroxidation leads to altered membrane properties, decreased fluidity and damage to membrane bound proteins (Beckman et al., 1998). Protein damage has been characterized by cross linking and adduction of amino acid residues. Reactive oxidative species are the fundamental cause of numerous negative biochemical changes that occur throughout an organism's life and are indicative of oxidative stress.

OXIDATIVE STRESS

Oxygen's vital role to life comes at a cost to every aerobic organism. It is the consumption of oxygen and the direct incorporation of it into biomolecules that leads to the production of ROS. Once created, radical oxidants can create a serious imbalance between antioxidant defense systems and the continuous production of ROS, commonly referred to as oxidative stress (Van

Dyke, 2002). This imbalance is used to illustrate the steady state level of oxidative damage done to various cellular components of an organism. Oxidative stress is determined by one of three factors, 1) a decrease in antioxidant production, 2) an increase in oxidant production, or 3) the inability of an organism to repair the damage caused by radical onslaughts (Sies et al., 2005). Overall, oxidative stress occurs when the number of oxidants produced exceeds the number of antioxidants produced. Generally, 21% is the amount of oxygen that aerobes are capable of protecting themselves against as far as antioxidant defense systems go. However, anything over 21% oxygen can cause numerous and even irreversible damage to cellular systems (Halliwell, 1998). The inability of an organism to correct this oxidative damage can result in numerous disease states such as cancer, tissue injury, arthritis, and diabetes. It has been hypothesized that continual exposure to low levels of ROS contributes to the aging process as evidenced by the gradual accretion in the markers of cellular injury (Halliwell and Gutterage, 1993). Under conditions of severe oxidative stress, markers of ROS accumulate at an accelerated rate and mediate cell injury and dysfunction (Sarafian and Bredesen, 1994). Ultimately, the overall effects of oxidative stress depends on variables like age, diet, exercise, temperature, antioxidant defense system capabilities and the metabolic rate of the individual organism. It has long been proven that species with increased metabolic rates age faster and have decreased life span potential (Beckman et al., 1998).

Antioxidant defense systems are responsible for maintaining balance against oxidative stress and increasing maximum life span potential. Because oxidative stress is induced naturally in the body, mild forms of it can be dealt with by simply upregulating antioxidant defense systems to restore equilibrium, and therefore decrease the production and damage caused by ROS (Benzie, 2000 and Halliwell, 1998). Such defense systems may include superoxide dismutase (SOD), peroxidases, uric acid, α -tocopherols, vitamin C and other antioxidant enzymes (Ames et al., 1981, Banerjee et al., 2003, and Benzie, 2000). Several forms of antioxidants must exist in organisms in order to combat the different pathways in which ROS are generated. Each pathway taken can lead to multiple forms of oxidative stress which can be broken down into metabolic oxidative stress, postprandial oxidative stress, environmental oxidative stress and many others (Sies et al., 2005). Eating foods rich in antioxidants can help lower oxidative stress by directly scavenging ROS via carotenoids and thiols (Sies et al., 2005). This in turn helps metabolism

because targets of postprandial oxidative stress are low density lipoproteins (LDL) which can be modified by ROS and lead to further damage such as atherosclerosis, strokes, diabetes and even obesity (Sies et al., 2005). Associated with these disease states is an increase in the accumulation of markers linked to ROS, which contributes to the pathology of each disease and further aggravates the disease process (Halliwell, 1998). For example, ROS are over produced at the sites of inflammation from the congregation of neutrophils, monocytes, and/or macrophages that generate large amounts of ROS (Halliwell et al., 1992) as a normal consequence of the physiology of these cells in the activated state.

Oxidative stress is also a consequence of tissue injury, such as excessive exercise or muscular disuse atrophy. During muscle atrophy, metal ions released from storage sites can stimulate the conversion of hydrogen peroxide into the hydroxyl radical. Heme protein release (myoglobin and cytochromes) occurs with muscle damage, discharging peroxides and iron (Hagberg, 1981). Mitochondrial damage can cause electron leakage, stimulating formation of superoxide, which further contributes to oxidative stress (Halliwell and Gutteridge, 1993).

Despite the multiple lines of defense that each organism has, oxidative stress will cause damage to various sites in the body. The measurement of antioxidant derivatives (ascorbate, urate and oxidation products) along with the depletion of specific antioxidants shows the protective mechanisms are working and that damage is being minimized by turnover and repair (Halliwell, 1998). Specific markers accumulate in ROS-mediated tissues and can indicate oxidative damage has occurred. Reactive oxidative species are overproduced during extreme bouts of oxidative stress that lead to DNA damage, direct damage to proteins, glutathione (GSH) depletion, increases in intracellular free iron and copper, and increased lipid peroxidation (Halliwell, 1998). Increases in free iron leads to peroxidation and destruction of membranes therefore releasing iron into the surrounding tissues causing injury to nearby tissues and cells. Over time oxidative damage will accumulate in vital organs due to a lack of DNA repair in cells, thus leading to injury, disease states, faster aging in short lived species and eventually death (Fridovich, 1999).

ANTIOXIDANTS

Reactive oxidative species are proficient in causing multiple forms of oxidative damage to various cellular components. Reactive oxygen species attack and oxidize key principal areas in the body such as DNA, proteins, lipids and other molecules, which in turn leads to mutations, disease states and tissue injury (Benzie, 2000). However, for every reaction there is an opposite reaction, and this is found in the form of antioxidants. Constant repair of oxidative stress is crucial for the survival and increased maximum life span of each particular species. It is antioxidant defense systems that preserve homeostasis and prevent damage in order for normal cell function to occur (Banerjee et al., 2003). “An antioxidant is any substance that, when present at low or high concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate” (Halliwell et al., 1990). In other words, an antioxidant is anything which can avoid oxidative stress by prevention, enzymatic diversion, scavenging or quenching the production of ROS before they have the ability to destroy multiple cellular mechanisms. It is a principal purpose of any antioxidant to help maintain the redox status of an organism in order to minimize damage associated with reactive oxidative species.

Antioxidant defense systems are universal across the broad spectrum of species that exist on this planet. While the first antioxidant defense systems were primitive, not much more than protective barriers, species of today have evolved to include an arsenal of antioxidant weapons that are specific to each type of ROS that might occur throughout a lifetime. As evolution progressed, antioxidant defense systems increased. Protection from ROS is a highly complex system that takes into consideration which reactive species is generated, how it is generated, where it is generated and what target is being damaged (Halliwell, 1998). Therefore, it is safe to say that no one antioxidant is 100% effective against all types of ROS. Each antioxidant plays a significant role in protecting every species from oxidative onslaughts. Their mechanisms of action can be divided into substrate-specific antioxidant enzymes, nonenzymatic antioxidants, and preventative antioxidants (Papežíková et al., 2006). Each of these actions further include the ability to minimize O₂ levels, scavenge ROS, inhibit ROS formation, scavenge ROS precursors, bind metal ions needed to catalyze ROS formation, and increase antioxidant defenses (Halliwell, 1998).

Superoxide dismutases are one of the most potent endogenous intercellular antioxidant enzymes capable of scavenging ROS. These substrate-specific enzymes are responsible for removing $O_2^{\cdot -}$ by increasing its conversion to H_2O_2 (Fridovich, 1983). Once this radical has been degraded, further generation of other ROS can be prevented. These types of redox reactions rely heavily on a certain substrate being produced in order to continue a chain of reactions. When a part of the chain has been removed the reactions can no longer continue and ROS are no longer produced, therefore a decrease in oxidative stress occurs. Other enzymes that aid SOD in the continued removal of ROS are catalases and peroxidases, which are found in the cytosol and peroxisomes and are dedicated to seeking out and removing peroxides (H_2O_2) from the cell and turning them into water (Beckman et al., and Halliwell, 1998). Superoxide dismutases, along with catalases and peroxidases, work together in order to form a unified barrier against oxidative stress.

Non-enzymatic extracellular antioxidants are known for having sacrificial interactions with ROS and reducing them into a product that is less harmful than the original (Papežíková et al., 2006). Such expandable antioxidants like uric acid, ascorbic acid (vitamin C) and α -tocopherol (vitamin E) are scavengers. They are also deemed replaceable or recyclable substrates, because they are usually found in great quantities, located and stored in body fluids and plasma (Papežíková et al., 2006). However, these important antioxidants, with the exception of uric acid, cannot be synthesized by most mammalian species and therefore need to be taken in through the diet. Vitamin E is a lipid soluble compound found in the inner cellular membrane (Ji, 1999). Because of its location in the body it has the ability to act as the most important chain breaking antioxidant within lipid bilayers (Banjeree et al., 2003 and Ji, 1999). Vitamin E has the ability to protect lipids against oxidative damage by scavenging peroxy radical intermediates (Halliwell, 1996). This stabilizer of protein-lipid membranes also functions as a scavenger of singlet oxygen and reactive nitrogen species (Traber, 1996). Water soluble vitamin C is found most often in the plasma, cytosol and other water containing compartments of the cell. This hydrophilic antioxidant is an electron donor to redox reactions, has the ability to help regenerate vitamin E, as well as react efficiently with superoxide and peroxy radicals (Cabelli et al., 1983 and Krakowska et al., 2006). Studies have shown that vitamin C will also react with O_3 ,

cigarette smoke and NO_2 ; all the while serving as cofactors for important metabolic reactions (Halliwell, 1996). Uric acid is a powerful antioxidant that is produced in the body as a product of purine degradation rather than consumed as a dietary requirement. The nitrogenous bases of guanine and adenine are broken down into uric acid. Uric acid exists in the form of urate which is a salt of uric acid, particularly for the avian species.

Uric acid is arguably one of the most important non-enzymatic extracellular antioxidants. However, it is also categorized as a preventative antioxidant because it not only intercepts strong oxidants like hydroxyl radicals, peroxy nitrite, singlet oxygen, lipid hydroperoxides, and hypochlorous acid, but has the ability to form redox inactive complexes with transition metal ions (Papežíková et al., 2006, Stinefelt et al., 2005). Preventative antioxidants have the ability to diminish the activity of metal ions by decreasing their ability to participate in Fenton chemistry, which leads to a decrease in oxidative damage. Preventative antioxidants include protein based albumin and transferrin along with non-protein based uric acid (Papežíková et al., 2006).

Exogenous antioxidants that can only be taken in through the diet are becoming an ever more popular movement. The increase in trying to identify these possible antioxidants rises from finding a treatment, or prevention, for diseases caused by ROS. Recent studies have shown that complementing diets with substances like fruits and vegetables can help aid individuals in fighting cancer, heart disease, and many other types of illnesses (Leonard et al., 2003). One such substance that has come into play is resveratrol, a polyphenol found in grapes, red wine, and other foods (Leonard et al., 2003, Borra et al., 2005). It has the ability to scavenge $\cdot\text{OH}$ and O_2^- while inhibiting lipid peroxidation and DNA damage brought on by Fenton reactions (Leonard et al., 2003). Resveratrol may also aid in a type of redox regulation in platelets due to the fact that ROS are present in blood platelets and act as second messengers (Olas et al., 2004). While recent studies have shown that resveratrol is a scavenger of ROS, they have also shown that it may also have many useful properties including anti-inflammatory, antibacterial activities, as well as help prevent cancer and coronary heart disease (Chen et al., 2005, Imamura et al., 2002, Leonard et al., 2003). Despite the fact that resveratrol has protective properties, its mechanism of action is not totally understood and requires further studies (Imamura et al., 2002).

THE VALUE OF URIC ACID

Reactive oxidative species are constantly being produced either by the body or the environment. The over production of these oxidants leads to a redox imbalance that must be corrected in order to avoid oxidative stress, which in turn, may lead to disease initiation. Overcoming these onslaughts that seem to jeopardize the defense systems of each organism is crucial to the survival and longevity of each species. Antioxidants seem to be the answer to most of the questions brought on by the oxygen paradox. However, questions and answers now become more precise and targeted toward specific ways to alleviate ROS and the continuous harm they cause. Uric acid may be a major portion of the answer to oxidative stress and the diseases that attack each organism as a result of it. Uric acid's antioxidant activity at physiological concentrations, it has been postulated that, in conjunction with lactoperoxidase, uric acid may be involved in the fight against cancer (Ames, 1983). Al-Bekairi et al (1991) confirmed that uric acid is also a potent antimutagen; it inhibits biochemical changes induced by cyclophosphamide in mice (Al-Bekairi et al., 1991). Overall, uric acid is considered a physiologically useful antioxidant and radical scavenger because it has the ability to interact with biological oxidants and radicals, the end products of these interactions are physiologically less harmful than the original components, and it is also found in high enough concentrations within the tissues to ensure significant reactions (Becker, 1993).

Uric acid is one of the most powerful circulating antioxidants available. In fact, it has been shown that uric acid comprises 30-60% of the radical quenching capabilities of blood plasma (Becker, 1993). Uric acid is ubiquitous; it can be found in numerous types of extracellular fluids which aids in its ability to react with oxidative species via electron transfer before the oxidant can react with any biological molecules in the body (Simic et al., 1989). Uric acid acts by forming redox inactive complexes with transition metal ions (Davies et al., 1986) and by intercepting strong oxidants including the hydroxyl radical, peroxyxynitrite, singlet oxygen, lipid hydroperoxides and hypochlorous acid (Ames et al., 1981, Becker et al., 1989, Iqbal et al., 1999, Whiteman et al., 2002). Uric acid functions as a non-catalytic binder of iron which aids in the protection of DNA and helps minimize the damage done by the hydroxyl radical generated by

the reaction between iron, adriamycin and hydrogen peroxide (Miura et al., 1993) and also has the ability to react with ozone to prevent oxidation of plasma lipids and lipoproteins (Cross et al., 1992). Although uric acid is unable to protect alcohol dehydrogenase from inactivation when exposed to superoxide, uric acid can inhibit superoxide-mediated DNA damage (Cohen et al., 1984).

Purine nucleotides play a large role in the evolution of uric acid. They are principle parts that are responsible for cellular energy, in the form of ATP, and engage in the formation of DNA as well as RNA. It is the breakdown of adenine and guanine based purines that lead to the formation of uric acid, which exists in the form of urate (Hediger et al., 2005, Benzie, 2000). In the avian species uric acid is formed primarily in the liver and is excreted by the kidneys into urine. Purine ring de novo synthesis starts from the activated ribose, 5-phosphoribosyl 1-pyrophosphate (PRPP), with the sequential addition of nitrogen and carbon containing units donated from either glutamine, glycine, aspartate, folic acid or CO₂. The first and committed step in purine synthesis is the donation of an NH₂ group by glutamine that displaces the pyrophosphate at ribose C₁ giving rise to 5-phosphoribosylamine. A series of reactions then ensue resulting in the production of the common purine precursor inosine monophosphate (IMP). IMP is converted to adenosine monophosphate (AMP) in two steps through the enzymes adenylosuccinate synthase and adenylosuccinate lyase. Purines are also produced by salvage mechanisms, while pyrimidine rings can be degraded completely to CO₂ and NH₃ (urea excretion), purine rings are degraded to the metabolically inert, uric acid. It is through this mechanism that birds fed ribose and/or purine precursors, such as inosine, may generate elevated concentrations of plasma uric acid. The exact opposite occurs when birds are fed inhibitors of the uric acid pathway. Such is the case in the treatment of gout, where individuals are given allopurinol. Allopurinol and its metabolite, oxipurinol, act as competitive inhibitors of xanthine oxidase, which in turn leads to a decrease in the amount of urate that is produced in the body (Hediger et al., 2005).

Humans, birds, reptiles and higher primates have increased levels of uric acid as compared to other mammals because they lack the primary terminal enzyme uricase, which is responsible for degrading uric acid into allantoin (Benzie, 2000, Hediger et al., 2005, Iqbal et al., 1999). However, in the presence of oxidative stress, urate can be converted to allantoin and other by-

products of oxidation (Hediger et al., 2005). In fact, allantoin concentrations have been measured in the plasma of birds, which suggests that non-enzymatic pathways are being exemplified by the reaction of uric acid and reactive oxygen species in order to generate this compound (Simoyi et al., 2003). Uric acid in its original form is almost completely insoluble; however, sodium urate, which is the major form, found in the body, is more soluble.

Uric acid has been shown to have a positive association with maximum life span across species (Hediger et al., 2005, Iqbal et al., 1999, Klandorf et al., 2002). Therefore, the greater plasma uric acid concentrations are, the greater the life span of the individual species will be. Evidence of this lies in the avian species, as they have greater longevity for life compared to their body size. Birds, when compared to other species of similar size, have increased metabolic rates, high body temperatures and high glucose levels which should make them more susceptible to oxidative stress and radical onslaughts, however the opposite occurs (Hediger et al., 2005, Simoyi et al., 2002). It has been hypothesized that uric acid is responsible for the increased lifespan of numerous species including primates, reptiles and birds.

ROS GENERATION IN BIRDS

Reactive oxygen species are produced in the body as a consequence of numerous metabolic processes. Aerobic metabolism is one such process that can cause oxidative damage throughout the body, which is a very important factor in aging and age related diseases like cancer, diabetes, heart disease and inflammatory response. However, free radicals are implicated in the aging process because of the inverse correlation between the specific metabolic rate (ml of O₂ consumed per gram of body weight per hr of a given species and the life span of that species) (Adelman et al, 1987). The explanation that Adelman et al, (1987) gives for this is that smaller animals have increased metabolic rates and will therefore consume more oxygen which will lead them to produce more ROS. As their consumption of oxygen increases, so does the damage to critical areas in the body. Overall, the increase in oxidative damage will produce a higher rate of aging in smaller species.

On the other hand, we have the avian species that seems to be the exception to the rule, that small animals have increased oxidative stress. The reason they are the exception is the fact that most birds are surprisingly longer lived than most mammals of comparable body size (Klandorf et al, 2001). This longevity for life comes at quite a shock because the avian species possesses many characteristics that, based on theories of aging, would render them more vulnerable to oxidative onslaughts from ROS (Simoyi et al, 2002). Such characteristics begin with an increased metabolic rate that can be 2-2.5 times greater than mammals of the same size (Lindstedt and Calde, 1976). This increase requires a higher rate of oxygen to be used and therefore causes a dramatic rise in the amount of ROS that are being produced within the system of the bird, leading to increased oxidative damage and aging. The next characteristic that sets the avian species apart is that, their plasma glucose levels are typically 2-6 times greater than mammalian counterparts (Iqbal et al, 1999). Glucose is responsible for helping generate Maillard reactions (Stinefelt et al, 2005), that aid in tissue crosslinking and polymerization, which are all signs of aging (Iqbal et al, 1999). The last characteristic that distinguishes the avian species is their elevated basal body temperature, which is about 3° C higher than other mammals (Holmes and Austad, 1995). When we mix all three of these traits together, the end product becomes a recipe for elevated ROS production, glycosylated proteins, oxidative damage and aging.

How does the avian species do it? What is it in their make-up that allows them to produce so many free radicals, yet not feel the effects of those radical attacks? The synergistic theory of aging states that the accumulation of these glycosylated end-products are what leads to the continual aging, oxidative stress and damage of DNA, proteins and lipids throughout the life of any organism (Iqbal et al, 1999). Despite all of this, the avian species are the only vertebrate animals that show a high basal metabolic rate and a high maximum life span (Herrero and Barja, 1998).

Improvements in overall lifespan of the avian species have been attributed to increases in antioxidant defense systems. Antioxidants have the ability to protect against the development of degenerative diseases and the aging process (Barja, 1998). Previous studies have concluded that the avian species has increased levels of antioxidants (α -tocopherols, carotenoids, and uric acids)

when compared to other mammals of similar size (Klandorf et al, 2002). These highly advanced antioxidant defense systems that birds have are complex and organized in order to fight off any type of ROS that attacks. Studies are now finding that uric acid may be one of the most important antioxidants when it comes to prevention and protection from free radicals. The problem with uric acid is that most animals have uricase, the primary terminal enzyme that is responsible for the breakdown of uric acid into allantoin (Benzie, 2000, Hediger et al., 2005, Iqbal et al., 1999). Humans, birds, reptiles and higher primates lack uricase which is responsible for the significant increase in plasma uric acid concentrations found in non-uricase producing mammals (Klandorf et al, 2001). Along with high plasma uric acid concentrations, birds have the ability to wrap uric acid in a protein coat, within the nephrons, so that uric acid crystals remain soluble and don't harm the kidneys (Braun, 1997 and Simoyi, 2002). These traits, along with an increased basal body temperature, allow the avian species to maintain a two-fold greater plasma uric acid concentration than that of humans (Iqbal et al, 1999). As a result, uric acid is associated with increased life spans and antioxidant defense properties. In previous studies involving uric acid manipulation, decreases in plasma uric acid lead to an increase in oxidative stress (Klandorf et al, 2002). Another study conducted by Simoyi et al (2002), showed that by supplementing feed with hypoxanthine and/or inosine, that plasma uric acid concentrations could be increased and oxidative stress could be lowered. Inosine is a naturally occurring purine that occurs during the breakdown of adenosine to uric acid (Mabley et al, 2002). Both of these studies indicate that uric acid is an extremely important antioxidant defense mechanism for the avian species.

JUSTIFICATION

Can increasing a bird's antioxidant defenses reduce oxidative stress and markers of free radical mediated damage? Better yet, can antioxidant defense systems be elevated prior to the onset of oxidative stress in order to provide protection from reactive oxygen species? Increasing our knowledge about various antioxidant defense systems can allow us to find various ways of dealing with the constant onslaught of free radicals and the cell mediated damage that follows. Previous studies have shown, that by increasing vitamin E, a free radical quenching antioxidant,

we can lower cardiac tissue thiobarbituric acid reactive substances (TBARS), an indicator of oxidative stress (Villar-Patino et al, 2002). However, it is necessary to fully understand uric acid metabolism and identify the most uricogenic compounds so that we can achieve the long-term goal of reducing oxidative stress by elevating uric acid levels. In a previous study by Simoyi et al 2002, they showed that elevating plasma uric acid levels in broilers, using an oral purine precursor, lead to kidney hypertrophy. The ability of elevated plasma uric acid level via inosine to decrease oxidative stress as well as protect against it, over a short period of time has not been investigated.

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CHAPTER ONE

Inosine Ameliorates the Effects of Hemin Induced Oxidative Stress

INTRODUCTION

Molecular oxygen is arguably the most important element of the periodic table. Without it there would be no water, no air to breathe and most species on earth would cease to exist. Although oxygen is important to life on earth, it acts as a two edged sword providing numerous advantages on one side, but on the other hand, has the potential to create multiple forms of toxicity (Fridovich, 1983). It is the consumption of oxygen and the direct incorporation of it into biomolecules that leads to the production of reactive oxygen species (ROS). Simple metabolic processes that naturally occur during aerobic energy catabolism are responsible for creating ROS, which will ultimately lead to the oxidative damage of proteins, lipids and DNA (Benzie, 2000). Reactive oxygen species are the fundamental cause of numerous negative biochemical changes that occur throughout an organism's life and are indicative of oxidative stress. Once reactive oxidative species are created, the bodies own natural defense systems come into play in order to minimize the generation and help counteract the damages that are brought on by these highly reactive and unstable species. Over time, constant exposure to these deleterious species leads to cellular damage and life threatening diseases.

Evolution's answer to this oxygen paradox relies on a wide range of antioxidant defense mechanisms that are extensive, diverse, coordinated and effective on many different levels (Benzie, 2000). Protection from ROS is a highly complex system that takes into consideration which reactive species is generated, how it is generated, where it is generated and what target tissue is being damaged (Halliwell, 1998). Therefore, it is safe to say that no one antioxidant is 100% effective against all types of ROS (Papežíková et al., 2006). However, each antioxidant plays a significant role in protecting every species from oxidative onslaughts. Overcoming these oxidative attacks is crucial to the survival and longevity of each species.

Exogenous antioxidants that can only be taken in through the diet are becoming an ever more popular movement. The increase in trying to identify these possible antioxidants rises from finding a treatment, or prevention, for diseases caused by ROS. Recent studies have shown that

complementing diets with substances like fruits and vegetables can help aid individuals in fighting cancer, heart disease, and many other types of illnesses (Leonard et al., 2003). One such substance that has come into play is resveratrol, a polyphenol found in grapes, red wine, and other foods (Leonard et al., 2003, Borra et al., 2005). It has the ability to scavenge $\cdot\text{OH}$ and $\text{O}_2\cdot^-$ while inhibiting lipid peroxidation and DNA damage brought on by Fenton reactions (Leonard et al., 2003). Resveratrol may also aid in a type of redox regulation in platelets due to the fact that ROS are present in blood platelets and act as second messengers (Olas et al., 2004). While recent studies have shown that resveratrol is a scavenger of ROS, they have also shown that it also has many useful properties including anti-inflammatory, antibacterial activities, as well as prevention of cancer and coronary heart disease (Chen et al., 2005, Imamura et al., 2002, Leonard et al., 2003). Despite the fact that resveratrol has protective properties, its mechanism of action is not totally understood and requires further studies (Imamura et al., 2002).

Uric acid is arguably one of the most important non-enzymatic extracellular antioxidants (Ames 1993). It is considered a useful antioxidant and radical scavenger because it has the ability to interact with biological oxidants and radicals, the end products of these interactions are less harmful than the original components, and it is also found in high enough concentrations within the tissues to insure significant reactions (Becker, 1993). However, uric acid is also viewed as a preventative antioxidant because it not only intercepts strong oxidants like hydroxyl radicals, peroxyxynitrite, singlet oxygen, lipid hydroperoxides, and hypochlorous acid, but has the ability to form redox inactive complexes with transition metal ions (Papežiková et al., 2006, Stinefelt et al., 2005). Such defensive antioxidants have the ability to diminish the activity of metal ions by decreasing their ability to participate in Fenton chemistry, which leads to a decrease in oxidative damage.

Humans, birds, reptiles and higher primates have increased levels of uric acid as compared to other mammals because they lack the primary terminal purine oxidative enzyme uricase, which is responsible for degrading uric acid into allantoin (Benzie, 2000, Hediger et al., 2005, Iqbal et al., 1999). However, in the presence of oxidative stress uric acid can be converted to allantoin and other by-products of oxidation (Hediger et al., 2005). In fact, allantoin concentrations have been measured in the plasma of birds, which suggests that non-enzymatic pathways are being

exemplified by the reaction of uric acid and reactive oxygen species in order to generate this compound (Simoyi et al., 2003).

Uric acid has been shown to have a positive association with maximum life span across species (Hediger et al., 2005, Iqbal et al., 1999, Klandorf et al., 2002). Therefore, higher plasma uric acid concentrations produce a greater life span of the individual species. Evidence of this occurs in the avian species, since they have greater longevity for life compared to animals of comparable body size. Birds, when compared to other species of similar size, have increased metabolic rates, increased body temperatures and elevated glucose levels that make them more susceptible to oxidative stress and radical onslaughts, however the opposite occurs (Hediger et al., 2005, Simoyi et al., 2002). It has been hypothesized that uric acid is the antioxidant responsible for the increased lifespan of numerous species including primates, reptiles and birds.

In the current study, altered uric acid levels are linked inversely to measurements of oxidative stress. The specific objective was to determine if the oral inosine treatment, which increases uric acid production, might serve as a potential therapy for oxidative stress in broiler chickens. Inosine is a naturally occurring purine that can be derived from the oxidative deamination of adenosine (Mabley et al., 2005), and is directly involved in the formation of uric acid via hypoxanthine and xanthine production via xanthine oxidase, which increases plasma uric acid levels when supplemented in the diet. Inosine is thus hypothesized to decrease oxidative injury whether it is supplemented before or after the onset of oxidative stress.

MATERIALS AND METHODS

Birds and Management: One day old broiler chicks (n=40; Ross X Cobb; mixed sex) were obtained from Pilgrim Farms in Moorefield, WV and maintained under standard husbandry practices. Broiler chicks were floor reared and given a starter diet *ad libitum* in pan feeders, drinking water, and temperatures were maintained at recommended levels for a six week rearing period. Provisions for space, temperature, light, and husbandry were rigidly followed (Ross

Breeders, 1996). Starter feed was given to the broiler chicks during the first three weeks. Afterward, the birds were given a standard grower feed. Blood samples were obtained from the wing vein and placed into heparinized tubes for measurement of plasma uric acid (PUA) and leukocyte oxidative activity (LOA). Body weights (BW) were taken biweekly until termination of the experiment. Feed intake was observed throughout the experiment. Broilers were euthanized at 6 weeks of age.

Uric Acid Determination: Blood samples were collected and plasma uric acid (n=5 per treatment group) was determined using a commercially available Uric Acid Reagent kit (Sigma Chemicals, St. Louis, MO).

L-012 Based Chemiluminescence as a Measurement of Oxidative Stress: Chemiluminescence techniques are functional assays to quantify the release of oxidants from cells or tissues (Van Dyke, 1987). L-012-based chemiluminescence (L-BCL) was used to determine the amount of LOA as described by Iqbal et al, 1999. Two milliliters of blood from 4-and 5-week-old birds (n=20) was carefully layered on 2ml of mono-polyresolving medium in a 13 X 100 mm #10 Falcon tube and leukocytes were isolated by centrifugation at 2000 X g for 20 minutes. To a 3ml luminometer tube was then added 100µl of leukocytes, 100µl L-012 solution, 200µl PBS (pH=7.4) and 100µl phorbol myristate acetate (PMA). The reaction tube was then placed into the luminometer (Berthold model LB 9505C) with the temperature set at 37°C. Oxidative stress was determined by measuring the integrated luminescence generated over 20 min using KINB software. Results were reported as counts per minute (CPM). Measured luminescence was corrected based on the number of leukocytes present in each reaction tube.

Buffer Mixture: Throughout both studies a buffer solution was used in order to solubilize hemin. The hemin buffer solution contained 1ml of dimethylsulfoxide (DMSO), 1ml of PMA (pH=7.4), and an amount of hemin (20mg/kg BW) adjusted for each bird, according to weight.

Preliminary Study for Effect of Inosine on Kidney Morphology: Throughout this preliminary study ten (n=10), four-week old broilers, were divided into two groups consisting of five inosine treated (n=5) and five control birds (n=5). On day one inosine was included in the grower diet of

the inosine birds, at 0.6M/kg of feed/day, and control birds were given a grower diet. Birds in both control and inosine treatments were given feed and water *ad libitum*. After seven days birds were killed by cervical fracture, and the abdominal cavity opened ventrally to reveal the kidneys. The dorsal aorta just cranial to the kidneys was cannulated, and 20 ml of 0.1M Phosphate buffer (pH 7.2) was injected to clear the kidneys of blood. Immediately following this procedure, 20 ml of 10% neutral buffered formalin (NBF) was injected to fix the kidneys. The kidneys were carefully dissected out of the synsacrum and immersed and stored in 10% NBF.

The tissue was then processed routinely for light microscopy through a series of graded alcohols, toluene then into paraffin wax. The tissue was embedded in paraffin wax and cut at 5 μm in an unbiased manner at 10 equally-spaced intervals along its length (Mayhew, 1991). The resulting sections were stained with hematoxylin and eosin.

Volumes of the kidney components (cortex, medulla, major blood vessels) and nephron components (renal corpuscle, proximal tubule, loops of Henle, distal tubule, collecting ducts) were estimated by point counting using the Cavalieri Principle (Gundersen et al., 1988).

Experimental Design Study 1: At four weeks of age, 20 broilers were weighed, individually banded, and randomly divided into four groups: control, hemin injected (20mg/kg body weight (BW) w/ buffer), inosine fed (0.6M/kg of feed/day), and hemin/inosine. The broilers received a grower diet and drinking water *ad libitum*. On day one, each bird (n=20) was injected daily with 2mL of buffer solution or hemin buffer solution. Throughout the study control birds (n=10) were injected daily with a buffer solution, while hemin birds (n=10) were injected daily intraperitoneal with a 20mg/ kg BW hemin buffer solution. Blood was drawn from the wing vein of each bird on day eight and measured for levels of leukocyte oxidative activity (LOA) and plasma uric acid (PUA). On day 10, control and hemin birds were subdivided into inosine fed birds (n=5) and hemin/inosine fed birds (n=5). These birds were provided 0.6M/kg of feed/ day of inosine mixed in with the feed. Blood was then taken on day 15 and measured for levels of LOA and PUA. On day 16 birds were euthanized.

Experimental Design Study 2: Four week old broilers (n=20) were weighed, individually banded, and divided into four groups: control, hemin injected (20mg/kg BW w/ buffer), inosine fed (0.6M/kg of feed/day), and hemin/inosine. The broilers received a grower diet and drinking

water *ad libitum*. Beginning on day one, inosine/hemin birds (n=5) were pretreated with 0.6M/kg of feed/day inosine mixed in with their feed while control birds (n=15) were given a standard grower diet with no supplementation. On day three, blood samples were drawn and measured for LOA and PUA levels. After the bleeding, control birds were further divided into control, hemin and inosine groups. Hemin buffer was then injected into hemin and hemin/inosine groups and the standard buffer was injected into the control and inosine groups. At the same time, inosine was added to the diet of the inosine group. On day five, blood samples were obtained and once again measured for levels of LOA and PUA. On day six, the birds were euthanized.

Statistical Analysis: One way ANOVA's were used to compare the volumes of the kidney and nephron components. Significance was set at the 95% confidence interval for the preliminary study done on inosine dosage.

Data was analyzed by analysis of variance (ANOVA) and performed with PC-SAS software (SAS Institute, 2000). A general linear models procedure was used to determine significant differences among treatment means. Fisher's least significant difference test was used to compare group means. Contrasts were also run with hemin against all other treatments. Differences were considered significant at $P \leq 0.05$.

RESULTS

Preliminary Study on Dosage of Inosine on Kidney Morphology: One way ANOVA's revealed that there was no difference in percent of cortex, medulla or major blood vessels between treatment groups. Further, our results indicated no differences between treatment groups in the components of the nephrons (renal corpuscle, proximal and distal tubule, loop of Henle, cortical collecting tubule, collecting ducts and capillaries) (Fig. 1 & 2).

Study 1

Growth Response: In contrast to the preliminary studies done on inosine dose-response in which hemin was added to the diet, differences in body weight (BW) were significant between

treatment groups over a 16 day period (Table 1). On day one, birds were weighed for the first time revealing no significant differences in BW between treatments. On day six, the birds were weighed revealing significant differences in BW between treatments ($P=0.02$). The inosine group had increased BW which differed significantly from both hemin treated groups. Significant differences also occurred among the control group and the inosine/hemin group. No significant differences were found between the hemin-treated groups. No significant differences were found between the control and inosine-treated groups. Again, on day 10, birds were weighed and a significant difference in BW was found ($P=0.01$). A significant difference occurred among the hemin and hemin/inosine treated groups when compared to the control and inosine-treated groups. No significant difference was found between the control and inosine groups. No significant difference was found between the hemin-treated groups. On day 16 the last measurements were made revealing a significant difference in BW between treatments ($P=0.004$). The control treatment differed significantly from both hemin-treated groups. However, it did not differ from the inosine-treated group. The inosine-treated group differed from the hemin/inosine treated group, but did not differ from the hemin group or the control group. Throughout the experiment, control and inosine treated groups never significantly differed in BW and Hemin and hemin/inosine groups never differed in BW.

Feed Intake: Feed intake varied between treatments (Table 2). The control group, as well as the inosine group consumed more feed (kg) than did the hemin or hemin/inosine groups over a period of 16 days. Overall, differences in feed intake were not significant.

PUA and LOA: On day 8 the PUA concentration of hemin and hemin/inosine treatments were not different when compared to control and inosine treatments ($P=0.11$) (Figure 3). On day 15 after the administration of inosine into the feed, PUA concentrations of inosine and hemin/inosine treatments were significantly higher ($P=0.0001$) compared to control and hemin treatments as shown in Figure 5. Hemin treatment was contrasted against all other treatment groups and found to have lower PUA concentrations ($P=0.0007$).

The LOA on day 8 for hemin and hemin/inosine treatments were increased compared to control and inosine treatments ($P=0.0333$). The hemin groups were significantly higher in LOA concentrations when compared to control and inosine groups ($P\leq 0.05$) (Figure 4). Once inosine

had been added to the diet, results on day 15 indicated that the hemin/inosine treatment decreased LOA to levels measured in inosine fed birds on day 8 (Figure 4 & 6). Control and inosine treatments continued to have low levels of LOA ($P \leq 0.0044$). Figure 6 also shows that the hemin group maintained higher levels of LOA when compared to all other treatments ($P \leq 0.001$)

Study 2

Growth Response: Within the course of a week no significant differences in BW were found between any of the four groups (Table 3).

PUA and LOA: On day three, the PUA concentration of the pretreated hemin/inosine group was significantly increased ($P \leq 0.05$) when compared to the control birds (Figure 8). On day five, after the hemin injections and inosine supplementation, the PUA concentration of inosine and hemin/inosine groups were significantly different when compared to control and hemin groups ($P < 0.000$) (Figure 10). Inosine and hemin/inosine groups had increased levels of PUA when compared to both control and hemin groups.

The LOA on day three for the control and hemin/inosine groups were different. Hemin/Inosine birds had decreases levels of LOA when compared to the control birds (Figure 9). On day five, after the hemin injections and supplementation of inosine, control, inosine, and hemin/inosine treatments had significantly lower levels of LOA when compared to the hemin treatment ($P < 0.0033$). Inosine, as well as hemin/inosine groups had levels of LOA that were not significantly different than that of the control group (Figure 11).

DISCUSSION

Oxygen's vital role in life comes at a cost to every aerobic organism. It is the consumption of oxygen and the direct incorporation of it into biomolecules that leads to the production of ROS. Reactive oxidative species are proficient in causing multiple forms of oxidative damage to various cellular components. They attack and oxidize key structural and

genetic molecules of the body such as DNA, proteins and lipids which in can leads to mutations, disease states and tissue injury (Benzie, 2000). However, for every reaction there is an opposing reaction, and this is found in the form of antioxidants. Constant inhibition of oxidative stress by antioxidants is crucial for the survival and increased maximum life span of each particular species. It is antioxidant defense systems that preserve homeostasis in order for normal cell function to occur (Banerjee et al., 2003). Uric acid (UA) is considered a physiologically useful antioxidant and radical scavenger because it has the ability to interact with biological oxidants and radicals; the end products of these interactions are physiologically less harmful than the original components. Uric acid is also found in high enough concentrations within the tissues to insure significant protective reactions (Becker, 1993).

It has been established that birds have remarkable longevity for their body size (Holmes & Austad, 1995) despite the fact that they also have much higher metabolic rates, body temperatures and blood glucose concentrations when compared to mammals (Ogburn et al., 1998). Theoretically, birds should sustain more damage from processes that lead to ROS. However, the exact opposite occurs which would suggest that birds have a highly developed mechanism for dealing with oxidative stress. Studies have shown that uric acid plays a significant role in limiting oxidative stress and thus increasing uric acid concentrations via inosine supplementation reduces measures of oxidative stress in chickens (Simoyi et al., 2002).

In previous studies, the inclusion of inosine (0.6 mol/kg) in the diet of broilers for 3 wks increased plasma concentrations of uric acid and decreased oxidative stress (Simoyi et al., 2002). Our studies were in agreement with previous studies because the inclusion of inosine in the diets of broilers at a dose of 0.6mol/kg for 7 days resulted in an increase in concentrations of plasma uric acid concomitant with a decrease in oxidative stress. However, Simoyi et al (2002) showed that inosine, given over a three week period of time, increased kidney weight three fold compared to controls. Our results indicated that the administration of inosine for a seven day period did not affect kidney weight or ultrastructure. (Figures 1& 2). In the avian kidney, uric acid is bound to a protein in the proximal tubule, and chronic increased uric acid levels may lead to an increase in morphology of the kidney (Simoyi et al., 2002). This suggests that short bouts of inosine included in the diets of broilers may be advantageous in treating oxidative damage.

In a previous study, Klandorf et al (2001) showed that hemin was associated with an enhanced elevation of oxidative stress in broilers. Hemin is a rich source of metal ions like iron

and has the ability to generate ROS via Fenton chemistry. A simple mixture of an iron salt and H_2O_2 can set into motion, a chain of events that will lead to the generation of free radicals (Halliwell et al., 1990). From this, we concluded that by supplementing broiler diets with hemin we could increase oxidative stress and therefore could potentially develop a model for testing the efficacy of certain compounds to determine whether or not they could serve as a useful antioxidant or treatment against oxidative damage.

In the preliminary studies the graded administration of hemin to the diets of the broiler chicks resulted in characteristic dose dependent increases in oxidative stress as well as a significant decrease in concentrations of uric acid. However, after 3 to 7 d the levels of oxidative stress decreased and uric acid returned control levels again. The reduction in oxidative stress was associated with a decrease in food intake, which resulted in a decline in body weight. A possible explanation for this was that the palatability of the diet was reduced, which led to the decline in food intake. Once they stopped consuming the diet, they reduced the consumption of hemin, which led to a decrease in oxidative stress. For each of these preliminary studies we found inconsistent measurements of oxidative stress for all hemin birds, which led to the conclusion that the intake of hemin was variable among the birds. For this reason we needed to ensure a more reliable way to administer the correct doses of hemin in order to increase oxidative stress. Thus in the two principal studies we decided to inject the birds with hemin rather than administer it to them in the feed. Birds were injected daily (i.p.) to insure that they each received the correct dose of hemin according to body weight. Once we established a steady state level of oxidative stress then we could supplement the inosine into the diet.

In study one we demonstrated that dietary supplementation of inosine effectively reduced hemin induced oxidative stress in vivo. During the first phase of the study hemin significantly increased the onset of oxidative stress and decreased uric acid levels in broilers injected with hemin before inosine was supplemented into the diets (Figure 4 & 5). Birds injected with hemin had significantly reduced body weight (Table 1) as well as decreased feed intake (Table 2) when compared to birds that were being injected with a placebo. After inosine was supplemented into the diet, concentrations of uric acid were increased (Figure 6) while oxidative stress declined almost to control levels (Figure 7).

Our second study demonstrated that pretreatment with inosine limited the development of oxidative stress in hemin-injected broilers. Birds were supplemented with inosine for 3 d, which

resulted in a significant increase in uric acid (Figure 8) and a reduction in baseline oxidative stress levels (Figure 9). Once the hemin birds were injected, concentrations of uric acid remained significantly elevated above the control and hemin groups (Figure 10). Pretreatment with inosine significantly attenuated the increase in oxidative stress in hemin injected broilers (Figure 11).

In conclusion, the present investigation supports the critical role of uric acid as an antioxidant in birds and specifically demonstrated that inosine-induced increases in uric acid concentrations in chickens is associated with a reduction in oxidative stress. Further, the administration of inosine, a precursor to uric acid, has the ability to either limit the increase in oxidative stress associated with a pro-oxidant or reduce the magnitude of a stress in an animal already oxidatively stressed. Inosine may thus represent a treatment modality for certain avian disorders associated with inflammation and oxidative stress. These may well represent a majority of avian acute and chronic diseases.

Figure 1: Effects of Inosine on the Vasculature of the Kidney

Preliminary study of Inosine and its effects on kidney volume with comparison of cortex, medulla and major vasculature within the kidneys of the birds fed control and inosine diets for 1 wk.

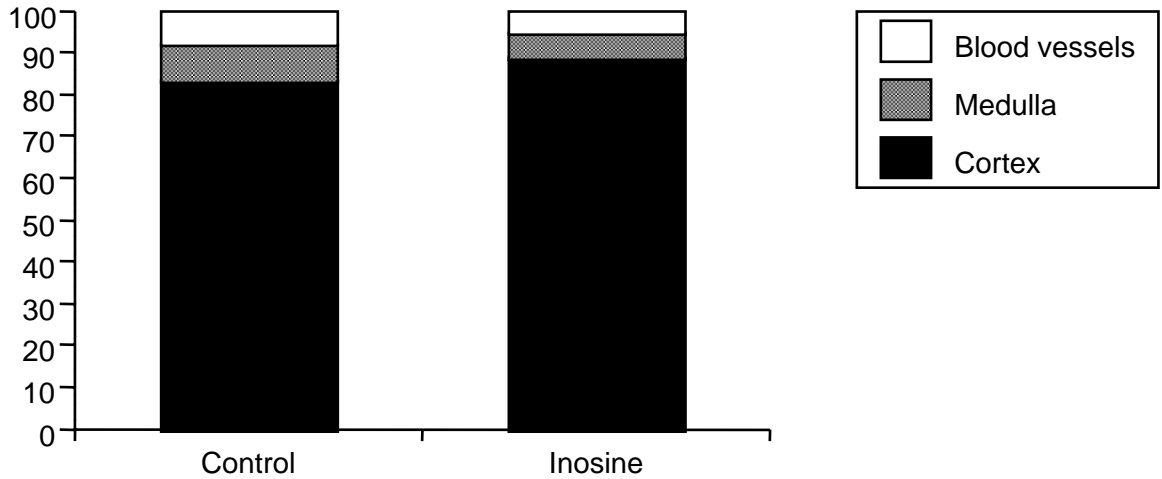
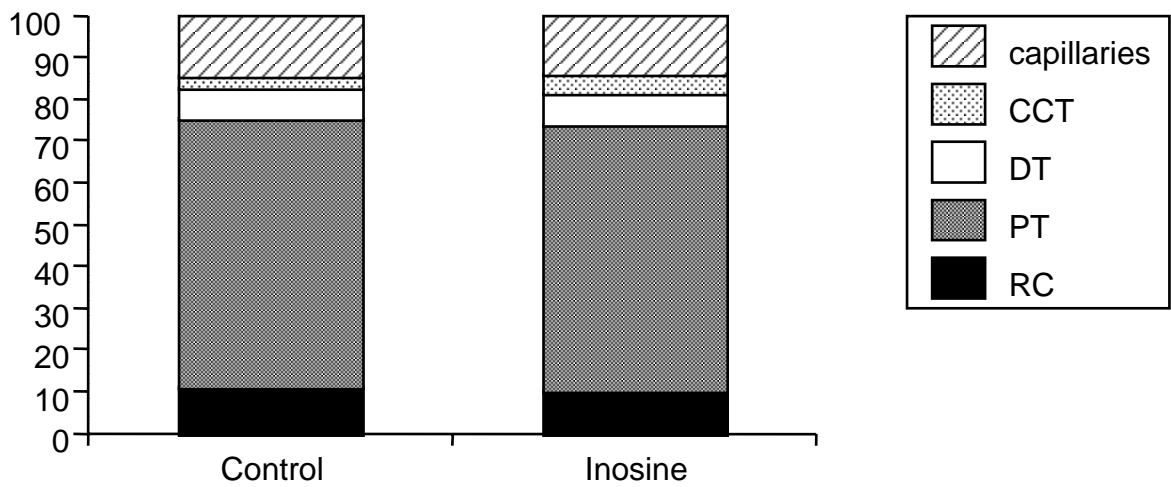


Figure 2: Effects of Inosine on the Nephron Components of the Kidney

Preliminary study of Inosine and its effects on kidney volume in the nephron components within the cortex of the birds fed control and inosine diets for 1 wk.



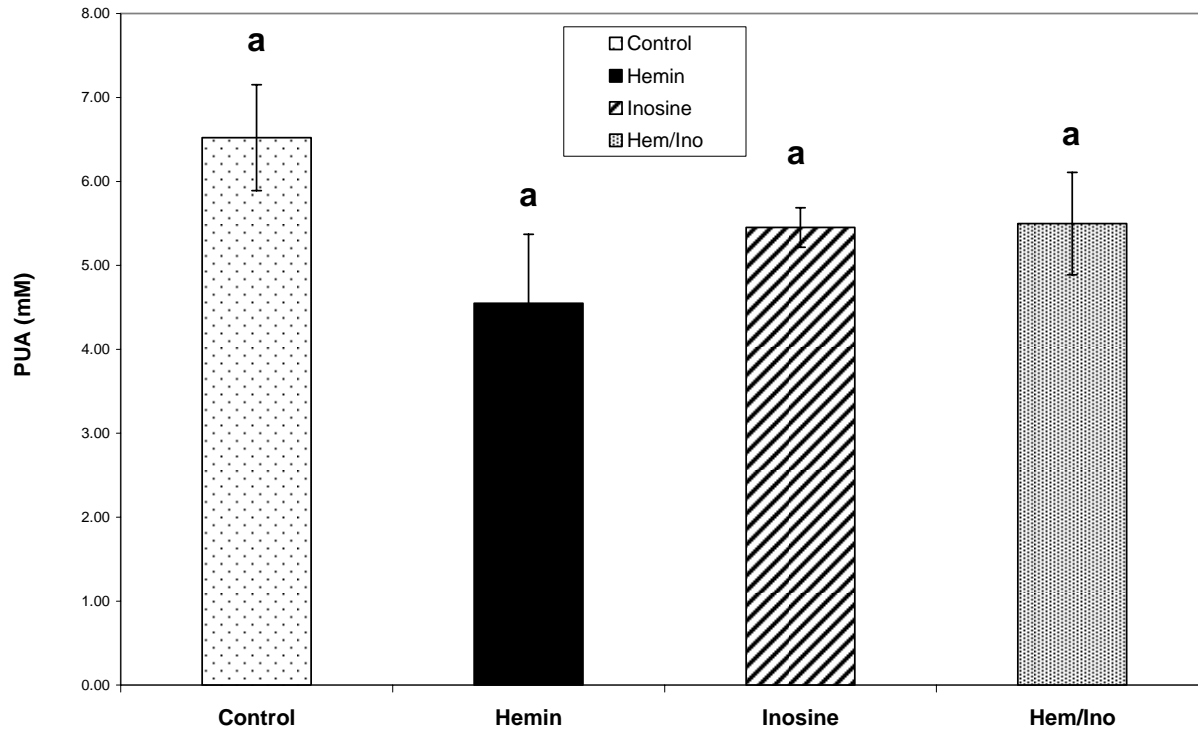


Figure 3: Study 1: Effects of Hemin on Plasma Uric Acid Concentrations (PUA) (Day 8)

All broilers fed a grower diet and injected (i.p.) with a control or hemin injection for 8 days.

Treatments with different letters differ significantly ($P \leq 0.05$).

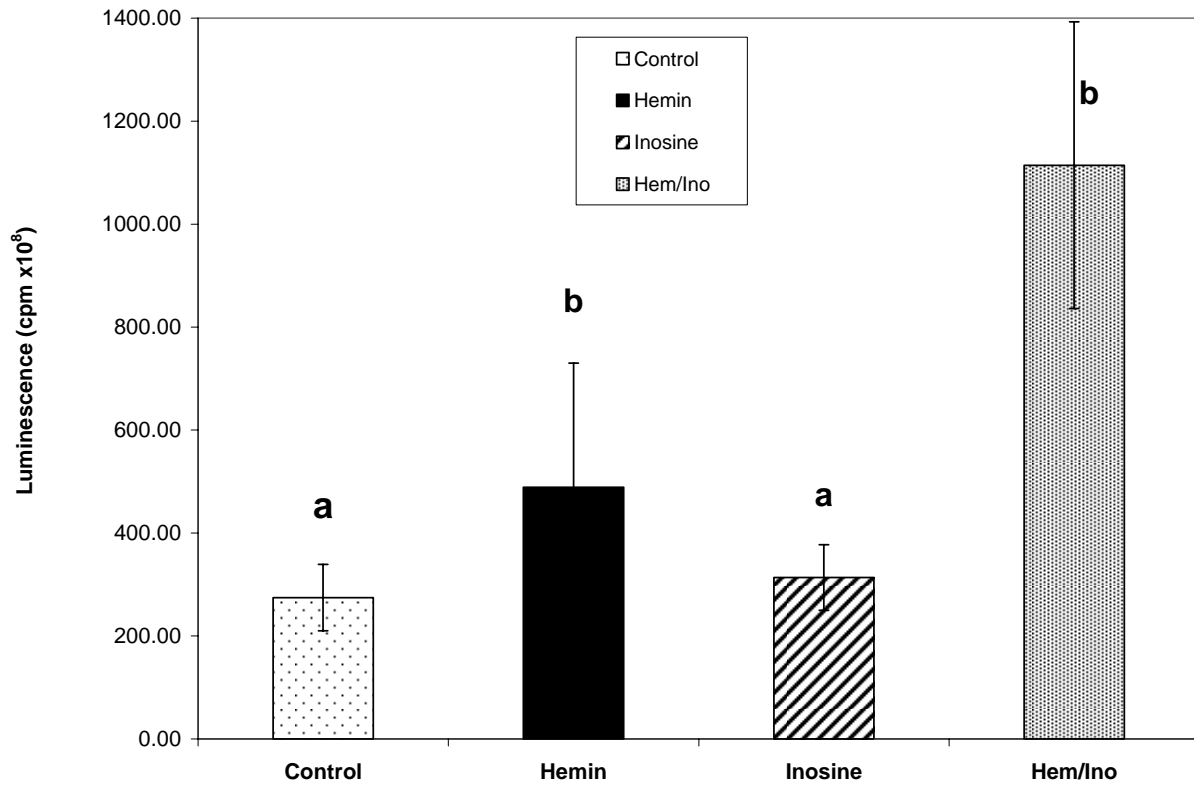


Figure 4: Study 1: Effects of Hemin on Leukocyte Oxidative Activity (LOA) (Day 8)

All broilers fed a grower diet and injected (i.p.) with a control or hemin injection for 8 days.

Treatments with different letters differ significantly ($P \leq 0.05$).

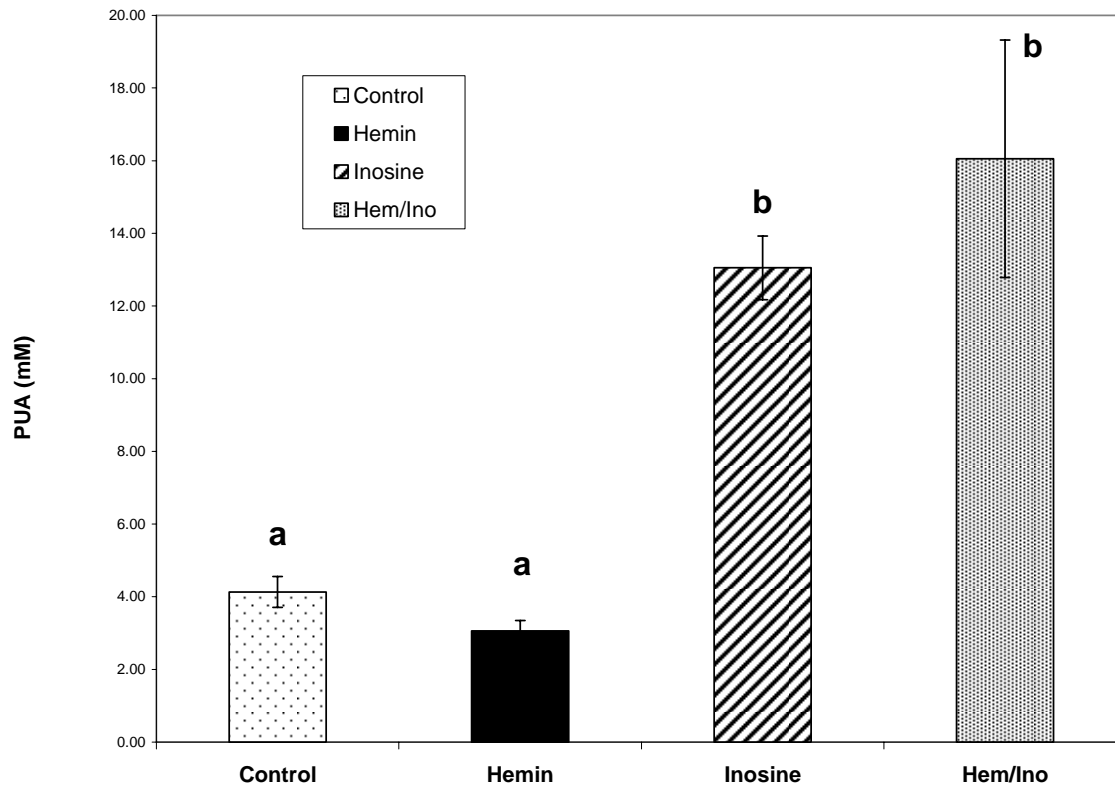


Figure 5: Study 1: Effects of Hemin and Inosine on PUA Concentrations (Day 15)

All broilers fed a grower diet and injected (i.p.) with a control or hemin injection for 15 days.

All inosine treatments had inosine supplemented in the feed for 1 wk. Treatments with different letters differ significantly ($P \leq 0.05$).

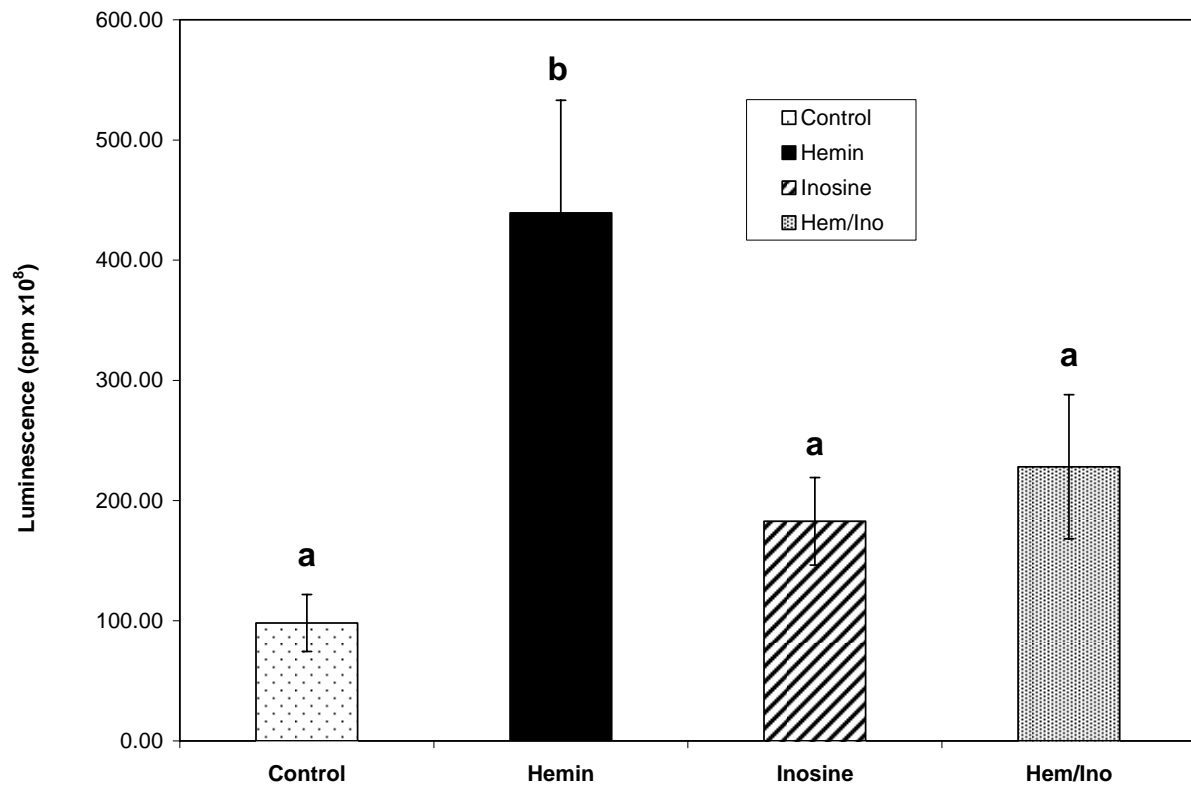


Figure 6: Study 1: Effects of Hemin and Inosine on LOA (Day 15)

All broilers fed a grower diet and injected (i.p.) with a control or hemin injection for 15 days.

All inosine treatments had inosine supplemented in the feed for 1 wk. Treatments with different letters differ significantly ($P \leq 0.05$).

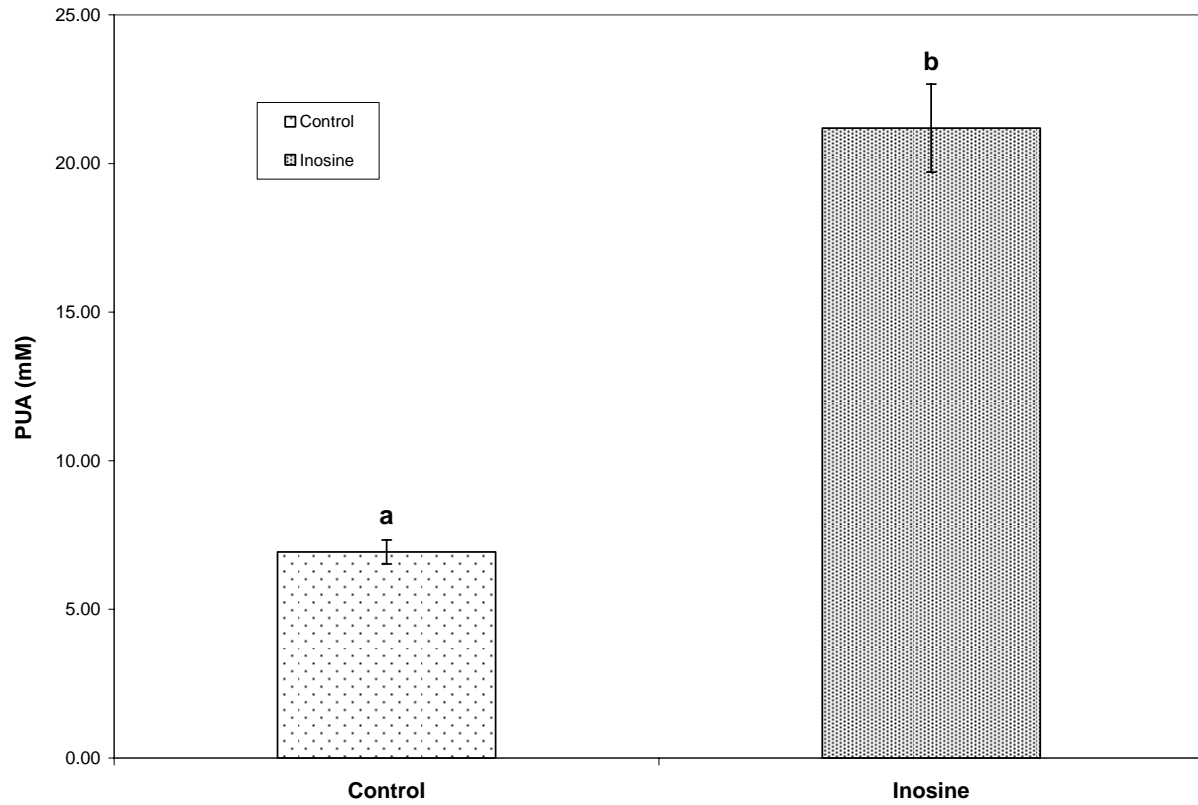


Figure 7: Study 2: Effects of Inosine on PUA Concentrations (Day 3)

All broilers fed a grower diet while inosine treatment was pretreated with purine supplemented feed. Treatments with different letters differ significantly ($P \leq 0.05$).

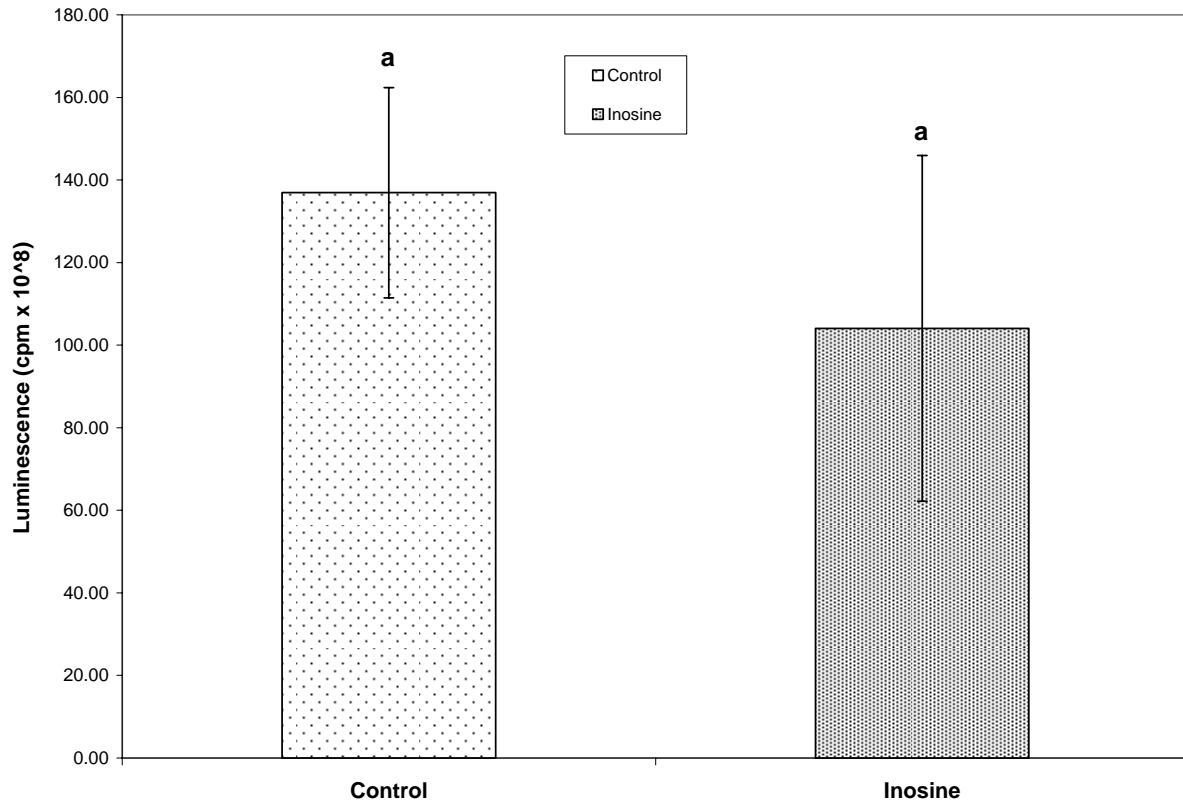


Figure 8: Study 2: Effects of Inosine on LOA (Day 3)

All broilers fed a grower diet while inosine treatment was pretreated with purine supplemented feed. Treatments with different letters differ significantly ($P \leq 0.05$).

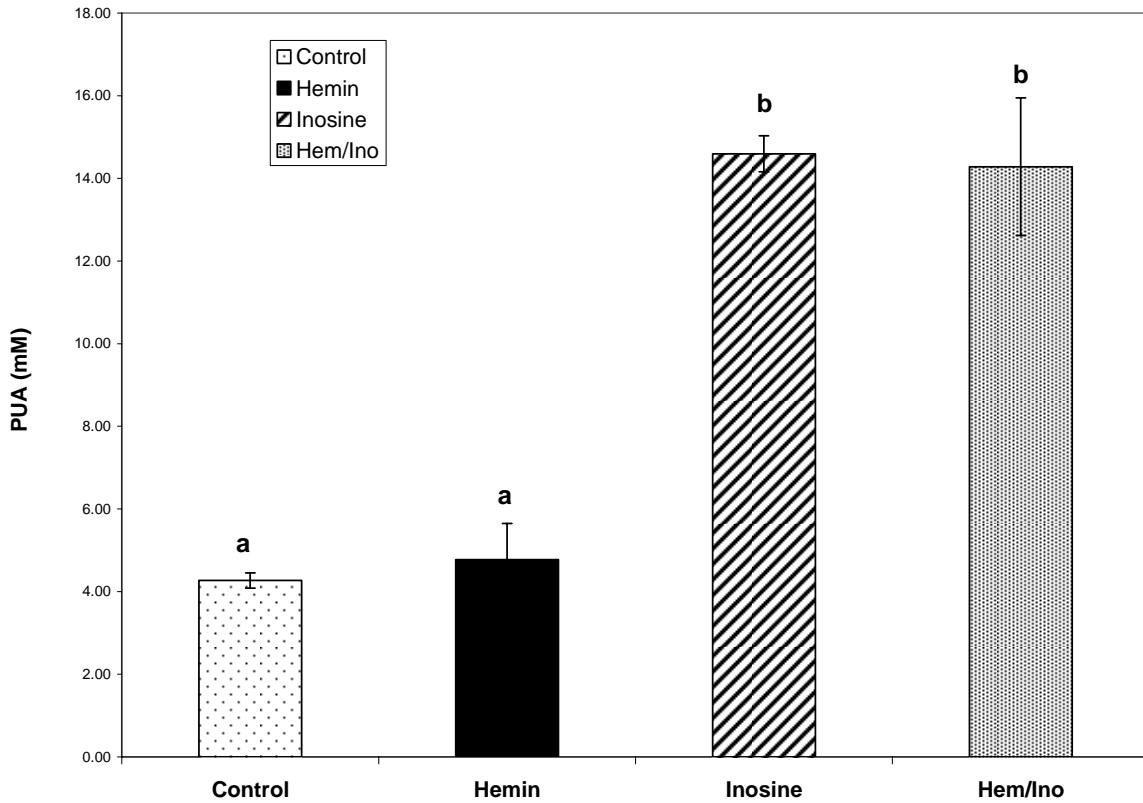


Figure 9: Study 2: Effects of Inosine and Hemin on PUA Concentrations (Day 5)

All broilers fed a grower diet and injected (i.p.) with a control or hemin injection for 2 days. Inosine treatment was pretreated with purine supplemented feed for 5 days. Treatments with different letters differ significantly ($P \leq 0.05$).

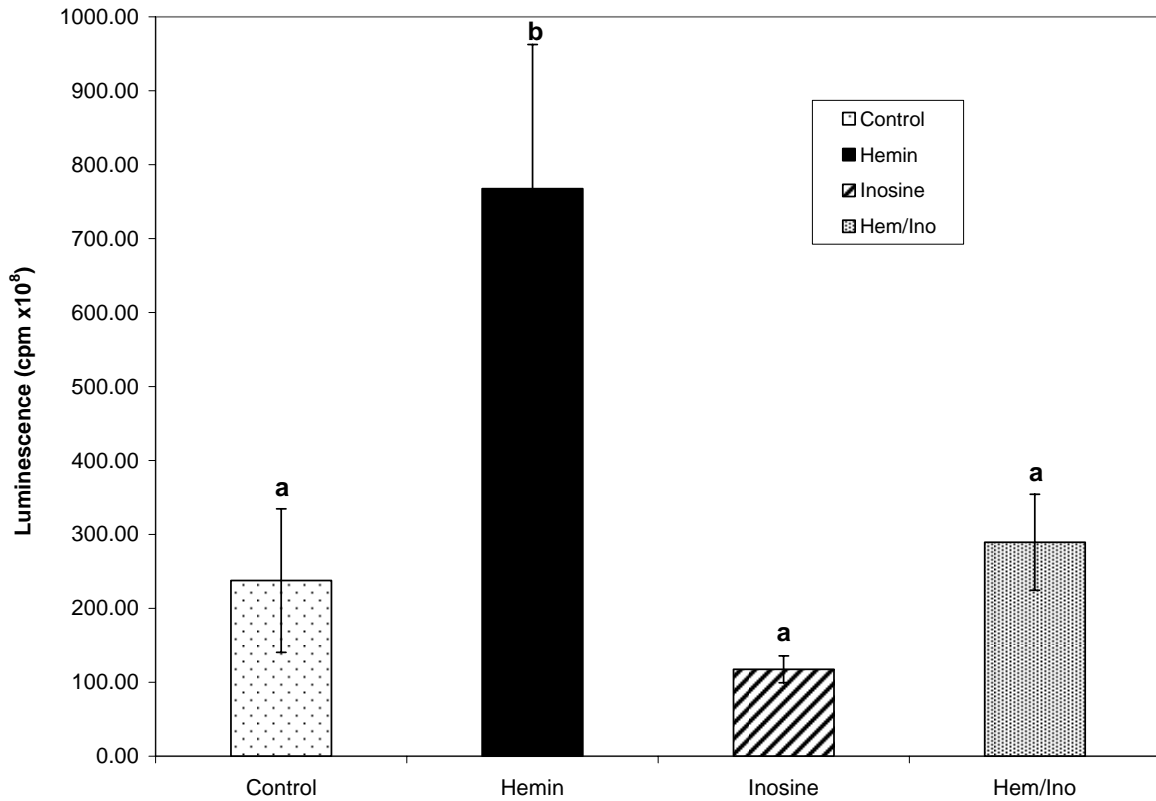


Figure 10: Study 2: Effects of Inosine and Hemin on LOA (Day 5)

All broilers fed a grower diet and injected (i.p.) with a control or hemin injection for 2 days. Inosine treatment was pretreated with purine supplemented feed for 5 days. Treatments with different letters differ significantly ($P \leq 0.05$).

Body Weight (kg) \pm SD*				
Treatment	Day 1	Day 6	Day 10	Day 16
Control	1.4416 \pm 0.1095	1.6532 ^{ab} \pm 0.0867	2.1252 ^a \pm 0.0633	2.6404 ^a \pm 0.1492
Hemin	1.3692 \pm 0.1171	1.4864 ^{bc} \pm 0.1084	1.7768 ^b \pm 0.2568	2.2388 ^{bc} \pm 0.2434
Hemin/Inosine	1.324 \pm 0.0944	1.4372 ^c \pm 0.1440	1.7736 ^b \pm 0.1777	2.0568 ^c \pm 0.2128
Inosine	1.3908 \pm 0.1222	1.6794 ^a \pm 0.1725	2.091 ^a \pm 0.2304	2.3692 ^{ab} \pm 0.2358
ANOVA P-value	0.4346	0.0237	0.0143	0.0041
Fischer's LSD value	-----	0.1771	0.2636	0.2863

*SD = Standard deviation

Table 1: Study 1: Effect of diet on growth performance with post-treatment of Inosine

Values are means \pm SD. Means with different superscripts within each column differ significantly ($P < 0.05$).

Feed Intake Over 16 Days	
Treatment	Feed Intake (kg)
Control	9.345
Hemin	6.985
Hemin/Inosine	7.330
Inosine	9.298

Table 2: Study 1: Effect of diet on feed intake with post-treatment of Inosine

Descriptive data on the overall feed intake for each treatment group over a 16 day period.

Pre-treatment of Inosine Bird Weights (kg)	
Treatment	Avg. Body Weight (kg)
Control	1.9328
Hemin	1.8640
Hemin/Inosine	1.8412
Inosine	1.7348

Table 3: Study 2: Effect of Diet on Growth Performance with Pre-treatment of Inosine

Descriptive data on average body weight of broilers pretreated with inosine for 1 wk.

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APPENDIX

Appendix 1: Diet Formulation for Broiler Grower Diets

Starter Diet for Swks.			<i>200 lbs</i>	<i>1/12/06</i>		
LEAST COST NUTRITIONAL ANALYSIS						
Custome	: WVU	- Joe Moritz	Page...	1		
Address	: Morgantown, WV	26506	Date...	1/12/2006		
			Time...	10:02 AM		
			Date Created	: 2/18/2003		
R&D	: West Virginia University		Rep. Phone No. :	293-2631 (4435)		
Rep	: Joe Moritz					
=====						
Lot Number:	1 - Broiler chickens					
Ration Number:	3 - NRC starter w/ drugs					
Number of Animals:	Breed: BROILER		Animal Code	: DEKALB		
Days on Ration :	Times Fed/Day	: 1	Ration Status	: FEASIBLE		
Production (%) :	50.00	Temperature	Feed Conversion:			
=====						
Lbs/Batch	Percent	Ingredient	Cost	Price Range	Ingredient	
A.F.	Dry	A.F.	Code Name	\$/CWT (As Fed)	Low High	Min Max
=====						
1180.6	1038.9	59.03	79 Corn, Ground	4.00		13.76
651.1	586.0	32.56	216 Soya Ml - 50	12.75	1.60	770.92
98.1	97.9	4.90	724 Soybean Oil	25.00		4788.08
39.5	39.5	1.97	416 Defl. Phosph	1.00		1405.66
13.8	13.8	.69	410 Limestone	1.00		10968.0
5.0	4.9	.25	NB3000 NB3000	100.00		5.0000 5.0000
4.8	4.7	.24	305 Methionine99	100.00		1205.27
4.5	4.5	.22	488 Salt	5.00		19623.9
1.6	1.6	.08	COBAN 60 Coban 60	100.00		1.6000 1.6000
1.0	1.0	.05	BMD BMD	100.00		1.0000 1.0000
=====						
2000.0	1792.8	100.00	Ration Cost \$/Ton: 167.97			
=====						
Num	Name	Unit	Minimum	Maximum	Sug Amt	Percent
=====						
1	Weight	Lbs	.2067	.2067	> .2067	1.00/Wt
2	Dry Matter	Lbs			.1853	89.64/Wt
107	Met. Energy-Poultry	KCAL	299.9767	299.9767	299.9767	1451.5/Wt
110	Crude Protein	Grams	18.7488		19.7262	21.04/Wt
111	Lysine	Grams	1.0312		1.0473	1.12/Wt
112	Arginine	grams	1.1718		1.2724	1.36/Wt
114	Isoleucine	grams	.7500		.8075	.86/Wt
115	Leucine	grams	1.1249		1.6948	1.81/Wt
118	Meth + Cystine	grams	.8437		.8437	.90/Wt
119	Phenylalanine	grams	.6750		.9245	.99/Wt
122	Threonine	Grams	.7312		.7312	.78/Wt
123	Tryptophan	grams	.1875		.2591	.28/Wt
124	Valine	grams	.8437		.8989	.96/Wt
127	Linoleic Acid	grams	.9374		3.6980	3.94/Wt
130	Calcium	GRAMS	.9374	.9374	.9374	1.00/Wt
132	Phosphorus - Avail	GRAMS	.4218	.4218	> .4218	.45/Wt
134	Sodium	GRAMS	.1875	.1875	.1875	.20/Wt
=====						
Percent Moisture in Ration: 10.4						
=====						
****	The information on this report represents our best estimates.					****
****	Since many factors other than the feed may affect production,					****
****	actual performance cannot be guaranteed.					****

Appendix 2: Diet Formulations for Broiler Grower Diet

Ingredients	Conventional Grower
Corn	59.13
Soybean	29.82
Soybean Oil	3.67
Corn Gluten	4.50
Defluorinated Phosphate	1.20
Limestone	1.10
Salt	0.18
Poultry Premix ^A NB3000	0.25
Methionine	0.02
Coban 60 ^B	0.08
BMD 50 ^C	0.05
Calculated Nutrients	
ME (kcal/kg)	3200
Crude protein (%)	21.07
Methionine (%)	0.38

^ASupplied per kg of diet: manganese, 0.02%; zinc, 0.02%; iron, 0.01%; copper, 0.0025%; iodine, 0.0003%; selenium, 0.00003%; folic acid, 0.69mg; choline, 386mg; riboflavin, 6.61mg; biotin, 0.03mg; vitamin B₆, 1.38mg; niacin, 27.56mg; pantothenic acid, 6.61mg; thiamine, 2.20mg; manadione, 0.83mg; vitamin B₁₂, 0.01mg; vitamin E, 16.53 IU; vitamin D₃, 2133 ICU; vitamin A, 7716 IU.

^BActive drug ingredient Monensin Sodium 60gpb (90g/ton inclusion)- Elanco Animal Health, Indianapolis, IN. As an aid in the prevention of coccidiosis caused by *Eimeria necatrix*, *Eimeria tenella*, *Eimeria acervulina*, *Eimeria brunette*, *Eimeria mivati*, and *Eimeria maxima*

^CBacitracin Methylene Disalicylate 50g/lb (50g/ton inclusion)- Alpharma, Fort Lee, NJ. For increased rate of weight gain and improved feed efficiency.