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Modulation of Aging Process by Diet and Crosslinking Inhibitor and their Effects on Production in Poultry

Muhammad Iqbal, D.V.M., M.Sc. (Hons)

Dissertation submitted to the Faculty of the College of
Agriculture, Forestry and Consumer Sciences
West Virginia University
in the partial fulfillment of the requirements for the degree of

Doctor in Philosophy

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Modulation of Aging Process by Diet and Crosslinking Inhibitor and their Effects on Production in Poultry

Muhammad Iqbal

(Abstract)

The factors which influence the age-related decline in production performance and increases in meat toughness in broiler breeder hens are poorly understood. We have hypothesized that the elevated plasma concentration of glucose contributes to the nonenzymatic attachment of glucose to proteins, and generates glycoxidation crosslinks in tissues which can ultimately impair function, is responsible for loss of production. One such crosslink, pentosidine, is an established indicator of both glycative and oxidative tissue damage in mammals. Specifically, the role of diet restriction (DR) and a nucleophilic hydrazine compound, aminoguanidine (AG), a crosslinking inhibitor, were examined on tissue crosslinking with the view towards both extending the production period and improving carcass quality of broiler breeder hens. Additionally, isolation of pentosidine from skin and tendons of hens and their relationship with tendon breaking strength (TBS), time (TBT) and meat tenderness (SF) were carried out. Chicks were randomly divided into four dietary groups from eight to 125 wks after hatch: *ad libitum* (AL); diet restricted (DR, fed 60% of *ad libitum*); AL and DR groups supplemented with 400 ppm AG (1.35 mg/kg BW/day) each (AL+ AG and DR+AG respectively).

The results showed that pentosidine was present in the skin (P_s) and tendon (P_t) of the broiler breeder hens, and that there was an age-related, linear increase in its concentration ($p < 0.001$, $r^2 = 0.94$) for skin and ($p < 0.0001$; $r^2 = 0.79$) for tendon in AL-fed hens. DR and supplementation of AG with AL-fed hens (AL+AG) retarded P_s ($p < 0.001$; $p < 0.001$) and P_t ($p < 0.001$; $p < 0.01$) respectively as compared to AL-fed hens. Concurrently, there was also an age-related parallel increase in TBS ($p < 0.0001$; $r^2 = 0.56$), in TBT ($p < 0.0001$; $r^2 = 0.67$), and in shear force (SF) ($p < 0.0001$; $r^2 = 0.95$) in AL-fed hens. Similarly DR significantly retarded the overall TBS ($p < 0.01$) TBT ($p < 0.02$), and SF ($p < 0.0001$) values as compared to AL-fed hens over the period of this study. Supplementation of AL-fed hens with AG (AL+AG group)

significantly retarded the concentration of P_t , P_s and SF values and was similar to those of the DR group. Although, supplementation of DR with AG (DR+AG) retarded the rate of accumulation of P_s ($p<0.001$) over the laying cycle, the reduction in P_s did not significantly affect the mean production. It is therefore concluded that the rate of P_s and P_t accumulation and the age-related increase in meat toughness can be retarded by either DR or AG. P_s can also be used as a predictor of age-related changes in meat. The same approach has the potential to be initiated in other animal production systems. Because of the linear, age-related increase in the concentrations of tissue pentosidine, it can be used as biomarker for the study of aging.

Dedication

To my beloved parents (late) whose inspirations
lead me to pursue this study
and
brother, Ijaz Ahmad (Late)

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First of all I am grateful to Almighty Allah who gave me courage, health and opportunity to accomplish the requirements for this degree.

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LIST OF ABBREVIATIONS

ACP	Aldol condensation product
AG	Aminoguanidine
AGE-R	Advanced glycosylation endproducts receptor
AGEs	Advanced glycosylation endproducts
AH	Aldol histidine
AL	<i>Ad libitum</i>
Br. W	Breast weight
BW	Body weight
CB	Cynomide bromide peptide
CML	N ^ε -(Carboxymethyl)-lysine
CPM	Count per minute
CT	Cook time
CY	Cook yield
DHNL	Dihydroxynorleucine
DHLNL	Dihydroxylysineonorleucine
DR	Diet restriction
FFI	2 Furoyl-4(5)-2(furanyl)-1 H-imidazole
Gluc-lys	Glucitolyllysine
Gluc-hyl	Glucitolylhydroxylysine
HD	Hen day
HHMD	Histidinohydroxymerodesmosine
HNL	Hydroxynorleucine
HLNL	Hydroxylysineonorleucine
LOX	Lysyl oxidase
LNL	Lysineonorleucine
LP	Lsyl pyridinium

MDA	Malanodialdehyde
NFC-1	Non fluorescent crosslinks
NADH	Nicotinamide adenine dinucleotide (reduced)
NO	Nitric oxide
OHP	Hydroxypyridinium
P	Pentosidine
PA	Pyridinium analogue
P _s	Skin pentosidine
P _t	Tendon pentosidine
SF	Shear force
TBS	Tendon breaking strength
TBT	Tendon breaking time

Chapter 1

INTRODUCTION

A common feature of the life cycle of virtually all multicellular organisms is the progressive decline in the efficiency of various physiological processes (Sohal and Weindruch, 1996). A variety of strategies and models have been used to understand the nature of the mechanisms underlying the phenomenon of senescence. One hypothesis for aging postulates that the senescence-associated loss of functional capacity is due to the accumulation of molecular oxidative damage (Sohal and Orr, 1995). Nonenzymatic glycosylation or glycation of tissue protein, and subsequent formation of advanced glycosylation endproducts (AGEs) has become of increasing interest in gerontological research for the last two decades, because these processes have been implicated as mediators of the aging process (Cerami, 1985; Monnier, 1989). The glycation theory of aging suggests that modification of proteins by glucose and associated browning or Maillard reaction leads to the gradual crosslinking, polymerization and characteristic development of brown color and fluorescence (Cerami, 1985; Monnier, 1989). These crosslinks are postulated to lead to the deterioration in the structure and function of tissue protein in a manner similar to the mechanism by which the pathophysiology of diabetes generates tissue specific complications (Monnier, 1989; Bucala and Cerami, 1992; Vlassara *et al.*, 1994). One such crosslink, pentosidine, has been isolated and characterized in diabetic subjects and is an imidazole [4, 5b] pyridinium molecule comprising a lysine and an arginine residue crosslinked by a pentose (Sell and Monnier, 1989). Pentosidine is a glycoxidation product, as both glycation and oxidation reactions are involved in its biosynthesis (Baynes, 1991). In mammals, pentosidine has been found to increase in skin, tendons and other tissues over the life span of the individual, and hence can be used as a sensitive biomarker for aging studies (Grandhee and Monnier, 1991; Dyer *et al.*, 1991 b; Cefalu *et al.*, 1995). A comparable increase in biomechanical strength of collagens has been found *in vitro* after incubation of rat tail tendons

with glucose (Andreassen and Oxlund, 1985; Kent *et al.*, 1985; Andreassen *et al.*, 1988; Menzel and Reihnsner, 1991) and *in vivo* in animals with experimental diabetes (Galeski *et al.*, 1977; Andreassen *et al.*, 1981). In another study, Verzar (1963) demonstrated that the resistance to thermal denaturation of rat tail tendons, measured as tendon breaking strength (TBT), increased with advancing age. Recent theories have suggested that nonenzymatic glycation reactions play a key role in the phenomenology of aging, either alone or in combination with other processes, such as free radical generation (Masoro *et al.*, 1992; Kristal and Yu, 1992). Such stochastic processes may, in turn, exert a regulatory effect on gene expression during aging (Sohal and Allen, 1990).

Meat of spent fowls becomes objectionably tough with increasing age, and this toughness limits its use in whole muscle foods resulting in considerable economic loss to the poultry industry (Nakamura *et al.*, 1975; Shimokomaki *et al.*, 1972). For this reason, meat has been traditionally used in less-profitable, comminuted or retorted products where the small particle size or thermal processing is used to reduce toughness (Nurmahmudi and Sams, 1997). This problem is of particular importance to the poultry industry which produces spent fowl as a by-product of table and hatching egg production. Approximately 130,000,000 spent fowl are disposed of in the USA annually (Vandepopuliere and Lyons, 1996); thus, the potential economic benefit to be gained by tenderizing this meat is tremendous. Presently, there is no economically feasible way to increase meat tenderness by decreasing collagen crosslinking in spent fowl meat (Kondaiah and Panda, 1992; Woods *et al.*, 1997).

Advancing age also adversely affects the reproductive success of many species of animals including poultry. A very common observation in broiler breeder hens is the rapid, age-related decline in reproductive performance once the genetic potential for maximum production of eggs has been reached. Furthermore, eggs that are laid during the later period of the reproductive life span are less likely to be fertilized and, if fertilized, are less likely to hatch (Lerner *et al.*, 1993). The factors that influence these age-related decreases in reproductive success are poorly understood. However, there is evidence in male humans that adult onset diabetes accelerates the

age-related decline in reproductive function and fertility (Kaiser and Korenman, 1988).

Hypothesized that the age-related decline in production and meat quality may be, in part, due to the nonenzymatic glycosylation process, because naturally plasma glucose concentrations in birds are 2-6 times higher than mammals (Holmes and Austad, 1995; Beuchat and Chong, 1996; Iqbal *et al.*, 1998 a). In addition birds also have metabolic rate 2-2.5 times as high as those of mammals of similar body sizes (Lindstedt and Calde, 1976; Austad and Fischer, 1991) which presumably expose them to a higher rate of free oxygen radical production and, consequently, accelerated tissue damage (Harman, 1956; Del Maestro, 1980); 2) comparatively elevated body temperature (about 3°C higher than mammals) (Holmes and Austad, 1995). Both of these latter factors should accelerate the formation of advanced Maillard products. Hence, birds can provide a novel model for ascertaining the role of tissue glycosylation in the age-related functional decline of tissues.

The principal objective of these studies was to understand the role of nonenzymatic glycosylation in birds and to find ways to delay this process so as to improve the productive performance of livestock. Previous studies have shown that diet restriction (DR) is one of the most commonly used measures. It has been shown to increase the mean and maximum life span, decrease age-associated physiological changes, and delay or prevent many of the age-associated diseases (Weindruch and Salford, 1988; Synder, 1989). For example, the reproductive life span of *ad libitum*-fed broiler breeder is both shorter and less productive than their DR counterpart; DR is the only established means of delaying the decline in their productive performance. The ability of DR to modulate reproductive aging and slow or prevent the onset of a number of major age-associated diseases, makes DR a tremendously powerful and versatile tool (Yu, 1994b). DR also is used successfully in the poultry industry to achieve the desired body weight without affecting reproductive performance (Fontana *et al.*, 1992).

A nucleophilic hydrazine compound, aminoguanidine (AG), that acts as a crosslinking inhibitor and is the most extensively used pharmacological compound (Khatan *et al.*, 1988;

Odetti *et al.*, 1990; Oxlund and Andreassen, 1992; Wu, 1995). *In vivo*, AG has been shown to lessen the formation of collagen-linked fluorescence associated with the production of advanced glycosylation endproducts (AGEs). AG has been postulated to block pathological tissue alterations via inhibition of deleterious long-term changes to proteins associated with hyperglycemia (Edelstein and Brwonlee, 1992 ab). AG has been referred to as a virtually nontoxic nucleophilic hydrazine ($LD_{50} = 1800$ mg/kg in rodents) (Ou and Wolff, 1993). AG has been successfully used in poultry to retard the fluorescence in skeletal muscle associated with aging collagen (Klandorf *et al.*, 1996).

Objectives:

The objectives of these studies were to:

1. isolate and quantify the glycoxidation product, pentosidine, from the skin (P_s) and tendon (P_t) of broiler breeder hens.
2. determine if pentosidine accumulates in the tissues of the hens with advancing age.
3. determine if dietary modulation and supplementation of AG can:
 - a. retard the rate of accumulation of P_s and P_t over the period of time.
 - b. affect the mechanical properties of tendon.
 - c. prevent the age-related increase in meat toughness.
 - d. improve the production performance of broiler breeder hens.

Chapter 2

REVIEW OF LITERATURE

Aging

Aging is an inevitable biological process that affects living organisms. In general, aging is characteristically described as a time-dependent functional decline, leading to the cell's incapacity to withstand external and internal challenges (Yu, 1996). The causal factors that underlie the time-dependent, deleterious processes of aging have not yet been well defined, and no single adequate molecular explanation for aging is currently available. The consensus among researchers views the concept of biological aging as an organism's failure to maintain homeostasis (Holliday, 1992).

Bases of Aging Hypotheses

Through the decades, the extent and complexity of aging process has been reflected by a plethora of hypotheses which have inundated the field of aging. According to a recent review, there are more than 300 theories on aging (Medvedev, 1990). An in-depth treatment of these theories is beyond the scope of this review. Most aging theories fall into two broad categories based on putative casual forces: (1) intrinsic factors referring to genomic components and genetic processes; and (2) extrinsic factors, encompassing various external environmental influences on the organism.

Intrinsic Factors of Aging

The long-held depiction of aging as a programmed force (Holliday, 1992) is based on observations of various species with finite life spans. The fixed life span of a given species was believed to be predetermined by their genetic makeup, suggesting that the course of

senescence is actively programmed in much the same manner as the development and maturity phases of the life cycle. The fact that strong genetic influences underlie the course of the aging process is undeniable, as seen in life span differences among species and in cases of accelerated human aging, such as Werner's syndrome. Yet, at present, no compelling reason supports the view that aging is actively programmed by genetic mechanisms (Kowland and Kirkwood, 1994). Crucial unanswered questions then are, to what extent do intrinsic genetic forces play in setting the course of aging?. And, how do these forces interact with environmental factors (i.e., extrinsic forces) to influence the rate of aging?. One emerging clue elucidated from recent molecular studies indicates that intrinsic genomic factors can be readily influenced by environmental factors, such as oxidative stress and DR (Van Remmen *et al.*, 1995; Yu, 1995). A better understanding of the nature of these interactions should provide further insights for many unresolved questions about the causality and process of biological aging.

Extrinsic Factors of Aging

Most popular theories today postulate a role for extrinsic factors in the aging process. These theories have centered around the notion that age-related deterioration is primarily related to structural and functional modifications of cellular constituents. Among them are four related hypotheses that implicate free radicals, glycosylation reactions, and /or Maillard reactions as causative factors in the aging process.

The Free Radical Theory of Aging. Investigations over the last two decades have focused a great deal on identifying the extrinsic factors of aging (Eichhorn, 1979). Among them, two of the most likely factors are diet (Nolen, 1972; Holehan, 1986) and free radicals (Orr and Sies 1985; Saul *et al.*, 1987; Porta, 1988; Kehrer and Smith, 1994; Sohal, 1994). These extrinsic factors are particularly significant, because they have characteristic ways of influencing the aging process (Porta, 1988; Farmer and Sohal, 1989; Mote *et al.*, 1991; Kehrer and Smith, 1994; Pahlavani *et al.*, 1994). First, both are inseparable components of an organism's normal metabolism: diet for energy and generation of free radicals during an oxygen metabolism. Second, interaction between these two factors results in deleterious effects directly and indirectly

on cellular structure and functions. Third, and perhaps more important, both have the capability to interact with intrinsic genetic components which can modify the ultimate course of aging (Farmer and Sohal, 1989; Esterbauer and Schaur, 1990; Mote *et al.*, 1991; Dean *et al.*, 1992; Chaudhry *et al.*, 1994; Pahlavani *et al.*, 1994). Thus, in contrast with programmed aging theories, aging hypotheses based on extrinsic factors recognize the organism's genotypic susceptibility as well as its ability to adjust to a changing environment in the face of external forces (Corpas *et al.*, 1993). Evidence supports the view that abnormal cellular constituents such as DNA, (Cathcrat *et al.*, 1984; Vaca *et al.*, 1988; Brandy and Davison, 1990; Chou *et al.*, 1991; Chung *et al.*, 1992) proteins, (Oimomi *et al.*, 1986; Stadtman, 1988; Baynes and Monnier, 1989; Masoro *et al.*, 1989; Sohal *et al.*, 1993), carbohydrates (Kristal and Yu, 1992; Cadenas *et al.*, 1994; Brownlee, 1995; Wolff, 1995), and lipids are oxidatively damaged by free radicals (Greshman *et al.*, 1954; Yagi, 1991; Yu *et al.*, 1992; Laganriere and Yu, 1993; Chen *et al.*, 1995). Data on cellular damage imply that oxidative modifications of genetic components by these extrinsic factors are consistent with what has been observed in aging and disease processes.

The concept of free radical involvement in aging and disease was proposed by Greshman *et al.* (1954) and Harman, (1956). These investigators envisioned free radical species causing irrecoverable functional deterioration associated with aging. Gerschaman provided clues to the possible cause of cellular damage from X-ray irradiation, oxygen poisoning, and radiation. In formulating the free radical theory of aging, Harman deemed oxygen free radicals as the primary underlying cause of age-related deterioration and disease leading to death. This aging theory is meritorious for having provided modern gerontologists with both a conceptual base and the experimental opportunities necessary to explain the biological basis of senescence and disease.

More knowledge gained in the field of free radical biochemistry over the last decade necessitated an expansion and modification of the original free radical theory of aging (Yu and Yang, 1996). Free radicals are defined chemically as atoms or molecules that are highly reactive due to an unpaired electron in an outer orbit (Del Maestro, 1980). Because of the reactivity due to the unstable electronic configuration, free radical reactions proceed randomly by chain

reaction (Oldham *et al.*, 1988). Although not itself a free radical, the biologically important molecule H_2O_2 can undergo the trace metal (Fe/Cu) catalyzed Haber-Weiss (Aust, 1985) and Fenton reactions (Halliwell and Gutteridge, 1984), leading to the generation of highly reactive hydroxyl radicals.

Most biological substances are targets of free radical attack, and the ubiquitous, non-selective, and random nature of free radical reactions make the free radical theory of aging among the most plausible mechanisms to explain both aging processes (Harman, 1956; Porta, 1988) and major age-related diseases such as cancer (Sun, 1990) and atherosclerosis (Del Maestro, 1980). The most important factor implicating free radicals in the aging process is the fact that under normal physiological conditions, the majority of these free radicals are generated endogenously from inspired O_2 molecules as by-products of the mitochondrial electron transport reactions involved in energy production. This inherent nature and absolute oxygen requirement for biological systems make it extremely difficult to eliminate the possibility that free radicals do not play causal roles in aging processes.

Although major cellular sources of free radicals are mitochondria, microsomes, and peroxisomes, sources of reactive species are widespread throughout the body, (Freeman, 1984; Boje and Arora, 1992; Moncada *et al.*, 1992; Masters, 1994), for example, intracellular enzymes reactions formed by oxidases, dehydrogenases and oxygenases and extra cellular sources of oxidant species form glycation and Maillard reactions (Kristal and Yu, 1992). Nitric oxide synthase has been shown to be another significant oxidant source (Boje and Arora, 1992; Moncada *et al.*, 1992 Masters, 1994). Emerging evidence shows the generation of reactive aldehyde compounds from the lipid peroxidation process (Esterbauer and Schaur, 1990; Chen *et al.*, 1995; Kristal *et al.*, 1996). An estimated 1-2% of mitochondrial oxygen consumption is converted, by simple electron transfer, to the formation of the superoxide radical (Bandy and Davison, 1990). Mitochondrial production of superoxide has been estimated to be as high as 10^7 superoxide radicals/day (Nohl and Hegner, 1990; Guarnieri *et al.*, 1993). It has been estimated that a single cell in a living organism may be exposed to 10^{10} molecules of superoxide radical

(O[•]) per day (Ames *et al.*, 1993) representing 1.75 kg of O[•] produced in a 70 kg human over a one year period (Frei, 1994). This would roughly translate to a range of 2 to 5 gm of O[•] being produced in a broiler from 0 to 49 days of age (Bottje *et al.*, 1995).

Consequences of Free Radical Attack. Although free radical production per se does not appear uniformly increased with age, the damage induced, e.g., lipid peroxidation, is detected very widely (Yu, 1994). Lipid peroxidation can have numerous detrimental effects on cell function (Horton and Fairhurst, 1987) and membrane fluidity (Yu, 1994). For example, there is evidence that Na⁺/K⁺ ATPase activity is disrupted by lipid peroxidation (Thomas and Reed, 1990), resulting in an inability of the cell to maintain the electrical gradient required for cell viability. An increase in cytosolic Ca²⁺ following oxidative induced damage to membrane Ca²⁺-ATPase causes the loss of cytosolic Ca²⁺ regulation and has been suggested as a key step leading to cell death (Thomas and Reed, 1988). Free radicals can also oxidize proteins and DNA within the cell, while extracellular release of reactive oxygen species (ROS) can damage neighboring cells. Another group of toxic compounds formed during lipid peroxidation are the 4-hydroxyalkenals which react with sulfhydryl groups of proteins, and along with malondialdehyde (MDA), can convey the toxic effects of lipid peroxidation throughout the cell (Bottje *et al.*, 1995).

The Glycosylation Theory of Aging. The implication of glycation or glycosylation reactions in the aging process (Monnier and Cerami, 1981; Cerami, 1985) can be considered as an expanded version of the collagen crosslinking theory of aging, and is based on the crosslinking of numerous proteins by modified glucose residues. Indeed, at the heart of the glycation theory (Cerami, 1985) is the hypothesis that these crosslinks impair both cell and tissue function, which thus leads to age-associated tissue deterioration. The glycosylation theory came in the aging field somewhat circuitously through observations concerning the increased development of specific pathologies in diabetic patients and their similarities to those in the aged population. This

association lead to the linkage between the glycation process and Maillard reactions as possible mechanisms underlying the accelerated aging of diabetics.

Types of Collagen Crosslinks

Collagen crosslinking is a complex post-translational modification that occurs extracellularly (Yamauchi and Mechanic, 1987; Fujimoto, 1988; Reiser *et al.*, 1992). Collagen crosslinks appear to arise from one of two general biosynthetic pathways (Reiser, 1991; Yamauchi and Mechanic, 1987). Crosslinks may be classified into two major groups: those mediated by the enzyme lysyl oxidase (Siegel, 1979; Reiser *et al.*, 1992) and those derived from the nonenzymatic addition of glucose adducts (Baynes and Monnier, 1989; Reiser, 1991).

Enzymatic Collagen Crosslinks. A crosslink pathway begins with the lysyl oxidase-mediated oxidative deamination of certain lysine and hydroxylysine residues. The resultant aldehyde moieties may undergo further reactions with each other or with lysine, hydroxylysine and histidine residues to form a series of di-, tri- and tetrafunctional crosslinks in collagen (Robins, 1982).

In some tissues and species, aging is associated with an increase in non-reducible mature crosslinks, the reaction products of difunctional enzymatic crosslinks and unmodified residues (Fujimoto, 1982; Yamauchi *et al.*, 1987). However, in many tissues the generation of mature crosslinks tends to plateau with age, rather than increase in a linear fashion, and thus are unlikely to account for the progressive physiochemical changes associated with age. In contrast, there is considerable evidence that nonenzymatically mediated collagen crosslinks derived from glucose adducts increase with age in virtually all tissues studied. The accumulation of such compounds, so called advanced glycation endproducts (AGEs), is associated with alterations in mechanical properties, solubility, ligand binding and conformation of collagen (Reiser, 1991). Recent theories have suggested that nonenzymatic glycation reactions play a key role in the phenomenology of aging, either alone or in combination with other processes, such as free

radical generation (Masoro *et al.*, 1989; Kristal and Yu, 1992). Such stochastic processes may in turn, exert a regulatory effect on gene expression during aging (Sohal and Allen, 1990).

Glucose Derived Nonenzymatic Collagen Crosslinks. The second pathway begins with the nonenzymatic glycation of lysine and hydroxylysine residues. These early glycation products, glucitolyllysine and glucitolylhydroxylysine, are believed to undergo a series of reactions to form complex fluorophores and chromophores, collectively referred to as advanced glycation endproducts (AGEs) (Reiser, 1991). There is now considerable evidence that collagen crosslinks arise from nonenzymatic glycation accumulate in virtually every tissue of diabetic subjects (Reiser, 1991), and that such abnormalities are associated with changes in the physiochemical properties of collagen and ultimately with long-term clinical complications.

The nonenzymatic reaction between glucose and proteins is collectively known as Maillard reaction. On the basis of the recent *in vitro* and *in vivo* studies, it is proposed that this nonenzymatic glycosylation of certain proteins in body triggers a series of chemical reaction that culminate in the formation, and eventual accumulation of irreversible crosslinks. It begins with the formation of schiff bases when an aldehyde group (CHO) of glucose and an amino group (NH₂) of a protein are attracted to each other. This combination is unstable and quickly rearranges itself into a stabler, but still reversible, substance known as an Amadori product. If a protein persists in the body for months or years, some of its Amadori products slowly dehydrate and rearrange themselves yet again- into new glucose-derived structures. These can combine with various kinds of molecules to form irreversible structures named AGEs. Most AGEs are yellowish brown and fluorescent and have specific spectrographic properties. More important for the body, many are also able to crosslink with adjacent proteins. The precise chemical structure of advanced glycosylation end products and of most AGE-derive crosslink is still not known. Nevertheless, some evidence suggests that AGEs are often created by the binding of an Amadori product to glucose or another sugar. Such end products would form bridges to other proteins by binding to available amino groups. In some instances two Amadori products may instead merge, creating an AGE that is also a crosslink. The first such crosslink isolated is 2-

furoyl-4(5)-(2-furanyl)-1H-imidazole, or FFI, mixture of amino acid lysine, albumin and glucose (Cerami *et al.*, (1987), but since been shown to be an artifact of the isolation procedure.

The Maillard Reaction Theory of Aging. Monnier (1989) and Monnier *et al.*, (1991) expanded the glycation theory of Aging to include all Maillard reactions, the class of chemical reactions to which glycation reactions belong. The Maillard reaction represents a series of complex processes that include initiation, propagation, and termination processes similar to those seen in free radical reaction (Monnier *et al.*, 1991). Maillard reactions may be initiated by any compound containing an α - hydroxy aldo or keto group reacting with the ϵ -amino group of an amino acid. A typical reaction producing glycated proteins is the formation of Amadori products via a Schiff base resulting from a condensation reaction between the amino groups of proteins and aldo or keto moieties of reducing sugars (Wolff *et al.*, 1987). The Amadori product can degrade further to various ketoaldehydes, each of which contains reactive carbonyl groups (Monnier, 1990). In some cases, these reactions require transition metals such as Fe and Cu, as do some free radical reactions (Mullarkey *et al.*, 1990). The secondary substances are often more reactive than the parent compound which can then generate from macromolecular complexes. The propagation processes, including fragmentation and crosslinking, eventually lead to advanced Maillard products (Monnier *et al.*, 1991) termed as AGEs (Monnier and Cerami, 1981).

Synergistic Theory. This recent theory proposed by Kristal and Yu, (1992) is comprised of the free radical, glycation, and Maillard reaction theories of aging: the essence of the proposal is based on the consideration that each of these three processes share common chemical reactions that involve free radicals. This new theory of aging is based on the prediction that age-related deterioration is produced by the sum of the damage induced by free radicals, by glycation and other Maillard reactions, and by their interplay. By combining the free radical, glycation, and Maillard theories of aging, (Yu, 1993) this theory postulates that these separate processes, which differ significantly in their site of generation and their primary targets, might interact to cause age-associated deterioration. The synergistic interactions suggested by this theory offers

a stronger scientific basis for the direct involvement of a Maillard reactions, including glycation, in the aging process, rather than their indirect involvement coupled to their association with age-related diseases.

Roles of Glycation and Oxidation in Aging Process

The extent of glycation or glycosylation of tissue proteins depends, in part, glucose concentrations and is increased in proportion to the degree of hyperglycemia in diabetes. Although glycation is relatively constant with age (Dyer *et al.*, 1992), other products of reactions with sugars and proteins accumulate in long-lived tissue protein (Dunn *et al.*, 1989; Dunn *et al.*, 1991). These compounds, such N^ε-(carboxymethyl)lysine (CML) and pentosidine (P), as well as a characteristic Maillard-type fluorescence are generated during the Maillard or browning stages of the glucose-protein reaction. CML and P are termed as glycoxidation products because both glycation and oxidation reactions are required for their formation from reducing sugars (Dyer *et al.*, 1991; Baynes, 1991). Strong correlations have been noted between CML, P, and fluorescence in the control and diabetic populations, providing evidence of age-dependent chemical modification of skin collagen via the Maillard reaction and acceleration of this process in diabetes. (Dyer *et al.*, 1992).

Oxygen may have a special role as a fixative of chemical damage to proteins by the Maillard reaction *in vivo*, as glycoxidation products are the only products of Maillard reaction known to accumulate in tissue proteins (Dyer *et al.*, 1991; Baynes, 1991). The increase in the rate of their accumulation in collagen in diabetes is consistent with the description of diabetes as a disease of accelerated chemical aging of tissue proteins. The accumulation of CML and P is also consistent with the free radical theory of aging which considers aging to be the result of cumulative oxidative damage to biomolecules. Despite the difference between the structures of CML and P, the two compounds accumulate in concert with normal aging and their rates of accumulation are comparably accelerated in diabetes. For this reason, they provide a consistent index of protein exposure to oxidative stress AGEs (Reiser, 1991). Glycoxidation products appear to be particularly sensitive indicators of oxidative stress, perhaps because of ease of

oxidation of reducing sugars and their adducts to protein. Although CML and P probably account only for a fraction of the carbohydrate adducts and crosslinks formed nonenzymatically in protein, both appear to be useful as biomarkers of the extent to glycative and oxidative damage of protein via the Maillard reaction (Dyer *et al.*, 1991; Baynes, 1991).

Pentosidine. This compound is the most widely described Maillard structure in the context of extracellular matrix glycation. First isolated by Sell and Monnier (1989a) its structure was described soon afterwards as an imidazopyridinium compound (Sell and Monnier, 1989b), comprised of single lysine and arginine moieties crosslinked by a pentose. FAB-high resolution mass spectrometry showed a m/z of 379,2069 compatible with the empirical formula $C_{12}H_{27}N_6O_4$. Pentosidine could be synthesized *in vitro* by reacting L-lysine with L-arginine and D-ribose of collagen or with D-ribose or its isomers i.e., the subsequent crosslink was given the name of pentosidine. However, preliminary data suggested that plasma ribose levels are very low in patients with diabetes. It has now been identified as the endproduct of the Maillard reaction elicited by hexoses, pentoses, ascorbate and a variety of Amadori compounds *in vitro* (Sell and Monnier, 1989a, 1989b; Dyer *et al.*, 1991; Grandhee and Monnier, 1991). The proposed mechanism of pentosidine formation from D-ribose, D-glucose, D-fructose, and L-ascorbate the common intermediate I. However, the major *in vivo* carbohydrate source leading to pentosidine formation is not known. What clear is that oxidation reactions are required at some stage in the reaction as the formation of pentosidine is inhibited in the absence of oxygen, a property that prompted Baynes to coin the phrase ‘glycoxidation product’ to describe pentosidine and other glycation compounds similarly affected by oxidation availability (Baynes, 1991).

Detection of pentosidine in the tissues of animals has been greatly facilitated by its acid stability, and more significantly by its fluorescent properties. Consequently, it has now been identified in numerous collagenous and non-collagenous tissues and shown to exhibit a linear increase with age in normal tissue (shrew, monkey, dog, pig, cow, rat, human) (Sell and Monnier

1989; Sell *et al.*, 1996) whereas in patients with diabetes, there is an accelerated rate of increase. However, its significance as a major crosslinking component has been largely discounted. Dyer *et al.*, (1991) initially presented evidence that pentosidine accounts for <1% of the total crosslinks formed during the *in vitro* browning reaction of proteins with glucose. This figure is based on a yield of only one pentosidine per 100 collagen molecules, representing 1% of the assumed potential of 1 crosslink per collagen molecule. It is now regarded mainly as a biomarker in the

assessment of cumulative damage to proteins by nonenzymatic reactions of the Maillard reaction. In view of the low yields of fluorescent crosslinks observed during *in vitro* and *in vivo* investigations, Bailey *et al.*, (1995) turned their attention to a detailed analysis of non-fluorescent components associated with highly crosslinked collagen. Using a modified technique developed for the detection of lysyl oxidase mediated crosslinks (Sims and Bailey, 1992) they were able to identify a non-fluorescent component, NFC-1, the concentration of which could be correlated to a change in physicochemical properties associated and is detected in HPLC analysis as a major ninhydrin positive peak eluting after lysine. NFC-1 is generated following short incubations of collagen with ribose (3 days) or glucose albeit after more extended incubation (3 month). Data from Paul and Bailey (1996) indicated that NFC-1 is composed of lysine and arginine moieties. It can be formed by the reaction of N-acetyl-lysine, N-acetyl-arginine with either ribose or glyoxal, a retro-aldol cleavage product of the Maillard reaction. The concentrations of NFC-1 are about 50 times greater than that of pentosidine in aorta (Sims *et al.*, 1996)

Location of Glycation Crosslinks. The location of the potential crosslinks in the collagen fiber, has not been elucidated. The decreased susceptibility of collagen to enzymes and the increased thermal denaturation temperature following glycation is consistent with inter helical crosslinking, which may involve lysines at random location along the length of the molecule, or preferentially glycated lysine. It is these lysines that might also be the site of the crosslinks. Studies on the location of the initial hexosyllysine in young and old rat tendon collagen by Reiser *et al.*, (1992), rather surprisingly indicated a preferential glycation of particular lysines in the cyanogen bromide peptide (CB-3) in the middle of the collagen molecule, since one might expect

the lysines in the rod-line molecule to be reasonably equivalent throughout the length of the molecule. Wess *et al.*, (1993) provided evidence from neutron scattering for increased glycation in the gap region of the fibre. The gap region of the collagen fiber is formed as a result of the end-overlap quarter stagger alignment of the molecules in the fiber. The end-overlap is a region of more tightly bound molecules whilst the gap or hollow region contains less and loosely bound molecules. This latter region might be accessible to glucose and the formation of glycation products as shown by Wess and his colleagues.

Collagen in Crosslinking Studies

In the past 15 years, considerable efforts have been devoted to understanding the nature of postsynthetic collagen modifications associated with diabetes (Monnier *et al.*, 1996). The focus on collagen is warranted for several reasons. First, collagen is present ubiquitously (25 to 30% of mammalian proteins), and changes in collagen structure occur in several tissues that are critically affected by diabetes, such as joints, arteries, the capillary bed in the retina, and the renal glomerular system. Second, the long half-life of collagen increases its susceptibility to accumulate molecular lesions. Thus, the biochemical nature of these changes in structure, if understood, may provide a window into the pathogenesis of diabetic complications in general. Third, the determination of specific collagen changes in skin may provide a much needed marker to monitor midterm efficacy of therapeutic intervention. (Monnier *et al.*, 1996).

Effect of Glucose on Collagen Structure

A number of changes occur at the tissue level in diabetes involve collagen. In humans, these include the rapid development of atherosclerosis (Minaker, 1987), thickening of capillary basement membranes (Kilo *et al.*, 1972), increased arterial wall stiffness (Pillsbury *et al.*, 1974), decreased lung elasticity (Schuyler *et al.*, 1976), sclerosis of renal glomeruli (Bilous *et al.*, 1989), stiffening of the heart (Regan *et al.*, 1977), periarticular rigidity (Grgic *et al.*, 1975), and osteoarthritis (Hadley, 1993). Some of these changes also are observed in the diabetic and galactosemic animal, such as capillary basement membrane thickening (Robison *et al.*, 1983; Chandler *et al.*, 1984; Klandorf *et al.*, 1996) and glomerulosclerosis (Velasquez *et al.*, 1990).

Data from animal studies, especially the diabetic rat, reveal profound abnormalities in the mechanical properties of tail tendons such as tensile behavior, stress strain behavior, and crimp length, as well as a tendency toward a larger fibril diameter (Galeski *et al.*, 1977). Thermal stability is increased in collagen from tendons (Yue *et al.*, 1983) and skin (Yosha *et al.*, 1983) of diabetic rats. Skin collagen solubility to acid and pepsin also is decreased (Ramanurthy *et al.*, 1972), and its strength and stiffness are increased (Andreassen *et al.*, 1981). Similarly, extractability of collagen by acid and pepsin from aortas of diabetic rats is decreased (Brownlee *et al.*, 1986), concomitant with an increase in the tensile strength of the aortic wall is increased (Andreassen and Oxlund, 1987).

At the molecular level, collagen in various tissues from diabetic individuals is less digestible by collagenase and cyanogen bromide (Francis *et al.*, 1974; Hamlin *et al.*, 1975; Kohn and Schnider, 1982; Schnider and Kohn, 1982; Kohn, 1983), and the pepsin releasable fraction of skin collagen contains more high molecular weight material than that from age-matched control subjects (Kohn and Schnider, 1982). X-ray diffraction studies of toe tendons from diabetic individuals reveal alterations in the lateral packing and axial structure of collagen molecules in diabetes (James *et al.*, 1991).

Although most of the changes described above are non-specific parameters of crosslinking, a number of investigators were able to bring additional evidence for the presence of high molecular crosslinked material in collagen extract using SDS-PAGE (Schnider and Kohn, 1982; Brownlee *et al.*, 1986). Thus, there is strong evidence that intra- and intermolecular crosslinks form at an accelerated rate in the extracellular matrix in diabetes.

Role of Lysyl Oxidase in Age-related Collagen Crosslinking

Lysyl oxidase (LOX) is the enzyme involved in the formation of the so-called physiological crosslinks that are needed for the stabilization of collagen fibrils. Drugs that inhibit the formation of LOX induce a condition called lathyrism in which collagens are poorly crosslinked with the result that arteries are at increased risk of rupture (Narayanan *et al.*, 1982).

Thus, the absence of the enzyme may have profound effects on development (Butler *et al.*, 1987). The finding that LOX activity is increased in lungs from diabetic rats (Madia *et al.*, 1979) has intrigued several investigations in this field.

Evidence supporting a role for LOX in collagen crosslinking in diabetes includes the inhibition of acid-soluble crosslinkages by β -aminopropionitrile (a LOX inhibitor) and D-penicillamine, which complexes lysine- and hydroxylysine-aldehyde groups (Chang *et al.*, 1980). It has been demonstrated that some of the specific LOX-dependent crosslinks are increased in experimental (Chang *et al.*, 1980) as well as human diabetes (Buckingham and Reiser, 1990).

Whereas LOX crosslinks may well contribute to the increased stability of newly synthesized collagen, such as in granulation tissue and perhaps during sclerosis, a number of observations suggest that these crosslinks are unlikely to completely explain the dramatic stiffening of tissues and crosslinking of collagen in diabetes. First, LOX-mediated crosslinks appear to form in newly synthesized collagen in very specific sites and are progressively replaced by more stable physiological crosslinks (Fujimoto, 1982; Reiser *et al.*, 1992). There is no evidence that diabetes can catalyze LOX crosslink formation in sites other than those involved during physiological maturation of collagen. Second, the tensile strength of aortic wall collagen of diabetic rats is increased, but LOX-mediated crosslinks are unchanged as compared with controls (Andreassen and Oxlund, 1987). Because collagen content in several tissues such as skin (Rodnan *et al.*, 1979; Vishwanth *et al.*, 1986), arteries (Rehker *et al.*, 1993), kidneys (Klein *et al.*, 1975), and heart (Regan *et al.*, 1977) is increased, findings of elevated LOX crosslinks in such tissues in diabetes is best interpreted as a reflection of newly deposited collagen.

Role of The Maillard Reaction in Collagen Crosslinking

The proposition that the Maillard reaction would play a role in collagen crosslinking was first formulated by Bensusan (1965) but went unnoticed. Similarly, earlier findings that collagen contains glucitolyl-lysine adducts (Robins and Bailey, 1972) did not trigger an investigation into the role of the Maillard reaction in diabetes and aging until nonenzymatic glycosylation was

proposed as a mechanism for diabetic complications (Cerami *et al.*, 1979). By this time, several studies by Kohn and Schnider (1982) and others demonstrated that diabetes has several features of accelerated aging, both at the tissue level and at the level of collagen itself. Thus, the concept that reducing sugars such as glucose could explain the progressive crosslinking of collagen during normal aging and at an accelerated rate in diabetes (Monnier and Cerami, 1981; Monnier *et al.*, 1984) warranted in-depth studies into the role of glucose as a crosslinking agent of the extracellular matrix. Many studies confirmed the fact that incubation of collagen with glucose or other glycating sugars could reproduce conditions associated with diabetes and normal aging. For example, the increase in thermal rupture time in diabetic and aging rats is duplicated by incubation in glucose (Yue *et al.*, 1983; Andreassen and Oxlund, 1985) or glucose-6-phosphate solutions (Kohn *et al.*, 1984).

The implication of glycated lysine residues or some protein-bound carbonyl intermediates in the formation of crosslinks suggest the role of Amadori products in collagen crosslinking. Several studies have firmly established that collagen glycation is increased during exposure to high glucose *in vitro* (Fujimoto 1977; Guitton *et al.*, 1981; Rogozinski *et al.*, 1983; Guitton *et al.*, 1984; Perejda *et al.*, 1984; Fujimoto and Horiuchi, 1986; Andreassen *et al.*, 1988; Fu *et al.*, 1994) or *in vivo* (Kohn and Schnider, 1982; Perejda and Uitto, 1982; Yue *et al.*, 1984; 1985; Davis and Stehbens, 1984; Le pape *et al.*, 1984; Oimomi *et al.*, 1986; Vishwanth *et al.*, 1986; Brennan, 1989; Monnier *et al.*, 1988; Lyons *et al.*, 1991; Miksik and Deyl, 1991; Miksik *et al.*, 1991) and that collagen glycation increases with age in several tissues (Kohn and Schnider, 1982; Andreassen and Oxlund 1985; Oimomi *et al.*, 1986; Miksik *et al.*, 1991). Yet, other studies have failed to document anything more than a loose relationship between crosslinking and glycation, either *in vitro* or *in vivo*. For example, collagen glycation, although short-term, does not inhibit collagenase digestibility (Lyons and Kennedy, 1985). Increased mechanical stability of tail tendons during glycation in phosphate buffer is prevented when glycation carried out in Tris buffer in spite of equal extent of glycation in both buffers (Andreassen and Oxlund, 1985; Lyons and Kennedy, 1985; Andreassen *et al.*, 1988). Treatment of diabetic rats with aspirin or naproxen, a nonsteroidal anti-inflammatory agent, prevents the

increase in thermal rupture time without decreasing glycation (Yue *et al.*,1985; Yue *et al.*,1984). Similarly, treatment of diabetic rats with vitamin E limits collagen crosslinking without affecting glycemia or glycated hemoglobin levels (Aoki *et al.*,1992).

Role of Glycooxidation in Collagen Crosslinking. Studies by Baynes and colleagues (Baynes, 1991) demonstrated that proteins that are oxidatively stressed generate a fluorescent product similar to those formed during incubation of proteins with reducing sugars. In subsequent studies, Baynes and colleagues (Fu *et al.*,1994) showed that covalent crosslinks form concomitantly with increasing glycation, fluorescence, and carboxymethyllysine (CML) and pentosidine levels during incubation with glucose and that anaerobic conditions suppress all modifications except the formation of Amadori products, i.e., glycation itself. Addition of antioxidants and free radical scavengers have a similar effect. Thus, these experiments show that it is possible to uncouple glycooxidation and crosslinking from glycation (Fu *et al.*, 1994). When aminoguanidine (AG), a crosslinking inhibitor, was used in these studies, it also blocked glycooxidation, thereby implicating ketoaldehydes in addition to reactive oxygen species in the crosslinking process. The question, however, is whether the ketoaldehydes originate from oxidation of Amadori products (glycooxidation) or from an earlier step, i.e., glucose autooxidation (Wolff and Dean, 1987) or oxidation of Schiff base intermediates (Namiki and Hayashi, 1975). Based on kinetic analysis, Fu *et al.*, (1994) concluded that Amadori products are not likely to be the major precursors of collagen crosslinks, but that direct autooxidation of glucose, perhaps catalyzed by amino groups, may be as important as glycation. In contrast, Hunt *et al.* (1993), using a system comprising bovine serum albumin, glucose, and copper, came to the conclusion that oxidation of Amadori products plays a more important role in protein modifications than glucose autooxidation.

To clarify this discrepancy, Glomb and Monnier (1995) followed CML formation as a function of glycooxidation in a glucose/lysine incubation system and used AG and boric acid as trapping agents for free glyoxal and Amadori products, respectively. Results showed that when the reaction is initiated from glucose, AG traps 50% of CML. However, CML formation is

hardly suppressed when it originates from Amadori products. In contrast, boric acid totally suppressed CML formation from Amadori products, but suppressed only 37% of the glucose/lysine system. These and other experiments (Glomb and Monnier, 1995) suggest that in a glucose/lysine system ~50% of CML formed originates from oxidation of Amadori products and ~40-50% from a pre-Amadori stage largely independent of glucose autooxidation, the so-called Namiki pathway.

Role of Hydrogen Peroxide in Collagen Crosslinking. The studies from other laboratories (Jiang *et al.*, 1990; Mullarkey *et al.*, 1990), implicate oxygen-reactive species in protein modification during incubation with glucose. To further determine the role of glucose and Amadori products in collagen crosslinking, Elgawish *et al.* (1997) added 2 mmol/l glycated ϵ -amino-capric acid to rat tail tendons incubated with 5 and 30 mmol/l glucose. A dramatic increase in crosslinking as measured by thermal stability was observed. A substantial part of crosslinking could be suppressed with catalase, thereby implicating H_2O_2 in the crosslinking process.

To evaluate the role of H_2O_2 in collagen crosslinking *in vivo*, intact tail tendons were implanted into the peritoneal cavity of diabetic rats, which were then treated for 28 days with intraperitoneal injections of catalase or a control protein. Again, significant inhibition of tail tendon crosslinking was observed, which could also be duplicated with antioxidants (Elgawish *et al.*, 1997).

PREVENTION OF GLUCOSE-DERIVED TISSUE DAMAGE

Knowledge of the mechanisms involved in collagen crosslinkings has led to attempts to alleviate or inhibit the effects of AGEs. An interesting suggestion is that the body possesses a system for removing some of these AGEs and damaged cells from the body. For this reason, the protection against Maillard reactants can be divided into three categories: 1) natural ; 2) physiological and; 3) pharmacological intervention.

Natural Protection Against Maillard Reactants. Defense against toxic carbonyl compounds derived from sugars or other sources are present and this involves:

Glucose as a Sources of Energy. Bunn and Higgins (1981) demonstrated that glucose as the major carrier of energy in mammals, precisely because it is the slowest reacting carbohydrate. It is apparent that other sugars such as ribose are 130 times more reactive in the browning reaction than glucose. Thus, the lower the plasma and cellular concentration of these highly reactive sugars, the lesser the biological stresses to an animal system. Furthermore, both plasma and cells have high levels of free low-molecular-weight amines that can compete with glucose, and thus act as physiological inhibitors of glycation.

Protection by Oxidative Fragmentation of Amadori Products. Amadori products can undergo oxidative fragmentation in the presence of Fe^{2+} and oxygen to form carboxyalkylated lysine residues. Fragmentation of the Amadori product of glucose between C2 and C3 gives rise to N^ϵ -carboxyalkylated lysine and erythronic acid, whereas fragmentation between C3 and C4 results in the formation of 3-(N^ϵ -lysino)-lactic acid and glyceric acid. The net effect is that the protein has lost a positive charge and gained a negative charge. Furthermore, the modification is irreversible. Thus, the formation of carboxy-alkylated protein may be detrimental to protein function. On the other hand, however, it might prevent irreversible inter- or intramolecular crosslinking and protein trapping during advance Maillard reactions (Brownlee *et al.*, 1983; Brwonlee *et al.*, 1985). This mechanism of protection might be particularly important in the prevention of excessive crosslinking on the extracellular matrix, the hallmark of the aging process.

Protection against Maillard Reactants through Enzymatic Inactivation. Hata *et al.*, (1988) has shown that liver enzymes such as α -ketoglutaraldehyde dehydrogenase in the presence of NADH can use 3-deoxyglucosone as a substrate, thereby suggesting that Maillard reactants could be inactivated enzymatically. Although there is no evidence that Amadori

products themselves could be cleaved enzymatically, the notion that Maillard intermediates that propagate the reaction could be inactivated enzymatically is important.

Protection by Endogenous Amines That Can Trap Maillard Reactants. The concept that one might be able to interfere in the course of the Maillard reaction comes from the work of Brownlee *et al.* (1986) who showed that AG can block the formation of protein-linked fluorescence and crosslinking in model systems *in vitro* as well as in diabetic rat skin collagen. Based on this concept, it is thus logical to consider amines not only for their ability to initiate the Maillard reaction by reacting with sugars, but also for their potential as scavenging agents of the highly reactive Maillard intermediates.

Protection by Cellular Removal and Degradation of Advanced Maillard Molecules. Vlassara *et al.*, (1984 a; b; 1987; 1988) demonstrated that macrophages have the ability to remove molecules or cells modified by AGEs. From their work evolved the notion that some cells have a specific receptor (AGE-R) that recognizes the modified protein, thus preventing the accumulation of AGE proteins. When myelin was glycated with glucose or glucose 6-phosphate, it was more rapidly taken up by macrophages compared to myelin incubated without sugar. Similar observations were made with AGE-modified albumin.

Further study by Vlassara and Bucala (1996) demonstrated the presence of cell surface receptors that were specific for the recognition and degradation of AGE-modified proteins also have been identified on circulation monocytes, lymphocytes, endothelial and renal mesangial cells, and other cellular systems to partake in both normal tissue remodeling and tissue damage. Interaction of AGE-modified proteins with AGE-R complex serves not only to degrade AGE proteins but also to induce the synthesis and release of cytokines (Vlassara *et al.*, 1988) and growth factors (Kirstein *et al.*, 1990, 1992), suggesting a dual purpose: disposal of senescent AGE-modified molecules and initiation of tissue repair and protein turnover.

Physiological Intervention. Throughout history, humans have sought a means to a longevity, (Busse, 1973; Comfort, 1979) but few have been successful in their pursuit of successful antiaging strategies (Yu, 1995). Thus, far, DR is the only life-prolonging means accepted by experimentalists. DR has consistently proven to extend both median and maximum life spans of laboratory animals, owing probably to its ability to intervene in both biological aging and life-shortening pathogenesis (Weindruch and Walford, 1988). In addition, evidence from the last two decades of DR research has established the credibility of DR as the most potent modulator of both physiological and pathologic processes. The following sections explain the unique abilities of DR as a research tool to further define the aging process.

Mechanisms of Action of DR. The following are the different hypotheses presented to explain the underlying mechanism of DR. The ability of DR to modulate life span and retard pathogenesis has merited it the gold standard of experimental gerontology in corroborating or disproving several major aging hypotheses. (Masoro, 1988; Weindruch and Walford, 1988).

The Growth and Development Hypothesis. The growth and development hypothesis is based on one of the oldest notions of aging, i.e., that the rate of growth is positively correlated with the rate of aging (i.e., the slower the growth, the longer the life span). The phenotypic consequence of slow growth under various conditions was taken as evidence of life-prolonging action. The validity of this hypothesis was tested by implementing DR after sexual maturity, or in mid life, in order to avoid the period of slow growth and to not interfere with development (Weindruch and Walford, 1988; Yu, 1994). Amazingly, the results of these studies clearly indicated that the rate of growth has little bearing on a life span; thus, DR provided evidence refuting the growth retardation theory.

The Glucocorticoid Cascade Hypothesis. A more recent hypothesis of aging is the glucocorticoid cascade hypothesis (Sapolsky *et al.*, 1986). The basic tenet of this proposal contends that chronically elevated glucocorticoid levels are the underlying factor of chronic

stress leading to neuronal death. Several studies support this claim by linking chronic stress to experimentally induced accelerated aging. Other supporting evidence shows that the hippocampus undergo a neural cell reduction with age. If this theory holds true, one could predict that glucocorticoid levels will be higher in *ad libitum*-fed rats than in DR rat. Studies by Sabtino *et al.*, (1991) examined rats at various ages by taking blood samples several different times during the day to detect diurnal hormone secretions. Surprisingly; throughout their life, DR rat had significantly higher levels rather than the predicted low levels of the stress hormone. Although further investigation is needed, the results from the DR studies suggests that the major role of glucocorticoid in aging is not as an accelerator of the aging process.

Metabolic Rate Hypothesis. Another theory of aging examined by the DR paradigm is the metabolic rate hypothesis. This theory has been deeply rooted in gerontological literature (Masoro *et al.*, 1982). The original proposal has based on the rate of living theory, which states that an organism's longevity is inversely related to its metabolic rate (Holliday, 1992; Comfort, 1979), suggesting that organisms with higher metabolic rates have shorter life spans. This view seems to be in line with another outdated idea, the "wear and tear" notion of aging. The validity of the metabolic rate view is now being questioned by recent revelations from several studies on homoiothermic animals, including those subject to the DR paradigm (Masoro *et al.*, 1982).

One major finding generated from DR studies reveals that DR rats have metabolic rates similar to their non-restricted counterparts, yet have much longer life spans (Masoro *et al.*, 1991). Similar trends are expected in primates, whose life spans are longer than predicted from their metabolic rate as calculated by oxygen intake. These findings call for a reassessment of metabolic rate as the major determinant in the aging process of all organisms (Holmes and Austad, 1995). Further, evidence casting doubts on the validity of this theory emanates from comparative studies of various species (Holmes and Austad, 1995; Barja *et al.*, 1994). These studies reveal that birds and bats also have substantially longer life spans from what would be predicted based on their metabolic rates (Holmes and Austad, 1995; Barja *et al.*, 1994).

DR as a Modulator of Oxidative Stress. The possibility that nutritional intervention, other than dietary antioxidant supplementation, stave off oxidative damage was first suggested by the work of Enesco and Kurk (1981). These authors restricted dietary protein (4% compared to 26%) at 3, 5, 7, and 12 months of age, finding that lipofuscin, found in the brain and heart of older mice, accumulation significantly reduced. Their work was confirmed by a recent work of Youngman (1993) who demonstrated that restriction of protein intake alone can reduce oxidatively damaged DNA and other oxidative damage. The first study of DR showing its ability to suppress oxidative reactions was conducted by Chipalkatti *et al.* (1983). Here, the authors observed that brain lipofuscin and brain malondialdehyde is significantly lower in 50% DR mice. Furthermore, they found that DR also reduces age-related lipid peroxidation in liver homogenate from 12- and 24-month-old mice. More recently, Sohal *et al.* (1994) used 40% caloric restricted mice at 9, 17 and 23 months of age, and obtained data demonstrating reduced protein oxidation, mitochondrial State 4 respiration superoxide, and H₂O₂ production in the brain, heart, and kidney. The current perception holds that the antioxidative action of DR seems far more widespread than earlier suspected (Koizumi *et al.*, 1987; Yu, 1993). Findings on the deleterious aging effects of membrane structure and cell function have led a number of investigators to propose the membrane hypothesis of aging. Zs-Nagy (1994) and Hansford (1983) independently hypothesized age-related declines in cellular bioenergetics based on membrane structural deterioration. This was also revealed by Grinna (1977) who documented alterations of both the gross chemical composition and the quantitation of mitochondrial and microsomal membranes in aged animals. Subsequent detailed membrane lipid analysis showed that while the major phospholipid classes did not vary significantly with age (Zs-Nagy, 1994), characteristic changes in fatty acid composition of the phospholipids did occur. Data showed that increased polyunsaturated fatty acids (20:4, 22:4 and 22:5) and decreased linoleic acid (18:2) are present in membranes from older *ad libitum*-fed rats. This would help explain the age-related increased susceptibility of these membranes to free radical attack. Interestingly, DR modulates a change in membrane lipid composition on the opposite direction: older DR animals have decreased in polyunsaturated fatty acid and increases in 18:2 concentration compared to their younger

counterparts. Thus, it appears that two major membrane properties, unsaturation to saturation ratio and peroxidizability, were effectively modulated by DR to provide a well-preserved, intact membrane structure and integrity (Yu, 1994). Confirming those changes, Hegner (1980) was the first to note that membrane fatty acid profiles change with age and determined the detailed quantitative and qualitative shifts in saturated and unsaturated fatty acids, strongly suggesting that age affects the basic components to the membrane structure.

Effect of DR on Glycosylation Process. Variable effects of DR have been reported in the literature. For example, a 10% decrease in blood glucose concentrations in DR rats (Cefula *et al.*, 1995) decreases the glycation of proteins by 18-33%. A similar observation was noted in a separate study; an approximately 11% decrease in glucose in DR animals was associated with a 34% reduction in glycated Hb (Masoro, *et al.*, 1989). Although the reason for this is unknown, a decrease in the level of oxidative stress in birds (DR as compared to AL) limit the formation of glycoxidation products by increasing the formation of antioxidant enzymes (Younman *et al.*, 1982; Yu *et al.*, 1982; Yu, 1994), this limitation being present in spite of higher-than-mammalian basal temperatures and concentrations of glucose. Further, strengthening the antioxidant defenses of diabetics by dietary supplementation of Vitamins C and E has been reported to decrease protein glycation without an effect on glycemia (Ceriello *et al.*, 1991; Aoki *et al.*, 1992; Davie *et al.*, 1992). These observations suggest that a reduction in oxidative stress in DR animals contributes to the observed decrease in protein glycation. This might result from changes in ascorbate homeostasis or the ratio of Vitamin E to polyunsaturated lipid concentrations in DR compared to AL animals (Cefalu *et al.*, 1995). While several studies have established that collagen glycosylation is increased with elevated concentrations of glucose both *in vivo* and *in vitro* and that this glycosylation increases with age in several tissues, other studies have failed to demonstrate anything more than a loose relationship between crosslinking and glycosylation, either *in vivo* or *in vitro* (Guitton *et al.*, 1984; Le pape *et al.*, 1984; Lyons *et al.*, 1991; Fu *et al.*, 1994; Monnier *et al.*, 1996). Furthermore, Beuchat and Chong (1997) reported that, in spite of circulating concentrations of plasma glucose in excess of 650 mg/dl, the amount of glycated Hb in hummingbirds (2-5%) is lower as compared to mammals which have levels

ranging from 6-8%. These data suggest that class aves may also have a more efficient antioxidant system which limits the generation of glycoxidation products. Indirect evidence to support this view includes the observation that, in spite of an increased metabolic rate, the production of oxygen free radicals in avian (Red-tailed hawk and chicken) heterophils was significantly lower than in analogous bovine neutrophils (Conlon *et al.*, 1991). In another study comparing pigeons and rats, researchers found that mitochondrial generation of both oxygen free radicals and peroxides was lower in pigeons and that pigeons had higher antioxidant activities (Ku and Sohal, 1993). Antioxidants such as Vitamin A (retinol and retinyl esters) and α -tocopherol (Vitamin E) have been found in higher concentrations in birds than in mammals (Schweger *et al.*, 1991).

Role of Uric Acid in Birds. Uric acid is one of the circulating antioxidants that demonstrates a positive correlation with maximum life span across species (Ames *et al.*, 1981; cultler, 1984). Humans, the most long lived among primates, have comparably high levels of uric acid because they lack uricase, the terminal degradative enzyme present in monkeys and other mammals (Ames *et al.*, 1981; Schreiber *et al.*, 1986). In support of this concept, the lower levels of uric acid in macaques (3-fold as compared to humans) correlate with a 3-fold shorter life span as compared to humans (Short *et al.*, 1997). Uric acid not only demonstrates a stabilizing influence on ascorbate in human blood (Sevanian *et al.*, 1991), by enhancing its role as a free radical scavenger (Ames *et al.*, 1981; Cultler, 1984), but also functions as a noncatalytic binder of iron and iron-containing compounds that would otherwise catalyze the oxidation of ascorbate. This being the case, many species of birds must far surpass mammals in their ability to cope with free radical damage.

Uric acid has been proposed as a potent scavenger of free radicals in human and animal tissues (Becker, 1993; Hellsten *et al.*, 1997). Uric acid is ubiquitous; it is found in all types of extracellular fluids including the lymphatic, CSF, interstitial, synovial, intraocular, and amniotic fluids as well as in the lining of the respiratory tract (Becker, 1993). In fact, a reduction in uric acid concentrations is associated with an increase in reperfusion injury (infarct extension) in

humans following myocardial infarction (Parmely *et al.*, 1992). Urate demonstrates, *in vitro*, the ability to scavenge peroxides, various hydroxyl radicals, and hypochlorous acid (Becker, 1993; Hellstein *et al.*, 1997). Concentrations of plasma uric acid in birds are approximately two fold greater than the urate concentrations measured in humans (Bishop *et al.*, 1992; Shapiro *et al.*, 1997). Although several different antioxidants are present in the body system of birds, we suggest that uric acid may play an important role in coping with oxidative stress and formation of glycoxidation products (Klandorf *et al.*, 1998).

Pharmacological Intervention of AGE Accumulation. Pharmaceutical intervention would seem to be the most likely route and pharmacological agents were sought that could inhibit this process by selectively blocking reactive carbonyl group on ketoamine products as well as and on their derivatives such as 3-deoxyglucosone and glycolaldehyde. Different chemical agents have been tried to delay the AGEs formation of crosslinks. Some of these include: a) Aminopyrazoles; b) Aminosubstituted benzimidazoles; c) 1,2 Disubstituted benzimidazoles; d) Substituted 1,2,4-triazoles; e) Amino-benzoic acids and derivatives; f) 2 alkylidene-aminoguanidines and derivatives; g) Aminoguanidine (AG); h) Rutin; I) Vit. B₆ (pyridoxine); j) Vit. B₁ (thiamine pyrophosphate); k) Vit C; l) 1,25 (OH)₂ or Vit D₃; m) ALT 462 and 486, 711.

The nucleophilic hydrazine compound aminoguanidine-HCl has been the most extensively investigated compound of this type and effectively inhibits the formation of AGEs *in vitro* and *in vivo* (Brownlee *et al.*, 1986). However, in some case AG did not work effectively and the precise mechanism of action is still controversial (Booth *et al.*, 1997).

Aminoguanidine (AG). It has been proposed as an agent of potential benefit in prophylaxis of diabetic complications of kidney, nerve and eye (Brownlee *et al.*, 1986; Brownlee *et al.*, 1988; Hammes *et al.*, 1991; Kumari *et al.*, 1991; Edelstein and Brownlee, 1992 a; Edelstein and Brownlee, 1992 b). *In vivo*, AG has been shown to diminish the formation of collagen-linked fluorescence associated with the production of AGEs. AG has been postulated to block

pathological tissue alterations via inhibition of deleterious long-term changes to proteins associated with hyperglycemia (Edelstein and Brownlee, 1992). AG has been referred to as a “virtually non-toxic nucleophilic hydrazine ($LD_{50} = 1800$ mg/kg in rodents (Edelstein and Brownlee, 1992 a b)).

Mode of Action of AG. Numerous studies have demonstrated that the AG is an inhibitor of many manifestations of nonenzymatic glycation (Brownlee *et al.*, 1988; Khatan *et al.*, 1988; Odetti, 1990; Matuieu *et al.*, 1995; Klandorf *et al.*, 1996; Kochakian *et al.*, 1998), some studies did not find it useful at all (Oxlund and Andreassen, 1992; Crijns *et al.*, 1998). However, there is a disagreement over its mode of action as well (Booth *et al.*, 1997).

The primary mechanism by which AG inhibits the formation of AGE has not been determined. However, this small molecule, in the class of compounds called hydrazines (containing an active NH_2 group), reacts with Amadori products. It binds to carbonyl groups and in so doing prevents the Amadori products from becoming AGEs (Fig. 2.9) (Brownlee *et al.*, 1986; Oxlund and Andreassen, 1992). The controversy arose after the recent kinetic studies carried out by Booth *et al.*, 1997. The kinetic studies confirmed that AG is a potent inhibitor of antigenic AGE formation induced by high glucose concentrations (Booth *et al.*, 1996). However, the inhibition unexpectedly appeared to diminish at later stages of the kinetics studies i.e., 144 hrs of incubation with ribose (Booth *et al.*, 1996). Due to the slowness of the glycation with glucose, this surprising observation could not be completely established in earlier studies. Furthermore, the basis for this diminished inhibition is open to question due to the possible long term instability of aminoguanidine as well as its potential to produce hydrogen peroxide (Ou and Wolff, 1993). The kinetics of aminoguanidine inhibition of ribose glycation appear fully consistent with the earlier glucose findings. Ribose glycation results clearly demonstrate that AG slows the rate of antigenic AGE formation in the presence of sugar but has little effect on the final amount of AGE formed. In the case of glycation by ribose, the overall rate of AGE formation has been shown to be strongly dependent on ribose concentrations (Khalifah *et al.*, 1996). In terms of the classical pathway of glycation, AG decreased the rate of initial Schiff

base formation by complexing the acyclic free aldehyde forms of sugars (Ledl, 1990). Inhibition through formation of such an AG-sugar interaction was first advanced by Khatami *et al.*, (1988). In addition, a glucose-AG adduct was recently produced, isolated, and structurally characterized by Hirsch *et al.*, (1995). The hypothesis that AG does not significantly react with Amadori or later intermediates on the generation of AGEs could be directly tested by observing its possible effects on post-Amadori kinetics, i.e., starting with protein Amadori intermediates. Preliminary studies had indicated a limited interaction of AG with post-Amadori AGE formation RNase (Khalifah *et al.*, 1996). These results are especially noteworthy, since AG was originally proposed by its discoverers to be a nucleophilic blocker of Amadori glycation intermediates (Brownlee *et al.*, 1986; Brownlee *et al.*, 1988).

A novel hypothesis has recently been proposed that AG is a inhibitor of inducible NO synthase which is responsible for nitric oxide (NO) synthesis from L-arginine, as a free radical NO mediates the destruction of pancreatic β -cell in IDDM. Because AG delayed the onset diabetes, this data suggested an important role for NO in β -cell destruction. Finally, macrophages, which have been implicated in β -cell destruction, have been shown to increase NO production in diabetes-prone BB rats (Wu, 1995). So it was hypothesized that NO production may be enhanced in IDDM subjects before the onset of diabetes and that inhibition of NO production may prevent of delay the onset of diabetes.

Poultry as an Animal Model for the Study of Aging

Class aves offers unique advantage as an animal model for the study of biogerontology, particularly for the study of retarded aging and long life (Holmes and Austad, 1995). The chief features of this class relevant to aging research is that most species are dramatically longer-lived than mammals of equal body size (Lindstedt and Calde, 1976). The longevity of birds is somewhat surprising, since they exhibit several other traits which some modern theories of aging suggest should render them more susceptible to the degenerative processes of aging. These traits are (1) metabolic rates as much as 2-2.5 times as high as those of mammals of similar body sizes (Lindstedt and Calde, 1976; Austad and Fischer, 1991) which presumably expose them to a

higher rate of free oxygen radical production and, consequently, accelerated tissue damage (Harman, 1956; Del Maestro, 1980). (2) blood sugar levels typically 2-6 times as high as those of mammals, along with a comparatively elevated body temperature (about 3°C higher than mammals) (Holmes and Austad, 1995). Both of these latter factors should accelerate formation of advanced Maillard products, also hypothesized to be involved in aging-related tissue degeneration (Cerami, 1985) in conjunction with the synergistic effects of free radical products (Sohal and Allen, 1990; Holmes and Austad, 1995). Keeping in view the longevity of birds, they must have special mechanism to prevent the formation of such products, degrade them once formed, remove affected cells, or some combination of the three to live longer (Holmes and Austad, 1995; Monnier, 1990; Iqbal *et al.*, 1997).

Although poultry is an atypical representative of class aves, however, chicken can be effectively used to study its specialized protective mechanism against free radical production and Maillard reaction damage and subsequent AGEs formation, and may offer insight into medical intervention for retarding aging in general and increasing the productive period of poultry and other animals in particular.

Chapter 3

The results of this research are presented as four separate experimental areas. They are formatted in the style of journals in which they will appear.

This chapter covers:

- **Materials and Methods**
- **Results**
- **Discussion**

Study I

Protein Glycosylation and Advanced Glycosylated Endproducts (AGEs) Accumulation: An Avian Solution?

ABSTRACT

The objectives of this study were to determine the effect of diet restriction (DR) and the crosslinking inhibitor, aminoguanidine (AG), on oxidative stress, concentrations of uric acid, and the rate of pentosidine accumulation in the skin (Ps) of naturally hyperglycemic broiler breeder hens. Female chicks (n=450) were randomly assigned to four groups from 8 to 92 weeks after hatch: *ad libitum* (AL); diet restricted (DR); AL and DR groups supplemented with 400 ppm AG each (AL+AG and DR+AG). The accumulation of Ps in AL birds increased linearly with age ($P<0.001$) and was significantly retarded in all treatment groups ($P<0.001$). Ps in the AL+AG group was comparable to that in the DR group, while Ps was lowest in the DR+AG group ($P<0.001$). PMA-induced respiratory bursts in blood leukocytes were lower and uric acid higher in both the DR and DR+AG groups as compared to AL and AL+AG groups ($P<0.001$). We concluded that the accumulation of Ps can be retarded by DR and AG.

INTRODUCTION

Class aves offers many advantages in providing animal models for biogerontology, particularly for the study of retarding aging and promoting longevity (1). The preeminent feature of this class in regard to aging is that most species within it are dramatically longer lived than mammals of comparable body size (2). Avian longevity is somewhat surprising due to other, inherently avian, traits which, based on many theories of aging, should render them more susceptible to the degenerative processes of aging. These traits include: 1: metabolic rates as much as 2 - 2.5 times higher than similarly-sized mammals (2, 3), which should, presumably,

expose them to a higher rate of oxygen free radical production and, consequently, accelerated tissue damage (4-6); 2: concentrations of plasma glucose typically 2-6 times mammalian norms, which should accelerate the Maillard reaction and generate high concentrations of tissue crosslinks or advanced glycosylated endproducts (AGEs); and 3: an elevated basal body temperature (about 3 °C higher than mammals) (1), which should contribute to the nonenzymatic attachment of glucose to proteins. Both of these latter factors should accelerate the formation of advanced Maillard products and, hence, the process of tissue aging (1).

The glycation theory of aging suggests that the modification of proteins by glucose and the associated browning or Maillard reactions leads to the gradual crosslinking, polymerization, browning, and fluorescence changes in collagen that are characteristic of aging (7, 8). It is postulated that, ultimately, these crosslinks will lead to the structural and functional deterioration of tissues (8-10). One such crosslink, or AGE, pentosidine, has been isolated and characterized. It is an imidazole [4, 5b] pyridinium molecule comprised of lysine and arginine residues crosslinked by a pentose (11). In mammals, pentosidine has been found to increase linearly over the lifespan of the animal and, hence, has been used as a biomarker for aging studies (12-14).

According to a synergistic theory of aging (15), age-related deterioration of tissues is due to the interplay of free radical damage, glycosylation and other Maillard reactions. The specific *in vivo* carbohydrate moiety which leads to pentosidine is not known, but oxidative reactions are required at some stage in its formation. In support of this view, the generation of pentosidine is inhibited in the absence of oxygen, a fact that prompted Baynes (1991) (16) to coin the term “glycoxidation product” to describe pentosidine and other glycation compounds similarly affected by oxygen availability. Glycoxidation products are exceptionally sensitive indicators of oxidative stress due to the relative ease of oxidation of both reducing sugars and their adducts. Although pentosidine accounts for only a small fraction of the carbohydrate adducts and crosslinks formed nonenzymatically in protein, its measurement is useful as a biomarker of both the glycative and oxidative damage to proteins (17).

In contrast to the proposed progeric role of glycation and free radical formation, diet restriction (DR) has been shown to retard the aging process. In rodents, DR lowers concentrations of plasma glucose, increases longevity, decreases age-associated physiological changes, and delays or prevents many age-associated pathologies (18, 19). In addition to diet restriction, supplementing the diet with crosslinking inhibitors may have a positive effect on preventing age-related complications. The decrease in tissue functionality associated with nonenzymatic crosslinks can be delayed or somewhat ameliorated by supplements such as the nucleophilic hydrazine, Aminoguanidine Hydrochloride (AG) (20-23). While numerous studies have demonstrated that AG is an inhibitor of many manifestations of nonenzymatic glycation, its specific mode of action is controversial (24).

The objectives of this study were two-fold: 1: to determine the effect of diet restriction (DR) as well as the crosslinking inhibitor, aminoguanidine (AG), on the rate of accumulation of the glycoxidation product, pentosidine, in the skin (Ps) of naturally hyperglycemic broiler breeder hens, and 2: to determine the plasma concentrations of the antioxidant, uric acid, and oxidative stress in blood leukocytes. Any reduction in oxidative stress and/or the accumulation of Ps and/or an increase in uric acid could be interpreted as a reduction or slowing of the aging process.

MATERIALS AND METHODS

Birds and Management. Day-old female broiler-breeder (CobbXCobb) chicks (n=450) were placed in electrically heated battery brooders and fed *ad libitum* until 4 wks of age. All birds were then fed a restricted diet (60% of AL) which optimizes production performance. A restricted diet is limited in calories only; all diet restricted birds receive the recommended amounts of vitamins, minerals and nutrients. Feed intake of AL-fed birds was determined on a weekly basis and adjustments in feed allocation to the DR birds were made accordingly. Photoperiod was set according to the Cobb Management Guide (Cobb-Vantress, Inc., Siloam Springs, AR 72761). All chicks were assigned randomly at 4 wks of age to one of four experimental groups: *ad libitum* (AL); diet restricted (DR); AL and DR supplemented in feed

with 400 ppm AG (1.35 mg/kg/day) (Aldrich Chemical Co., Inc., Milwaukee, WI 53233) (AL+AG and DR+AG, respectively). At this dose, a significant reduction in tissue fluorescence was recorded in poultry (25). Once assigned to a dietary group, the birds were fed accordingly throughout the study. All birds were fed daily between 0800 and 1000 hours and provided water *ad libitum*. The flocks were reared in floor pens until 20 wks of age at which time the hens were put into individual cages. Body weight was monitored weekly and recorded every 12 wks.

Skin samples and pentosidine determination. Hens (n=5) were randomly selected from each dietary group every 12 wks beginning at 8 wks of age and ending at 92 wks of age and killed by prior electrical stunning. Approximately 1 g of skin was removed from the abdominal area, washed with normal saline and stored at -80°C until assayed. The collagen digest for pentosidine determination was prepared according to a technique for 10 mg skin described by Monnier *et al.* (1986) (26) and Sell *et al.* (1992) (27). Briefly, this technique involved the removal of the epidermal and adipose layers, freezing in liquid nitrogen, and mincing. The minced samples were delipidated overnight in a chloroform-methanol (2:1) solution. Samples were then rehydrated in 50% methanol and hydrolyzed in 6 N HCl at 110°C for 18 h. All tubes were flushed with N₂ prior to capping for heating. Subsequent to the hydrolysis, the samples were placed into a SpeedVac centrifuge-type vacuum drier (Savant Instruments, Farmingdale, NY 11735) until dry. Samples were then reconstituted in 250 µl ddi H₂O and filtered using a Costar® Spin-X® centrifuge tube filter (Corning Costar Corp., Cambridge, MA 02140). A modified Stagman and Stalder method was used to estimate collagen via an hydroxyproline standard; it was assumed that hydroxyproline made up 14% of the total collagen (28).

The estimation of pentosidine was done by reverse phase HPLC (29). One mg of acid-hydrolyzed skin collagen digest in 100 µl water/0.01M heptafluorobutyric acid (HFBA) was injected into a 0.46X25 cm Vydac 218TP104 (10 µm) C-18 column (Vydac, Hesperia CA 92345) connected to a Shimadzu HPLC (Shimadzu, Inc., Columbia MD 21040). This apparatus consisted of two LC-600 pumps, an SIL-6B autoinjector, RF-551 fluorescence detector (excitation 325 nm, emission 370 nm). Separations were achieved by application of a linear

gradient of 12-42% acetonitrile from 0 to 20 min in water and HFBA. Quantification of pentosidine was made by comparison of peak areas with a pentosidine standard (Vincent Monnier, Cleveland, OH 44120) injected under identical conditions. A software package (Shimadzu CLASS-VP 4.2) was used to analyze the data.

Plasma glucose and uric acid determination. Blood samples (n=5) were collected from the wing vein of birds randomly selected for sacrifice as described above. Glucose was analyzed by a YSI 2700 Select biochemistry analyzer (YSI, Inc., Akron, OH 45387). Uric acid was determined using a kit from Sigma (procedure # 685, Sigma Diagnostics, MO, 63178).

Leukocyte isolation and chemiluminescent assay for oxidative stress. One milliliter of blood from 125 wk old hens (n=5 per group except for the AL group where only 2 birds remained) was suspended in mono-poly resolving medium (ICN 16-980-49) and leukocytes were isolated by centrifugation. The total number of leukocytes were counted using a routine hemocytometric technique. To a 3 ml luminometer tube were then added 100 μ l of leukocytes, 100 μ l luminol solution, 200 μ l PBS, and 100 μ l phorbol myristate acetate (PMA). The luminometer tube was placed into a luminometer (Berthold model LB 9505C) with the temperature control set at 37°C. Oxidative activity was determined by measuring the luminescence generated over 30 min; results were reported as counts per minute (CPM). The data was analyzed by a PC running KINB software. Luminescence was corrected for each group based on the number of leukocytes present.

Statistical Analyses

Data were analyzed by the general linear models procedure using a 2 x 2 x time factorial design (30). Regression analysis was performed to determine the correlation between the concentration of Ps and age. Oxidative stress was analyzed by a one-way ANOVA. The Student-Newman-Keuls Range method was used to test the significance of the difference between means.

RESULTS

Plasma Glucose and Body Weight

Hens in the DR groups weighed less at all ages monitored than those in the AL groups ($P<0.0001$; Fig. 1). At wks 44 and 68 DR increased ($P<0.05$) concentrations of plasma glucose as compared with AL birds (Fig. 2). AG did not affect body weight or plasma glucose concentrations.

Skin Pentosidine (Ps)

Ps accumulated linearly with age in AL-fed birds ($r^2=0.7768$); DR significantly reduced its accumulation in broiler breeder hens over the 92 weeks ($P<0.001$; Fig. 3). Concentrations of Ps in DR birds increased initially after hatch and then plateaued. For the AL group, concentrations of Ps were 0.018 ± 0.001 pmol/mg collagen for 8 wk old birds and 0.171 ± 0.018 pmol/mg collagen at 92 wks of age. Concentrations in the DR group were 0.009 ± 0.0007 pmol/mg collagen and 0.069 ± 0.008 pmol/mg collagen for 8 and 92 wks of age, respectively. Dietary supplementation with AG lowered the rate of Ps accumulation; concentrations of Ps in the AL+AG group were comparable to those of the DR group (Fig. 3). Aminoguanidine reduced ($P<0.001$) the accumulation of Ps in both dietary groups. The AL+AG group had concentrations of Ps ranging from 0.001 ± 0.0009 pmol/mg collagen at 8 wks to 0.049 ± 0.005 pmol/mg collagen at 92 wks, while the concentrations in the DR+AG group ranged from 0.012 ± 0.002 pmol/mg collagen at 8 wks to 0.036 ± 0.003 at pmol/mg collagen at 92 wks.

Oxidative Stress and Uric Acid

PMA-induced respiratory bursts as a measure of oxidative stress were lower ($P<0.0001$) and plasma uric acid higher ($P<0.0003$) in both the DR and DR+AG groups than in the AL and AL+AG groups ($P<0.001$). The AL+AG group showed a 10% increase in uric acid (Fig. 4) and a 302% decrease in oxidative stress (Fig. 5) as compared to the non-supplemented AL group. Plasma uric acid remained high at most time points and an overall increase in its mean concentrations was evident in DR-fed birds (Fig. 4). DR lowered oxidative stress and AG further enhanced that effect (Fig. 5).

DISCUSSION

Contrary to expectations, the higher-than-mammalian concentrations of glucose in AL birds were not associated with higher-than-mammalian concentrations of Ps. In fact, Ps concentrations in the hens (0.171 ± 0.018 pmol/mg collagen at 92 wks of age for AL-fed birds) were markedly lower than those reported in mammals (29 pmol/mg for human skin collagen and 10 pmol/mg for bovine tendon collagen (commercial Type I insoluble collagen)) (11). Thus, the formation of pentosidine, and, hence, possibly other AGEs, may not solely depend on high concentrations of plasma glucose per se, even in the presence of elevated basal temperatures and metabolic rates, as found in birds. In fact, glycosylated collagen may stimulate the oxidation process and lead to pentosidine formation (31) although only 0.4-20% collagen by weight is reported to be glycosylated (32, 33). In addition, whether the chemical composition of avian collagen with respect to arginine/lysine is responsible for reduced crosslink formation as compared to mammals remains to be established. The accumulation of Ps in AL birds was linear over the 92 wk sampling period (Fig. 6), comparable to the results reported in mammals (shrew, monkey, dog, pig, cow, rat, human) (11, 34).

One of our most interesting results is the finding that, despite the fact that DR reduced concentrations of Ps, it did not produce a corresponding reduction in concentrations of plasma glucose. In this study, in contrast to the prediction of the glycosylation theory of aging, which states that glucose or other products of carbohydrate metabolism are important contributors to the aging process so that DR (and a corresponding reduction in concentrations of plasma glucose) would be expected to reduce tissue protein glycosylation and AGE accumulation, DR birds had decreased concentrations of Ps ($P < 0.001$) coupled with increased average concentrations of plasma glucose ($P < 0.05$) when compared to AL birds.

Variable effects of DR have been reported in the literature. For example, a 10% decrease in blood glucose concentrations in DR rats (14) decreased the glycation of proteins by 18-33%. A similar observation was noted in a separate study; an approximately 11% decrease in glucose in DR animals was associated with a 34% reduction in glycated Hb (35). The reason for this is

unknown, however a decrease in the level of oxidative stress in birds (DR as compared to AL) may limit the formation of glycoxidation products by increasing antioxidant enzymes (36-38), this limitation being present in spite of higher-than-mammalian basal temperatures and concentrations of glucose. Strengthening the antioxidant defenses of diabetics by dietary supplementation of Vitamins C and E has been reported to decrease protein glycation without an effect on glycemia (39-41). These observations suggest that a decrease in oxidative stress in DR animals contributes to the observed decrease in protein glycation. This might result from changes in ascorbate homeostasis or the ratio of Vitamin E to polyunsaturated lipid concentrations in DR compared to AL animals (14). In the present study, a 4% increase in mean overall plasma glucose concentration was associated with a 99% decrease in mean Ps concentration. While several studies have firmly established that collagen glycosylation is increased with elevated concentrations of glucose both *in vivo* and *in vitro* and that this glycosylation increases with age in several tissues, other studies have failed to demonstrate anything more than a loose relationship between crosslinking and glycosylation, either *in vivo* or *in vitro* (42-46). Furthermore, Beuchat and Chong (1997) (47) reported that, in spite of circulating concentrations of plasma glucose in excess of 650 mg/dl, the amount of glycated Hb in hummingbirds (2-5%) is lower as compared to mammals which have levels ranging from 6-8%. These data suggest that class aves may also have a more efficient antioxidant system which limits the generation of glycoxidation products. Indirect evidence to support this view includes the observation that, in spite of an increased metabolic rate, the production of oxygen free radicals in avian (Red-tailed hawk and chicken) heterophils was significantly lower than in analogous bovine neutrophils (48). In another study comparing pigeons and rats, researchers found that mitochondrial generation of both oxygen free radicals and peroxides was lower in pigeons and that pigeons had higher antioxidant activities (49). Antioxidants such as Vitamin A (retinol and retinyl esters) and α -tocopherol (Vitamin E) have been found in higher concentrations in birds than in mammals (50).

Uric acid is one of the circulating antioxidants that demonstrates a positive correlation with maximum life span across species (51, 52). Humans, the most long lived among primates,

have comparably high levels of uric acid because they lack uricase, the terminal degradative enzyme present in monkeys and other mammals (51, 53). In support of this concept, the lower levels of uric acid in macaques (3-fold as compared to humans) correlate with a 3-fold shorter life span as compared to humans (54). Uric acid not only demonstrates a stabilizing influence on ascorbate in human blood (55), enhancing its role as a free radical scavenger (51, 52), it also functions as a noncatalytic binder of iron and iron-containing compounds that would otherwise catalyze the oxidation of ascorbate. This being the case, many species of birds must far surpass mammals in their ability to cope with free radical damage.

Uric acid has been proposed as a potent scavenger of free radicals in human and animal tissues (56, 57). Uric acid is ubiquitous; it is found in all types of extracellular fluids including the lymphatic, CSF, interstitial, synovial, intraocular, and amniotic fluids as well as in the lining of the respiratory tract (56). In fact, a reduction in uric acid concentrations is associated with an increase in reperfusion injury (infarct extension) in humans following myocardial infarction (58). Urate demonstrates, *in vitro*, the ability to scavenge peroxides, various hydroxyl radicals, and hypochlorous acid (56, 57). Because the concentrations of plasma uric acid in birds are approximately twice greater than the urate concentrations measured in humans (59, 60), we propose that the lower tissue concentrations of Ps in birds as compared to mammals are due to a more efficacious avian antioxidant system. Although several different antioxidants are present in the body system of birds, we suggest that uric acid may play an important role in coping with oxidative stress. However, the results from these studies do not support a predominant role for uric acid as the agent by which oxidative stress and Ps were reduced in DR and AG-supplemented birds.

The supplementation of an AL-fed animal with AG reduced the concentration of Ps to that measured in a DR animal. The reduction in concentrations of Ps in AL+AG birds was associated with a 10% overall increase in uric acid and a concomitant 302% reduction in PMA-induced respiratory bursts in blood leukocytes. On the other hand, in the DR+AG group the overall decrease in Ps was 43%, while the AL+AG birds showed an overall increase in uric acid

of 14% with an 85% reduction in PMA-induced respiratory bursts. DR has been shown to lower oxidative stress by modulating free radical production and increasing the concentration of antioxidant enzymes (36-38). Whether AG's mechanism of action in lowering oxidative stress mimics that of DR remains to be established. Reduced glycation as a result of reduced oxidative stress, in AL supplemented with AG as well as in DR, should increase longevity as suggested by the free radical theory of aging.

It is concluded that the rate of accumulation of Ps can be retarded by DR and AG and that birds appear able to retard the accumulation of glycoxidation products and their associated tissue damage in a more effective manner than mammals. This observation has important clinical applications in that a newly diagnosed diabetic may be able to limit or even prevent the accelerated accumulation of crosslinkages and the associated tissue degeneration. In regard to animal agriculture, the reduction in crosslinks may ultimately translate into an improvement in carcass composition and meat tenderness as well as an increase in production cycles for certain species.

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Figure 1: Effect of age and diet restriction (DR) and aminoguanidine (AG) on body weight (BW). Each point is the mean ($n=5$) $\pm$ SEM. * $P<0.0001$ between dietary groups (AL vs DR) at all time points.

Figure 2: Effect of age and diet restriction (DR) and aminoguanidine (AG) on concentrations of plasma glucose. Each point is the mean ($n=5$) $\pm$ SEM. * $P<0.02$ at those time points (AL vs DR).

Figure 3: Effect of age, diet restriction (DR), and aminoguanidine (AG) on skin pentosidine (Ps). Each point is the mean ($n=5$) $\pm$ SEM. Comparisons were within dietary groups (AL vs AL+AG and DR vs DR+AG). * $P<0.05$ and ** $P<0.001$.

Figure 4: Effect of age, diet restriction (DR), and aminoguanidine (AG) on concentrations of plasma uric acid. Each point is the mean ($n=5$) $\pm$ SEM. * $P<0.05$ and ** $P<0.0001$ at those time points (AL vs DR).

Figure 5: Effect of age, diet restriction, and AG on oxidative stress. Each point is the mean ($n=5$) $\pm$ SEM, except AL ($n=2$). Groups with different letters differ significantly ($P<0.0001$).

Figure 6: Correlation between age and pentosidine accumulation in the skin of *ad libitum*-fed broiler breeder hens. Each point is the mean ($n=5$) $\pm$ SEM.

Figure 5

Study II

Relationship between Mechanical Properties and Pentosidine in tendon: Effect of Age, Diet Restriction and Aminoguanidine in Broiler Breeder Hens

ABSTRACT

The objective of this study was to compare tendon breaking time (TBT) and tendon breaking strength (TBS) of the *flexor perforans et perforatus digiti iii* tendon with concentrations of pentosidine in tendons (P_t) of broiler breeder hens from 8 to 125 wk of age. In addition, effects of diet restriction (DR) and crosslinking inhibitor, aminoguanidine (AG) on P_t , TBS and TBT were determined. Female chicks (n=450) were randomly assigned to four treatment groups immediately after hatch: *ad libitum* (AL); feed diet restricted (DR; 60% of AL); feed of AL and DR groups supplemented with 1.35 mg/kg BW/day AG each (AL+AG and DR+AG, respectively). In AL-fed hens P_t increased with increasing age ($r^2 = 0.79$; $p < 0.0001$). Concurrently, there were age-related parallel increases in TBS ($r^2 = 0.56$; $p < 0.0001$), and TBT ($r^2 = 0.67$; $p < 0.0001$). The rate of accumulation of P_t in DR group was lower ($p < 0.0001$), as were TBS ($p < 0.01$) and TBT ($p < 0.02$) compared to AL hens. Concentration of P_t in AL+AG group was lower ($p < 0.0002$) as were TBS and TBT ($p < 0.01$ and $p < 0.01$, respectively). Supplementation of DR with AG did not affect ($p > 0.05$) P_t , TBS and TBT. In conclusion, the age-related increase in P_t and loss of elasticity in the tendon was retarded by DR. AG was effective in AL-fed hens where P_t , TBT and TBS declined compared to DR-fed hens.

INTRODUCTION

Abnormally high concentrations of plasma glucose over a period of many years may result in impaired tissue function, as found in diabetic patients. The cause of these complications appears to be multifactorial (1). One of the factors may be nonenzymatic glycation with subsequent formation of browning products resulting in irreversibly crosslinked protein (2-8). These crosslinks would be expected to affect the biomechanical properties of connective tissues, because collagens are long-lived proteins that possess ϵ -amino groups of lysyl and hydroxylysyl residues. Kohn (9) hypothesized that manifestations of aging are most pronounced in tissues with slow turnover, such as connective tissues of skin and tendon. In this regard, two established biomarkers of aging are rat tail tendon breaking time (TBT) (10-11) and the protein crosslink, pentosidine (12-14). Increased biomechanical strength and crosslinking of collagens were observed *in vitro*, after incubation of rat tail tendons with glucose (3,15-17), and *in vivo* in animals with experimental diabetes (18-19). Verzar (20) demonstrated that resistance to thermal denaturation of rat tail tendon, measured as TBT, increased with advancing age. Further work by Everitt *et al.* (21-22) showed that dietary restriction initiated at an early age in rats retarded aging rate of tail tendon collagen fibers, and inhibited the development of certain age-related disease processes such as renal disease, cardiac enlargement, and tumors. Further, Harrison *et al.* (23) and Harrison and Archer (24) established a strong correlation between age and TBT in inbred and hybrid strains of mice.

Since solubility of tail tendon collagen decreases with age, it was suggested by Verzar (20) that increased molecular crosslinking of collagen explains the age-related increase in TBT and the noted effects on collagen solubility. Ever since, the exact chemical structures of these putative markers of senescence have been sought (25). One such marker is a glycoxidation product, pentosidine, originally isolated from collagen of aged humans (26). Pentosidine has been found also in other mammals and in broiler breeder hens (13, 27-29). Pentosidine is an imidazopyridinium crosslink involving a pentose sugar crosslinked with arginine and lysine residues (26).

Class aves is an interesting model for biogerontology (30): concentrations of plasma glucose are typically 2 to 6 times mammalian norms (30-32), metabolic rates are as much as 2 to 2.5 times higher than similarly-sized mammals (33-34), and basal body temperature is about 3 °C higher than in mammals (30). All these factors should accelerate the formation of advanced Maillard products and hence, the process of tissue aging compared to normal aging (30, 35-37). Concentrations of pentosidine in skin of birds, however, are approximately 1000 fold lower than what is found in mammalian tissues (32).

The objective of this study was to compare the tendon breaking time (TBT) and tendon breaking strength (TBS) of the *flexor perforans et perforatus digiti iii* tendon in broiler breeder hens with concentrations of pentosidine in tendon (P_t) from 8 to 125 wks of age. Secondly, effects of diet restriction (DR) and crosslinking inhibitor, aminoguanidine (AG) on P_t, TBT and TBS were determined.

MATERIALS AND METHODS

Birds and Management

Day-old broiler breeder (Cobb X Cobb) female chicks (n=450) were placed in electrically heated battery brooders and fed *ad libitum* until 4 wks of age. At this time, chicks were randomly assigned to four treatment groups: *ad libitum* (AL); diet restricted (DR) fed at 60% of AL energy intake; AL with aminoguanidine (AG; Aldrich Chemical Co., Inc., Milwaukee, WI 53233) at 1.35 mg/kg BW/day (AL+AG); and DR with AG at 60% of that fed the AL group (DR+AG). AG was supplemented in feed of the hens. Birds were fed these diets throughout the study. The DR chicks received the recommended amounts of vitamins, minerals and nutrients according to the Cobb Management Guide (Cobb-Vantress, Inc., Siloam Springs, AR 72761). Feed and AG allowance for DR birds were calculated weekly based on the previous week's consumption by the AL group. At the selected dose of AG, a significant reduction in the accumulation of fluorescent endproducts occurred in *Biceps femoris* muscle of broiler breeder hens (38). All birds were fed daily between 0800 and 1000 hrs and had free access to water. The flock was reared in floor pens until 20 wks of age. At this time, hens were caged

individually. All birds were kept under light-tight conditions, and photoperiod was set according to the Cobb Management Guide.

Tendon Collection. *Flexor perforans et perforatus digiti iii* tendons were removed at 12 wk intervals from 8 to 92 wks and then again at 125 wks of age. This tendon is among the longest tendons, extending from the middle of the tibial region to the 3rd digit. Hens (n=5, except for the AL group at 125 wks where only 2 birds remained) from each group were randomly selected for tendon dissection. Tendons were removed by transverse cuts at the point of insertion and slightly proximal to the origin, washed with normal saline and stored at -80° C until physical and chemical analyses.

Measurement of Tendon Breaking Time and Strength. Tendon breaking time (TBT) and tendon breaking strength (TBS) was measured by a modification of the procedures described for the estimation of shear force in *pectoralis major* muscle in poultry (39). Prior to analysis each tendon was immersed in Ringer's solution, pH 7.4. Determination of TBT and TBS was on individual tendons mounted in an Instron Universal Mechanical Machine (Model TM, Instron Corp., Canton, MA 45419). The normal grip to grip length of each test specimen was 10 mm (40). Tendons were attached between the Instron's movable cross-head and rigid base such that its position remained centered between the jaws. The cross-head, attached to a 50 kg load cell (Model 152050; Daytronic, Miamisburg, OH 45342), moved away from the rigid base at a speed of 50.8 mm/min. Output from a LVDT conditioner (Model 9130, Daytronic Miamisburg, OH 45342) for the tensile deformation of the tendon was acquired by a computer equipped with a DT 2805 data acquisition board (Data Translation, Marlboro, MA 01752). Signals were processed with the HP-VEE software package (Hewlett Packard Co., Loveland, CO 80539-9929). The unloaded cross-sectional area at the mid-point of the tendon was used to normalize the TBS. Tendon breaking time was recorded when the movable cross-head started to lift up until the tendon was broken. Tendon breaking strength was expressed as peak force (pf) in kg/mm² and TBT as time in milliseconds (ms).

Tendon Pentosidine (P₁) Determination

Preparation of Collagen Digest. Broken *Flexor perforans et perforatus digiti iii* tendons were used for collagen digest for pentosidine determination. The collagen digest was prepared according to the techniques described by Monnier *et al.* (5) and Sell *et al.* (41). Briefly, 20 mg of tendon was frozen in liquid nitrogen, minced, and placed into a 13 X 100 mm screw-capped tube and then delipidated overnight in a chloroform-methanol (2:1) solution. Samples were rehydrated in a 50% methanol solution and hydrolyzed in 5 ml deaerated 6 M HCl at 110 °C for 18 h. All tubes were flushed with nitrogen prior to sealing with Teflon-faced rubber-lined caps. Subsequent to the hydrolysis, the samples were placed into a Speed Vac centrifuge-type vacuum drier (Savant Instruments, Farmingdale, NY 11735) until the HCl was evaporated. Samples were reconstituted in 250 μ l H₂O containing 0.01 ml heptafluorobutyric acid (HFBA) and filtered using a Costar® Spin-X® centrifuge tube filter (Corning Costar Corp., Cambridge, MA 02140). A modified Stegman and Stadler spectrophotometric method was used for estimation of collagen, using a hydroxyproline standard and assuming a collagen content of 14% hydroxyproline by weight (42).

Tendon Collagen Pentosidine. Tendon pentosidine (P₁) was measured by a modified reversed phase HPLC method (29). Samples of ~ 50 to 200 μ l volumes equivalent to 1 mg of collagen were injected into a 0.46X25 cm, Vydac 218TP104 (10 μ m) C-18 column (Vydac, Hesperia, CA 92345) connected to a Waters HPLC (Waters, Milford, MA 01757). The apparatus consisted of two pumps (Waters™ 600 Controller), an auto sampler (Waters™ 717_{plus}), and a scanning fluorescence detector (Waters™ 474_{plus}). Separations were achieved by a linear gradient of 12-42% acetonitrile from 0 to 25 min in water and 0.01M HFBA at flow rate of 1 ml/min. The pentosidine peak was monitored by an on line scanning fluorescence detector at excitation wavelength 325 nm/emission wavelength 370 nm. Quantitation of pentosidine was made by comparison with standard curve made by peak areas with a pentosidine standard (Vincent M. Monnier, Cleveland, OH 44120) injected under identical conditions. A software package (Millennium 2.1) was used for integration of peaks.

Statistical Analyses

Data were analyzed by the general linear models procedure using 2 X 2 factorial design. (43). Correlation between P_t , age, TBS and TBT were determined. The Student-Newman-Keuls Multiple Range test was used to estimate the significance of difference among means.

RESULTS

Pentosidine (P_t) of AL-fed broiler breeder hens increased with increasing age ($r^2=0.79$, $p<0.0001$). Concurrently, there was an age-related parallel increase in TBS ($r^2 = 0.56$, $p<0.0001$), and in TBT ($r^2 = 0.67$, $p<0.0001$). DR lowered ($p<0.001$) the accumulation of P_t (Fig. 1A). Similarly, DR retarded the overall TBS ($p<0.01$) and TBT ($p<0.02$) when compared to tendons from AL-fed hens (Fig. 1 B, C). The effect of DR was not consistent on these end-points; a greater decrease (112%) in the concentration of P_t was associated with a relatively lower decrease in both TBS (55%) and TBT (33%) respectively.

Supplementation of AL-fed hens with AG (AL+AG group) retarded ($p<0.0002$) P_t accumulation and resulted in decreases in TBS and TBT ($p<0.01$) (Fig. 2). As with DR, a 130% decrease in the concentration of P_t was measured in AL hens fed AG (AL+AG group) and was associated with 48% and 35% decreases in TBS and TBT, respectively. Supplementation of DR hens with AG (DR+AG group) did not affect ($p>0.05$) P_t , TBS or TBT (Fig. 3).

DISCUSSION

In the present study, age-related changes in P_t , TBS and TBT in broiler breeder hens were compared with the effects of DR and AG supplementation on these end-points. A parallel age-related increase in the concentration of P_t was associated with age-related increases in TBS and TBT in AL-fed hens. These observations are similar to those of Fu *et al.* (44-45) who found a parallel increase in pentosidine formation and collagen crosslinking when rat tendon tendons were incubated with glucose. Likewise, Richard *et al.* (46) showed a parallel increase in pentosidine and TBT when tail tendons were incubated with ribose. However, because the lag phase and the percent increase in pentosidine and TBT were not the same over the incubation

period in that study, there appeared to be some dissociation between the two events. These observations suggest that other crosslinks or factors may be responsible for the large increase in TBT during this type of incubation protocol. Undoubtedly, pentosidine would contribute to the observed increase in TBT if estimates of Vater *et al.* (47) are correct (14). The relationship between TBT and collagen crosslinks was further confirmed by the fact that the overall pattern of changes in TBT shared similarities with those of another glycoxidation product, carboxymethyllysine (CML) (48). Numerous studies have shown TBT to be highly correlated with age (20, 24, 49-50), whereas little information is available about TBS in the literature.

DR markedly affected age-related changes in P_t , TBT and TBS. In mammals, DR has been found to increase mean and maximum life span while delaying many age-associated disease processes such as tumorigenesis (51-52), leukemia (53), and renal diseases (22). Previous studies have shown that the physiological rate of collagen aging as determined by TBT is delayed by DR (1, 21-22). Similar observations were found in the present study. A significant delay of the age-related increase in TBS and TBT was observed in DR hens as compared to AL-fed hens (Fig. 1). Since DR delays the aging process, this intervention would be expected to also retard the age-related increase in the concentration of pentosidine. The rate of formation of P_t was significantly delayed by DR and these findings are comparable to those of earlier studies in rat tail tendons (14, 54).

The effectiveness of AG as an inhibitor of nonenzymatic glycation is controversial (1, 38, 55-60). There is also disagreement over its mode of action (61). In the present study, accumulation of P_t for the AL+AG group was retarded (Fig. 2A); whereas, AG had no effect in the DR+AG group (Fig. 3A). Supplementation of AL and DR hens with AG resulted in a reduction in P_t of 130% and 18%, respectively. The overall concentration of P_t , TBS and TBT for AL+AG group was comparable to those of DR-fed birds (Fig. 1, 2). The reduction in concentration of P_t (130%) for the AL+AG group was associated with a 48% decrease in TBS and a 35% decrease in TBT. In contrast to these observations, Oxlund and Andreassen (1), did not find any difference between TBT of tail tendons from diabetic rats treated with AG for 120

d and control rats. Results suggest that the detection of treatment effect is a likely explanation for these differences because a difference in TBT was recorded in the present study beginning at 20 wk (140 d) of age. In agreement with Oxlund and Andreassen (1), AG supplementation of DR did not affect P_t , TBS and TBT. These findings are consistent with those of Klandorf *et al.* (38) who reported no effect of AG supplementation in DR birds on the fluorescence of *Biceps femoris* muscles in poultry. Similarly, in a companion study (39), supplementation of DR with AG did not affect the shear force of the *pectoralis major* muscles over a 125 wk period. These results are consistent with the views that AG is relatively ineffective in DR-fed animals. Perhaps this is due to the total concentration of pentosidine in a given amount of collagen, and the extent of glycosylation in the body. Dyer *et al.* (62) reported that pentosidine accounts for <1% of the crosslinks formed from the *in vitro* Browning reaction between protein and glucose. Reports mention that glycosylation does not continue indefinitely and is limited in amounts that range from ~ 0.4 to 20% by weight of covalently attached carbohydrates, depending on the collagen's tissue of origin (63-65). Although DR significantly reduced crosslinking, it did not reduce the glycosylation (32). As a consequence, DR probably left little or no role to play for AG supplementation in reducing the glycosylation process and subsequently reduction in collagen crosslinks. Although the mode of action in lowering P_t , TBS and TBT in AL-fed hens is not clearly understood, AG lowers oxidative stress in broiler breeder hens (32). However, oxidative stress in that study has to be carefully interpreted because there were only two birds in the AL-fed group, and that was based only on one time point (125 wks) observation in broiler breeder hens.

Although rate of accumulation of P_t was similar to the age-related changes in TBS and TBT, there were differences in response of these end-points to DR and AG. A 130% decrease in mean overall P_t for AL group was reported by supplementing this group with AG which was associated with 48% and 35% decrease in mean TBS and TBT values, respectively. Similarly, a 112% decrease in mean overall concentration of P_t in DR birds, which was associated with 55% and 33% decreases in mean TBS and TBT values, respectively. These observations are in contrast to studies in mammals (44-46) which showed that TBT values were relatively higher as compared to the crosslinks present in rat tail tendons incubated with glucose or ribose,

suggesting other crosslinks than pentosidine might be involved in observed TBT values (14,47). A non-fluorescent component (NFC-1) has been isolated (66) and its concentration is also glucose dependent. NFC-1 concentrations are also found 50 times greater than that of pentosidine in aorta (67). We do not know the reasons for this discrepancy between these results and the results of our study. On the other hand, an 18% reduction in the concentration of P_t in DR+AG birds was associated with no changes in TBS and TBT (Fig. 3). Variable effects of DR and AG have been reported in literature on glycosylation (13,32,68). The results from our earlier studies (32) and other previous work do not support the view of increased crosslink formation as a result of hyperglycemia per se. We have previously observed that DR and AG lowered the crosslinking without any observable effect on plasma glucose concentrations. These findings are intriguing and imply that glycosylation may not be the primary cause of age-related crosslink formation (45,69-71). In support of this view, there are reports that Amadori products are not likely to be the major precursors of collagen crosslinks and that crosslinking agents are generated, in part, before Amadori rearrangement (72).

In summary, P_t , TBS and TBT increased over time in AL-fed hens. DR significantly retarded the rate of accumulation of P_t , TBS and TBT alone, not with AG supplementation. In conclusion, AG was effective only in AL and not in DR hens. The response of DR and AG was greater in lowering the concentration of P_t than those of TBS and TBT. The results support the view that it is not hyperglycemia per se that is responsible for the age-related changes in tissue crosslinking.

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Figure 1. Effect of age and diet restriction (DR) on (A) concentration of pentosidine (P_i), (B) tendon breaking strength (TBS), (C) tendon breaking time (TBT) in *flexor perforans et perforatus digiti iii* tendon of broiler breeder hens. Each point represents the mean (n=5; except at 125 wks (n=2) for AL group) $\pm$ SEM. Differences were significant, * p <0.05, ** p <0.001 and *** p <0.0001, between AL (closed squares) and DR (open circles) groups at those points.

Figure 2. Effect of age and aminoguanidine (AG) supplementation with *ad libitum*-fed (AL) on (A) concentration of pentosidine (P_i); (B) tendon breaking strength (TBS); (C) tendon breaking time (TBT) in *flexor perforans et perforatus digiti iii* tendon of broiler breeder hens. Each point represents the mean (n=5; except at 125 wks (n=2) for AL group) $\pm$ SEM. Differences were significant, * p <0.05, ** p <0.001 and *** p <0.0001 between AL (closed squares) and AL+AG (open triangles) groups at those points.

Figure 3. Effect of age, diet restriction (DR) and aminoguanidine (AG) on (A) concentration of pentosidine (P_i); (B) tendon breaking strength (TBS); (C) tendon breaking time (TBT) in *flexor perforans et perforatus digiti iii* tendon of broiler breeder hens. Each point represents the mean (n=5) $\pm$ SEM. Supplementation of DR with AG did not affect these end-points significantly (p >0.05) between DR (closed diamonds) and DR+AG (open circles) groups.

Figure 1

Figure 2

Figure 3

Study III

Age-related Changes in Meat Tenderness and Tissue Pentosidine: Effect of Diet Restriction and Aminoguanidine in Broiler Breeder Hens

ABSTRACT

The nonenzymatic glycosylation of tissue protein contributes to the formation of crosslinks that leads to structural and functional deterioration in the long-lived tissue protein, collagen. The accumulation of these crosslinks thus contributes to the objectionable toughness of meat from aged animals, decreases its economic value and limits its use in whole muscle foods. The objectives of this study were to determine the effectiveness of diet restriction (DR) and the crosslinking inhibitor, aminoguanidine (AG), on reducing the accumulation of crosslinks, thereby improving meat tenderness in broiler breeder hens. The glycoxidation product, pentosidine, was also measured in skin (Ps) to determine if changes in its concentrations correlated with the changes in shear force (SF). Chicks (n=450) were randomly assigned to four treatment groups from 8 to 125 wks after hatch: *ad libitum* (AL); diet restricted (DR); *ad libitum* and diet restricted groups supplemented with 400 ppm AG each (AL+ AG and DR+AG respectively). SF was measured with an Instron Universal Mechanical Machine. Ps was isolated by reverse phase HPLC. There was an age-related, linear increase in SF ($p<0.0001$, $r=0.96$) which correlated ($r=0.86$) with the age-related increase in Ps in AL fed hens. DR significantly retarded the SF ($p<0.0001$) over the sampling period. In general, SF values for AL+AG were similar to those measured in DR; whereas, no additive effect was observed for AG in DR birds. It was concluded that there was a linear increase in meat toughness (SF) with age that correlates with the accumulation of Ps, and that the decline in meat tenderness can be retarded by DR or

AG. Secondly, the effect of DR on accumulation of Ps was so pronounced that AG supplementation did not further enhance this effect.

Key words: Diet restriction, Aminoguanidine, Meat tenderness, Pentosidine, Aging.

INTRODUCTION

Meat of spent fowls becomes objectionably tough with increasing age and this toughness limits its use in whole muscle foods resulting in considerable economic losses to the poultry industry (Nakamura *et al.*, 1975; Shimokomaki *et al.*, 1972). For this reasons meat has been traditionally used in less-profitable, comminuted or retorted products where the small particle size or thermal processing is used to reduce toughness (Nurmahmudi and Sams, 1997). This problem is of particular importance to the poultry industry which produces spent fowl as a by-product of table and hatching egg production. Approximately 130,000,000 spent fowl being disposed of in the USA annually (Vandepopuliere and Lyons 1996); thus, the potential economic benefit to be gained by tenderizing this meat is tremendous.

The age-related decline in meat tenderness is due to many factors. Recent studies have suggested that glucose-derived, nonenzymatic collagen crosslinking plays a role in the normal aging process as well as the accelerated tissue aging associated with the hyperglycemic state (Brownlee *et al.*, 1988). The structural, long-lived tissue protein collagen is the major component of connective tissue and functions as the basic supporting structure of tissues and organs. Nonenzymatic collagen crosslinking in muscle may contribute to the decline in meat tenderness of aged animals; although, its importance in skeletal muscle has not been established (Bailey, 1989; Klandorf *et al.*, 1996). The glycosylation theory of aging suggests that modification of proteins by glucose and associated browning or Maillard reactions lead to the gradual crosslinking, polymerization, development of brown color and fluorescence characteristic of age-associated changes in tissue protein collagen (Cerami, 1985; Monnier, 1989). Accumulation of these crosslinks are postulated to lead to structural and functional deterioration of tissue protein and contribute to the pathophysiology of diabetic complications (Monnier, 1989; Bucala and Cerami, 1992; Vlassara *et al.*, 1994; Cefalu *et al.*, 1995). One such

crosslink, pentosidine, has been isolated and characterized in numerous animal species (Sell and Monnier, 1989; Iqbal *et al.*, 1997), and is an imidazole [4, 5b] pyridinium molecule containing a lysine and an arginine residue crosslinked by a pentose.

Presently, there is no economically feasible way to increase meat tenderness by decreasing collagen crosslinking in spent fowl meat (Kondaiah and Panda, 1992; Woods *et al.*, 1997). Numerous studies have demonstrated that diet restriction (DR) (Masoro *et al.*, 1989; Cefalu *et al.*, 1995) and the crosslinking inhibitor, aminoguanidine (AG) (Brownlee *et al.*, 1986; Khatan *et al.*, 1988; Oxlund and Andrassen, 1992; Wu, 1995), retard the accumulation of crosslinks over an animal's life time. Whether DR, AG or both (Klandorf *et al.*, 1996; Iqbal *et al.*, 1997), can improve meat tenderness (SF) as compared to *ad libitum* fed birds has not been determined. In addition, the glycoxidation product, pentosidine, was measured in skin (Ps) to determine if changes in its concentration correlated with internal changes in shear force (SF) values of the *pectoralis major* muscle.

MATERIALS AND METHODS

Birds and Management

Day-old broiler breeder (Cobb X Cobb) female (n=450) chicks were placed in electrically heated battery brooders and fed *ad libitum* until 4 wks of age. At this time, chicks were randomly assigned to four treatment groups: *ad libitum* (AL group); diet restricted (DR group); *ad libitum* with 400 ppm aminoguanidine¹ (AG); and diet restricted groups with 400 ppm AG in feed (AL+AG and DR+AG groups respectively). Birds were fed accordingly throughout the study. Diet restricted chicks were fed daily with a limited allowance diet according to the Cobb Management Guide². At the selected dose of AG, a significant reduction in the accumulation of fluorescent endproducts occurred in *B. femoris* muscle of broiler breeder hens (Klandorf *et al.*, 1996). All birds were fed daily between 0800 and 1000 hrs and had free access to water.

¹Aldrich Chemical Co., Inc., Milwaukee, WI 53233.

²Cobb-Vantress, Inc., Siloam Springs, AR 72761.

The flock was reared in floor pens until 20 wks of age. At this time, hens were caged individually. All birds were kept under light tight conditions, and photoperiod was set according to the Cobb Management Guide. Body weight was monitored weekly.

Meat Tenderness Evaluation

Breast muscle tenderness was determined with modification of the methods described by Kenney *et al.*, (1996).

Breast Muscle Collection. Tissue samples were obtained at 12 wk intervals from 8 to 92 wks and then again at 125 wks of age. Hens (n=5) from each group were randomly selected for slaughter. Birds were electrically stunned and bled using a modified kosher technique. The *pectoralis major* muscle was isolated after 4 h of refrigeration at 4 C, vacuum packed, and stored at -20 C until thermal processing.

Measurement of Shear Force. The *pectoralis major* muscle from each bird was cooked to an internal temperature of 70 C on a Farberware Smokeless Indoor Grill³. Endpoint internal temperature (CT) was monitored with an Industrial Datalogger⁴ equipped with a copper-constant thermocouple⁵. Cooked muscle was cooled to room temperature and refrigerated overnight at 4 C. Slices of 1.27 cm were cut perpendicular to the fiber orientation of the muscle. Four to six, 1.27 cm diameter cores were removed from these slices parallel to the fiber orientation through the thickest portion of the cooked muscle. Warner-Bratzler shear force (SF) was determined by using an Instron Universal Mechanical Machine⁶. A Warner-Bratzler apparatus was attached to

³Model 450N, Farberware, Inc., Bronx, NY 10462.

⁴Model 205, Beckman Instruments, San Diego, CA 92123-1898.

⁵Omega Technologies; Stamford, CT 06907.

⁶Model TM, Instron Corp., Canton, MA 45419.

a 50 kg load cell⁷ and tests were performed at a cross head speed of 127 mm/min. Output from a LVDT conditioner⁸ was acquired by a computer equipped with a DT 2805 data acquisition board⁹. Signals were processed with the HP-VEE software package¹⁰.

Breast Weight and Cook Yield. Raw and cooked breast weights (Br. W) were recorded. Cook yield (CY) was calculated by expressing cooked weight as a % of uncooked weight.

Pentosidine Determination

Preparation of Collagen Digest. Hens (n=5) from the AL group at all time points were randomly selected and sacrificed as described earlier. Approximately 1 g of skin was removed from the breast area, washed with normal saline and stored at -80 C until assayed. The collagen digest used for pentosidine determination was prepared according to the techniques described by Monnier et al. (1986) and Sell *et al.* (1992). Briefly, this involved the removal of epidermal and adipose layers from skin samples, freezing in liquid nitrogen, and mincing. The minced samples were delipidated overnight in a chloroform-methanol (2:1) solution. Samples were rehydrated in a 50% methanol solution and hydrolyzed in 6 N HCl at 110 C for 18 h. All tubes were flushed with N₂ prior to capping for heating. Subsequent to the hydrolysis, the samples were placed into a Speed Vac centrifuge-type vacuum drier¹¹ until the HCl was evaporated. Samples were reconstituted in 250 µL H₂O and filtered using a Costar® Spin-X® centrifuge tube filter¹². A modified Stegman and Stadler spectrophotometric method was used for collagen estimation, using a hydroxyproline standard (Maekawa *et al.*, 1970).

⁷Model 152050; Daytronic, Miamisburg, OH 45342.

⁸ Model 9130, Daytronic Miamisburg, OH 45342.

⁹Data Translation, Marlboro, MA 01752.

¹⁰ Hewlett packard Co., Loveland, CO 80539-9929.

¹¹Savant Instruments, Farmingdale, NY 11735.

¹²Corning Costar Corp., Cambridge, MA 02140.

Skin Collagen Pentosidine. Age-related changes in skin pentosidine (Ps) from 8 to 92 wks of age were determined in a separate study (Iqbal et al., 1998). For purposes of establishing a relationship with shear force in this study, tissue pentosidine is reported in AL-fed birds only from 8 to 125 wks of age. Estimation of pentosidine was done by a modified reversed phase HPLC method (Iqbal et al., 1997). One mg of acid-hydrolyzed collagen digest in 100 μ L of water/0.01M heptafluorobutyric acid (HFBA) was injected into a 0.46X25 cm Vydac 218TP104 (10 μ m) C-18 column¹³ connected to a Shimadzu HPLC ¹⁴. The apparatus consisted of two model LC-600 pumps, a model SIL-6B auto injector, a model RF 551 fluorescence monitor (excitation 325 nm, emission 370 nm). Separations were achieved by application of a linear gradient of 12-42% acetonitrile from 0 to 20 min in water and 0.01M HFBA. Quantitation of pentosidine was made by comparison of peak areas with a pentosidine standard¹⁵ injected under identical conditions. A software package (Shimadzu CLASS-VP 4.2) was used to analyze the data.

Statistical Analyses

Data were analyzed by the general linear models procedure using a 2 x 2 x time factorial design (SAS Institute, 1990). Regression analysis was carried out to determine the correlation between SF and Ps. Covariate analyses were carried out with age, CT and Br. W as covariates of SF in order to eliminate their potential effects on treatments. The Student-Newman-Keuls Range method was used to test the significance of difference between means.

RESULTS AND DISCUSSION

In the present study, the potential contribution of nonenzymatic crosslinks to meat tenderness has been addressed in broiler breeder hens over a 125 wks period. In both skin and

¹³Vydac, Hesperia, CA 92345.

¹⁴Shimadzu, Inc., Columbia, MD 21040.

¹⁵Vincent M. Monnier, Cleveland, OH 44120.

muscle Type I and III collagen are the two predominant genetic types of collagen (Sims and Bailey, 1982; Bailey and Light, 1989) and so a specific tissue crosslink, Ps, was used as an indicator of glycative and oxidative tissue damage in these tissues (Baynes, 1991). In mammals, pentosidine has been found to increase over the life span of the individual (Grandhee and Monnier, 1991; Dyer *et al.*, 1991b; Cefalu *et al.*, 1995). In agreement with these studies, there was an age-related, linear increase in Ps ($p < 0.001$, $r = 0.95$) that correlated ($r = 0.86$) with the linear increase in SF of *pectoralis major* muscle in AL-fed hens (Fig. 1). Nakamura *et al.*, (1975) reported that SF values of *pectoralis major* muscle increased with age ($r = 0.955$) in different strains of White Leghorn chicken up to 520 days of age. Similar observations were also reported by Awonorin and Ayoade (1992) in broilers; Robertson *et al.*, (1984) in buffalo and Brahman-Southorn steers; and Batcher *et al.*, (1962) on lamb cuts from different ages.

Because crosslinks play an important role in the normal aging process and diabetes, the utility of DR and a pharmacological agent (AG) to attenuate the formation of these crosslinks was investigated. Various methods have been used to degrade the intrinsic collagen network to improve the texture of meat from older animals. These approaches include: acid marinades (Arganosa and Marriott, 1989); injection of plant proteinases such as papain (Brooks *et al.*, 1985; Cronlund and Woychik, 1987); Ca^{+2} ion marinades and postmortem injections (Young and Lyon, 1989; Young *et al.*, 1989; Papa *et al.*, 1989; Koohmaraie *et al.*, 1988; 1989; 1990); postmortem injection of bacterial collagenases into muscle (Foegeding and Larick, 1986; Cronlund and Woychik, 1987); activation of postmortem collagen proteolysis by the action of neutral proteases (Koohmaraie *et al.*, 1987; Morgan *et al.*, 1991; Birkhold *et al.*, 1992; Nurmahmudi and Sams, 1997). However, most of these studies did not address changes in collagen and meat quality in older animals. DR is one of the most extensively used anti-aging measures (Weindrich and Walford, 1988; Synder, 1989) and has been reported to extend the life span in rodents (Berg and Simms, 1960; Yu *et al.*, 1982a), reduce glycosylation (Masoro *et al.*, 1989; Cefalu *et al.*, 1995; Beuchat and Chong, 1997) and delay the onset of a number of major age-associated diseases (Yu, 1994b). In our study DR decreased SF values of *pectoralis major* muscle: the effect became particularly pronounced ($p < 0.0001$) after 56 wks (Fig. 2). The mechanism whereby DR lowers

the SF values is not clearly understood. However, a decrease in the level of oxidative stress and glycation in DR animals might have been limited the formation of glycoxidation products by increasing the concentration of antioxidant enzymes (Yu *et al.*, 1982a; Youngman *et al.*, 1992; Yu, 1994b), thus indirectly limiting the age-related decline in meat tenderness in birds.

Morgan *et al.*, (1991) reported no differences in the thermal stability of collagen (total, heat-soluble, or heat-insoluble collagen) for CaCl₂-injected vs control muscles obtained from mature cows. However, in young animals, Birkhold *et al.*, (1992) reported increased meat tenderness due to structural degradation of the collagen network induced by calpains. However, these workers could not confirm that the proteolytic and tenderization were related. Measurement of calpain activity as a possible mechanism of action of DR in meat tenderness should be addressed in the future studies. Supplementation of AL with AG (AL+AG group) reduced ($p<0.0001$) SF values, however, no further decrease in SF values was recorded ($p>0.05$) for DR supplemented with AG (DR+AG group) (Fig. 2). This finding is consistent with a previous study in broiler breeder hens in which SF values of *B. femoris* muscle were not affected in DR birds supplemented with AG (Klandorf *et al.*, 1996). It appears that AG is relatively ineffective in DR animals. This could be due to the fact that glycosylation does not continue infinitively and is limited in amounts that range from ~0.4 to 20% by weight of covalently attached carbohydrates, depending on the collagen's tissue of origin (Eyre, 1980; Sims and Bailey, 1982; Bailey and Light, 1989). Furthermore, pentosidine accounts for < 1% of the crosslinks formed during *in vitro* browning reaction of protein with glucose (Dyer *et al.*, 1991). DR probably left little or no role to play for AG supplementation in reducing the glycosylation process and subsequently reduction in the meat toughness. Although the mode of action of AG in lowering SF values in the AL group is not clearly understood, AG has been found to lower oxidative stress (Iqbal *et al.*, 1998) in broiler breeder hens. Whether its mode of action is comparable to that of DR has yet to be established.

There was also a linear, age-related increase in Br. W ($p<0.0001$, $r=0.64$) and CT ($p<0.0001$, $r=0.95$) in AL fed hens. These observations are consistent with those of Awonorin

and Ayoade (1992) in broilers and turkeys, and Toumy and Lechnir (1964) in beef. DR significantly reduced Br. W and CT ($p<0.0001$). Covariate analyses did not eliminate the treatment effects when Br. W, CT and age were kept as covariates of SF. In general, there was no consistent effect of AG supplementation on breast weight and CT, however, a marked decrease in CT was observed at 80 wks in the AL+AG group (Fig.3, 4). No consistent effect of DR and AG was observed on cook yield (data not reported).

In summary, increased meat toughness (SF) with age correlates with accumulation of Ps, in AL-fed animals. The decline in meat tenderness can be retarded by DR or supplementation with AG. Furthermore, the effect of DR on crosslink accumulation is so pronounced that AG supplementation cannot further enhance this effect. The results of this study have potential implications, particularly for the use of skin as an indicator of age-related changes in muscle. Using this approach a skin sample can be used for pentosidine estimation and hence a predictor of meat tenderness. Secondly, the data support the use of AG in the diets of AL animals as to improve meat tenderness. Future studies could address pentosidine accumulation in specific muscles and relate these changes to SF values.

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Figure 1. Correlation between shear force (SF) in *pectoralis major* muscle and pentosidine (Ps) in the skin ($r=0.86$) of AL fed hens. Each point is the mean of 5 birds.

Figure 2. Effect of age, diet restriction (DR) and aminoguanidine (AG) on shear force (SF). Each bar represents mean ($n=5$) $\pm$ SEM. No common letters differ significantly ($p<0.001$) at 68 and 80 wks, and ($p<0.0001$) at 92 and 125 wks between AL and DR groups at those time points only. pf = peak force in kg.

Figure 3. Effect of age, diet restriction (DR) and aminoguanidine (AG) on breast muscle weight (Br. W). Each bar is the mean ($n=5$) $\pm$ SEM. No common letters differ significantly ($p<0.0001$) between AL and DR groups at all time points, and ($p<0.05$) in supplemented (AL+AG and DR+AG) as compared to non-supplemented groups (AL and DR) at those time points only.

Figure 4. Effect of age, diet restriction (DR) and aminoguanidine (AG) on cook time (CT). Each bar is the mean ($n=5$) $\pm$ SEM. No common letters differ significantly ($p<0.0001$) between AL and DR groups at all time points, and ($p<0.05$) in supplemented (AL+AG and DR+AG) as compared to non-supplemented groups (AL and DR) at those time points only.

Figure 1

Figure 2

Figure 3

Figure 4

Study IV

Effect of Dietary Aminoguanidine on Tissue Pentosidine and Reproductive Performance in Broiler Breeder Hens

ABSTRACT

Factors influencing the age-related decline in production parameters of broiler breeder hens are poorly understood. Elevated blood glucose concentrations measured in broiler breeder hens may contribute to this decline. The nonenzymatic attachment of glucose to proteins generates glycoxidation crosslinks in tissue proteins which can ultimately impair their function. One such glycoxidation crosslink, pentosidine, has been used as a biomarker for aging studies because of its accumulation on the structural protein collagen. The objectives of these studies were to determine if pentosidine accumulates with age in hens and if the crosslinking inhibitor, aminoguanidine (AG), could retard this accumulation. An additional objective was to determine if AG had any effect on production performance. In the first study broiler breeder hens (n=318) were randomly assigned to two groups: control and supplemented (400 ppm AG). Pentosidine was measured in the skin of the birds at 20 and 67 wks of age. Egg production was measured daily. In a second study, broiler breeder hens (n=60) were reared as previously described. Pentosidine was measured in the skin of the birds at 20 and 68 wks of age. Results showed that pentosidine was present in the skin of the hens, and that concentrations increased with age ($P<0.001$). Although pentosidine was reduced ($P<0.001$) in AG supplemented birds, production performance was not affected. In conclusion, AG retarded the rate of accumulation of pentosidine during lay in broiler breeder hens, but the reduction in pentosidine did not significantly affect production performance.

Key words: aging, diet restriction, aminoguanidine, pentosidine, broiler breeder

INTRODUCTION

Advancing age adversely affects the reproductive success of many species including poultry. Once the genetic potential for maximum production of eggs has been reached, there is a steady, age-related decline in performance. Furthermore, eggs that are laid during the latter period of the reproductive lifespan are less likely to be fertilized and, if fertilized, are less likely to hatch (Lerner *et al.*, 1993). The factors that influence these age-related decreases in reproductive success are poorly understood. However, there is evidence in male humans that adult onset diabetes accelerates the age-related decline in reproductive function and fertility (Kaiser and Korenman, 1988).

The reproductive lifespan of *ad libitum* fed broiler breeders is both shorter and less productive than their diet restricted counterparts; diet restriction (DR) is the only established means of delaying the decline in their productive performance. Extensive gerontological research within the last two decades has confirmed findings that restricting the food intake of laboratory animals extends their reproductive lifespan (Berg and Simms, 1960; Yu *et al.*, 1982). The ability of DR to modulate reproductive aging and slow or prevent the onset of a number of major age-associated diseases, makes diet restriction a tremendously powerful and versatile tool (Yu, 1994b). DR also is used successfully in the poultry industry to achieve the desired body weight without significantly affecting reproductive performance (Fontana *et al.*, 1992). However, the mechanism of action of diet restriction in chickens is not understood.

In animal models with hyperglycemia, DR is effective in retarding the accumulation of the fluorescent end-product, pentosidine, a glycoxidation crosslink in the long-lived tissue protein, collagen, that has been used as biomarker for aging studies (Cefalu *et al.*, 1995). In mammals, pentosidine has been found to increase over the lifespan of the animal; the rate of this accumulation of pentosidine, which is formed by the nonenzymatic glycosylation of lysine and arginine residues, is accelerated in animal models with hyperglycemia (Sell and Monnier, 1989; Grandhee and Monnier, 1991; Dyer *et al.*, 1991a,b; Sell *et al.*, 1992; Reiser, 1994; Nagaraj *et al.*, 1996).

In addition to DR, aminoguanidine hydrochloride (AG), a nucleophilic hydrazine which acts as a crosslinking inhibitor, delays the formation of collagen-associated crosslinks in the tissues (Brownlee *et al.*, 1988; Khatan *et al.*, 1988; Odetti, 1990; Matuieu *et al.*, 1995; Klandorf *et al.*, 1995). Klandorf *et al.* (1996) reported that the addition of AG to the diet of broiler breeder hens decreased the accumulation of fluorescent end-products of collagen crosslinks in skeletal muscle. Whether this compound, coupled with industry practices such as diet restriction, can effectively delay the decline in production performance of broiler breeder hens was the basis for this study. The specific objectives of this study were to determine if pentosidine is present in tissue of broiler breeder hens, and to determine if its concentrations change over a production cycle. Because of the links between hyperglycemia, collagen crosslinking, and reproductive function an additional objective was to determine if the crosslinking inhibitor, AG, affected the rate of accumulation of pentosidine and could improve production performance.

MATERIALS AND METHODS

Birds and Management

Two studies were undertaken in order to investigate the role of AG in the accumulation of pentosidine in the tissues of broiler breeder hens. In the first study, day-old broiler breeder (CobbXCobb¹⁶) female (n=318) and male (n=50) chicks were placed in electrically heated battery brooders and fed *ad libitum* until 4 wks of age. All birds were then fed daily with a limited allowance diet according to the Cobb Management Guide and chicks were randomly assigned to one of two experimental groups: control (diet restricted control) or supplemented (diet restricted supplemented with 400 ppm AG¹⁷) and were fed accordingly throughout the study. At this dose, a highly significant reduction in the accumulation of fluorescent end-products in the skeletal muscle of broiler breeder hens was reported by Klandorf *et al.* (1996). Roosters were

¹⁶ Cobb-Vantress, Inc., Siloam Springs, AR 72761.

¹⁷ Aldrich Chemical Co., Inc., Milwaukee, WI 53233.

reared separately on diet restriction according to the Cobb Management Guide. All the birds were fed daily between 0800 and 1000 hrs and body weight was monitored every two wks. The flocks were reared in floor pens until 20 wks of age. At this time, they were caged individually. At 18 wks the photoperiod was gradually increased over four wks time from 8L:16D to 14L:10D. At 34 wks of age the photoperiod was increased to 15L:9D, for the duration of the study. In a second study, day-old broiler breeder chicks (n=60) were reared as previously described. Since the production performance data in the first study were not significant, production data were not collected.

Pentosidine Determination

Preparation of Collagen Digest. In both studies, hens (n=5) at 20 wks (before the onset of lay) and 67 to 68 wks of age (end of the production cycle) from both the control and supplemented groups were randomly selected and sacrificed by the industry-standard method of electrical stunning. Approximately 1 g of skin was removed from the abdominal area, washed with normal saline and stored at -80 °C until assayed. The collagen digest used for pentosidine determination was prepared according to a modification of the technique described by Monnier *et al.* (1986) and Sell *et al.* (1992). Briefly, this involved the removal of epidermal and adipose layers from skin samples, freezing in liquid nitrogen, and mincing. The minced samples were delipidated overnight in a chloroform-methanol (2:1) solution. Samples were rehydrated in a 50% methanol solution and hydrolyzed in 6 N HCl at 110 °C for 18 h. All tubes were flushed with N₂ prior to capping for heating. Subsequent to the hydrolysis, the samples were placed into a SpeedVac¹⁸ centrifuge-type vacuum drier until the HCl was evaporated. Samples were reconstituted in 250 µL H₂O and filtered using a Costar® Spin-X® centrifuge tube filter¹⁹. A modified Stegman and Stadler spectrophotometric method was used for collagen estimation, using a hydroxyproline standard (Maekawa *et al.*, 1970).

¹⁸ Savant Instruments, Farmingdale, NY 11735

¹⁹ Corning Costar Corp., Cambridge, MA 02140

Skin Collagen Pentosidine. The estimation of pentosidine was by a modified reverse phase HPLC method described by Sell *et al.* (1992). One mg of acid-hydrolyzed collagen digest of skin in 100 μ L of water/0.01M heptafluorobutyric acid (HFBA) was injected into a 0.46X25cm Vydac 218TP104 (10 μ m) C-18 column²⁰ connected to a Shimadzu HPLC²¹. The apparatus consisted of two model LC-600 pumps, a model SIL-6B auto injector, a model RF 551 fluorescence monitor (excitation 325 nm, emission 370 nm). Separations were achieved by application of a linear gradient of 12-42% acetonitrile from 0 to 20 min in water and 0.01M HFBA. Quantitation of pentosidine was made by comparison of peak areas with a 58 pmol pentosidine standard²² in 50 μ L injected under identical conditions. A software package (Shimadzu CLASS-VP 4.2) was used to analyze the data.

Production Trial

Production Data. Individual egg production and total egg defects (extra large, small, soft, membranous, cracked, thin-shelled) were recorded daily, beginning with the first egg until 70 wks of age. Every 5 wks, beginning with the 25th wk and continuing to the 70th wk, 50 eggs were randomly selected from both groups for determination of eggshell thickness, egg weight, and specific gravity. Eggshell thickness was measured by Digimatic Indicator 543²³ at two sites. Specific gravity was calculated by the computer-assisted displaced-water method (CADW) (Hempe *et al.*, 1988).

Semen Collection, Artificial Insemination, and Fertilization Data. Semen was collected by a modified abdominal massage method (Burrows and Quinn, 1937) from CobbXCobb roosters. Beginning at 30 wks of age, 40 randomly selected hens from each group were artificially inseminated every 10 wks (at 30, 40, 50, 60, and 70 wks) with 50 μ L of fresh, undiluted semen. For each time point, artificial insemination was performed weekly beginning

²⁰ Vydac, Hesperia, CA 92345

²¹ Shimadzu, Inc., Columbia, MD 21040

²² Vincent Monnier, Cleveland, OH 44120

²³ Mitutoyo Corp., Japan; Charles Seifert Assoc., Inc., Carnegie, PA 15106 (U.S. reps).

two wks prior to the period in which the fertilization data were to be collected (i.e. beginning insemination at 28 wks for the 30 wk time point). Inseminations were accomplished by the in-cage technique described by Moultrie (1956).

From each treatment group, fifty eggs were randomly selected from the pool of eggs collected the 3rd and 4th days following each insemination and incubated in a forced-air incubator²⁴ at 37.5 °C and 60% relative humidity. The eggs were removed from the incubator after seven days for testing of fertilization and viability. Fertilization was calculated as the number of fertilized eggs per 50 eggs set. Embryo viability was calculated as the number of viable embryos on day 7 of incubation per 50 fertilized eggs. Embryo production was measured as the number of viable embryos on day 7 of incubation per 50 eggs set (fertilization x embryo viability) (Robinson *et al.*, 1991).

Statistical Analyses

One way ANOVA and Fisher's LSD test were done to compare the control and supplemented groups for each production parameter at each time point. The interaction between age and dietary groups for pentosidine was analyzed by a two-way ANOVA (SAS Institute, 1990).

RESULTS AND DISCUSSION

The Maillard hypothesis of aging proposes that aging results, in part, from chronic chemical modifications of proteins and other biomolecules by reducing sugars and by the accumulation of glycoxidation products and other advanced glycosylation end-products (AGEs) in tissue protein (Cerami, 1985; Monnier, 1990; Reiser, 1991). Because lifespan has been extended by DR in lab animals, we investigated the effect of DR, with supplementation of AG, on the formation of AGEs in the collagen of aging broiler breeder hens. The extent of crosslinking was quantified by measurement of the fluorescent end-product, pentosidine. While

²⁴ Jamesway Incubatory Co. Monroe, NC 28110

this compound has been characterized in mammals, no investigations involving pentosidine have been reported in birds prior to this study. In agreement with results from mammalian studies (Dunn *et al.*, 1989, 1991; Koizumi *et al.*, 1989; Sell and Monnier, 1989, 1990; Dyer *et al.*, 1991a,b; Sell *et al.*, 1992; Reiser, 1994; Cefalu *et al.*, 1995; Nagaraj *et al.*, 1996), the glycoxidation product, pentosidine was detected in the tissues of birds. We also found that the accumulation of pentosidine in skin collagen of control and supplemented broiler breeder hens increased with age ($P<0.001$) (Fig. 1). Skin pentosidine increased 75% and 78% in the control and supplemented groups, respectively. Aminoguanidine ($P<0.001$) retarded the accumulation of skin pentosidine (Fig. 1). Comparable results have been reported in mammals (Brownlee *et al.*, 1986; Hayase *et al.*, 1991; Odetti, 1990; Edelstein and Brownlee, 1992 a,b; Oxuland and Andreassen, 1992; Yagihashi *et al.*, 1992; Wu, 1995).

In spite of the elevated metabolism and plasma concentrations of glucose in birds (Holmes and Austad, 1995), the measured concentrations of pentosidine were markedly lower in diet-restricted broiler breeder hens as compared to age-corrected diet-restricted mammals (Sell and Monnier, 1989; Cefalu *et al.*, 1995). In a comparable study, the concentration of glycosylated hemoglobin (GHb) in hummingbirds is lower as compared to mammals in spite of circulating concentrations of plasma glucose in excess of 650 mg/dL (Beuchat and Chong, 1997). It appears that the bird has evolved mechanisms to limit dramatically the rate of accumulation of these crosslinks. It has been hypothesized that the mechanisms of free radical production and antioxidant defenses differ between birds and mammals (Holmes and Austad, 1995). Conlon *et al.*, (1991) reported that oxygen free radical production in avian (Red-tailed hawk and chicken) heterophils was significantly lower than in analogous bovine neutrophils. In a recent study using pigeons and rats, Ku and Sohal (1993) found that mitochondrial generation of both oxygen free radicals and peroxide was lower in pigeons, and that pigeons had higher antioxidant activities. Antioxidants such as vitamin A (retinol and retinyl esters) and α -tocopherol (vitamin E), have been found at higher concentrations in a variety of birds than in mammals (Schweigert *et al.*, 1991). In addition, uric acid and its' anion form, urate, are recognized as ubiquitous antioxidants in humans, monkeys, and insects (Souza *et al.*, 1996; Hellsten *et al.*, 1996). Because the

concentration of plasma uric acid in birds (8 to 12 mg/dL; Shapiro *et al.*, 1997) is approximately two-fold greater than the urate concentrations measured in humans (2.6 to 7.2 mg/dL; Bishop *et al.*, 1992), it may be speculated that the lower tissue concentrations of the glycoxidation product, pentosidine, in hens as compared to mammals is due to a more efficacious avian antioxidant system. Further exploration into this area may contribute to the understanding of the aging process.

Although AG effectively reduced skin pentosidine there were no consistent effects of AG treatment on the reproductive cycle. Production data (egg production, egg weight, eggshell thickness, egg defects, and specific gravity) in birds fed AG did not differ consistently from that of the control animals (Fig. 2, Tables 1 and 2). Fertility, viability and embryo production also were not significantly different between the two treatments (Table 3). It appears that the dose of AG (400 ppm) is effective in lowering the pentosidine from the skin collagen but not in ovarian collagen which suggests the use of higher AG dosage. Alternatively, there could be a negative correlation between increase of skin pentosidine concentrations and decrease in egg production over the period of time.

In conclusion, although supplementation of diet restricted broiler breeder hens with AG retarded the rate of accumulation of pentosidine over a laying cycle, the reduction in pentosidine did not significantly affect mean production performance.

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Table 1: Egg production and egg defects (Mean $\pm$ SEM)

Age (wks)	Hen Day Egg Production (%)				Egg Defects (%) [†]	
	n	Control	n	Suppl.	Control	Suppl.
25	106	34 $\pm$ 2	112	33 $\pm$ 2	46 $\pm$.3 *	51 $\pm$.3
30	105	80 $\pm$ 3	111	79 $\pm$ 3	11 $\pm$.3	6 $\pm$.2 *
35	105	75 $\pm$ 3	110	73 $\pm$ 3	10 $\pm$.3	9 $\pm$.2
40	104	75 $\pm$ 3*	110	70 $\pm$ 3	2 $\pm$.2	2 $\pm$.2
45	103	71 $\pm$ 3	109	71 $\pm$ 3	1 $\pm$.1	.49 $\pm$ 0
50	102	66 $\pm$ 2*	107	60 $\pm$ 3	5 $\pm$.2	4 $\pm$.1
55	101	58 $\pm$ 2	106	63 $\pm$ 2 *	3 $\pm$.1	2 $\pm$.1
60	101	51 $\pm$ 2	106	48 $\pm$ 2	4 $\pm$.1	5 $\pm$.1
65	100	45 $\pm$ 2	106	44 $\pm$ 2	3 $\pm$.1 *	6 $\pm$.2
70	99	41 $\pm$ 3	105	39 $\pm$ 3	3 $\pm$.1 *	7 $\pm$.2

* P<0.05 between dietary groups at those time points only

Control = diet restricted control

Suppl. = diet restricted supplemented with 400 ppm aminoguanidine

[†] % of extra large, small, soft, thin-shelled, membranous, deformed, cracked eggs.

Table 2: Eggshell thickness, egg weight, and specific gravity (Mean $\pm$ SEM)

Age (wks)	n	Shell thickness (mm)		Egg weight (g)		Specific gravity	
		Control	Suppl.	Control	Suppl.	Control	Suppl.
25	50	.36 $\pm$ 0.003	.36 $\pm$.0003	52.9 $\pm$ 0.94	49.1 $\pm$ 0.94	1.083 $\pm$ 0.001	1.081 $\pm$ 0.001
30	50	.35 $\pm$ 0.004	.34 $\pm$ 0.004	58.7 $\pm$ 0.51	57.9 $\pm$ 0.51	1.080 $\pm$ 0.001	1.083 $\pm$ 0.001
35	50	.38 $\pm$ 0.004	.37 $\pm$ 0.004	62.1 $\pm$ 0.60	61.0 $\pm$ 0.60	1.080 $\pm$ 0.001	1.079 $\pm$ 0.001
40	50	.38 $\pm$ 0.003	.37 $\pm$ 0.003	62.6 $\pm$ 0.85	64.8 $\pm$ 0.85	1.079 $\pm$ 0.002	1.082 $\pm$ 0.002
45	50	.35 $\pm$ 0.003	.36 $\pm$ 0.003	63.8 $\pm$ 0.60	63.8 $\pm$ 0.60	1.078 $\pm$ 0.006	1.078 $\pm$ 0.006
50	50	.37 $\pm$ 0.004	.39 $\pm$ 0.004	68.1 $\pm$ 0.56	67.0 $\pm$ 0.56	1.076 $\pm$.0002*	1.062 $\pm$ 0.002
55	50	.36 $\pm$ 0.004	.37 $\pm$ 0.004	68.0 $\pm$ 0.61	67.6 $\pm$ 0.61	1.061 $\pm$ 0.001	1.062 $\pm$ 0.001
60	50	.32 $\pm$ 0.003*	.29 $\pm$ 0.003	69.6 $\pm$ 0.55*	66.5 $\pm$ 0.55	1.065 $\pm$ 0.001	1.071 $\pm$ 0.001*
65	50	.37 $\pm$ 0.003	.38 $\pm$ 0.004	72.4 $\pm$ 0.47*	70.0 $\pm$ 0.47	1.049 $\pm$ 0.001	1.048 $\pm$ 0.001
70	50	.34 $\pm$ 0.003	.34 $\pm$ 0.003	72.4 $\pm$ 0.47*	70.0 $\pm$ 0.47	1.048 $\pm$ 0.00	1.047 $\pm$ 0.003

* P<0.05 between dietary groups at those time points only

Control = diet restricted control

Suppl. = diet restricted supplemented with 400 ppm aminoguanidine

Table 3: Fertility, viability and embryo production (Mean $\pm$ SEM)*

Age (wks)	n	Fertility (%)		Viability (%)		Embryo production (%)	
		Control	Suppl.	Control	Suppl.	Control	Suppl.
30	50	94 $\pm$ 2	88 $\pm$ 2	94 $\pm$ 1	84 $\pm$ 1	94 $\pm$ 1	84 $\pm$ 2
40	50	100 $\pm$ 2	96 $\pm$ 2	100 $\pm$ 1	94 $\pm$ 1	100 $\pm$ 2	94 $\pm$ 2
50	50	98 $\pm$ 2	96 $\pm$ 2	98 $\pm$ 1	90 $\pm$ 1	96 $\pm$ 2	90 $\pm$ 2
60	50	76 $\pm$ 2	74 $\pm$ 2	68 $\pm$ 1	74 $\pm$ 1	68 $\pm$ 1	74 $\pm$ 1
70	50	72 $\pm$ 2	70 $\pm$ 2	66 $\pm$ 1	64 $\pm$ 1	66 $\pm$ 1	66 $\pm$ 1

* Non significant difference between dietary groups (P>0.05)

Control = diet restricted control

Suppl. = diet restricted supplemented with 400 ppm aminoguanidine

Figure 1. Skin pentosidine in Control and AG-supplemented groups. Data is the mean of five birds.
***P<0.001.

Figure 2. Hen day egg production in Control and AG-supplemented groups.

Figure 1

Figure 2

Chapter 4

SUMMARY

The factors which influence the age-related decline in production performance and meat toughness in broiler breeder hens are poorly understood. A characteristic of broiler breeder hens is the rapid, age-related decline in production performance once the genetic potential for maximum production of eggs has been reached at 32 weeks. In addition, the meat of aged hens becomes objectionably tough, limiting its use in whole muscle food. Broiler breeder hens are characterized by both obesity and hyperglycemia (plasma glucose concentrations 2-4 fold higher than humans). We have hypothesized that the elevated plasma glucose concentrations contribute to the nonenzymatic attachment of glucose to proteins and generate glycoxidation crosslinks in tissues which can ultimately impair their function. One such crosslink, pentosidine, is an established indicator of both glycative and oxidative tissue damage in mammals. Because of its linear, age-related accumulation on the structural protein, collagen pentosidine can also be used as a biomarker for the study of aging. Diet restriction (DR) is the only known reliable measure for slowing the glycosylation process and, subsequently, the formation of advanced glycosylation endproducts (AGEs) in tissues. Specifically, we examined the role of DR and a nucleophilic hydrazine compound, aminoguanidine (AG), a crosslinking inhibitor, on tissue crosslinking with the view towards both extending the production period and improving carcass quality of broiler breeder hens. The objectives of these studies were to determine if: 1) pentosidine is present in the skin (P_s) and tendon (P_t) of broiler breeder hens (isolation and quantification) ; 2) pentosidine accumulates over the the life time of the animal; 3) dietary modulation and supplementation with the crosslinking inhibitor, aminoguanidine (AG), could retard the accumulation of pentosidine, improve production performance, and limit the age-related increase in toughness of meat and tendon. Chicks were randomly divided into four dietary groups from 8 to 125 wks after hatch: *ad libitum* (AL); diet restricted (DR, fed 60% of *ad libitum*); AL and DR groups supplemented with 400 ppm AG (1.35 mg/kg BW/day) each

(AL+ AG and DR+AG respectively) for studies I, II and III. Two experiments (study IV) were carried (n=318; 60 respectively) were carried out for reproductive performance (%HD egg production, egg defects, egg shell thickness, egg weight, specific gravity, fertility, viability, embryo production) until 70 wk of age. Collagen estimation was done by a hydroxyproline spectrophotometric method. Pentosidine was isolated and quantified by reversed phase HPLC. Plasma glucose and uric acid concentrations were estimated by standardized procedures. Oxidative stress was determined by a modified luminometric method. Tenderness of *pectoralis major* muscle was measured in terms of shear force (SF) using an Instron Universal Mechanical Machine. Tendon (*flexor perforatus et perforans digiti iii*) breaking time (TBT) and strength (TBS) were also determined by developing a new technique using an Instron Universal Mechanical Testing Machine. Egg production and egg defects were recorded daily. Egg shell thickness, egg weight, specific gravity (n=50) were measured every 5 wks. Fertility, viability and embryo production trials were carried out on fertile eggs obtained from artificially inseminated hens at an interval of 10 wk starting from 30th wk until 70th wk.

The results showed that pentosidine was present in the skin and tendon of the broiler breeder hens, and that there was an age-related, linear increase in its concentration ($p<0.001$, $r^2=0.94$) for skin and ($p<0.0001$; $r^2=0.79$) for tendon in AL-fed hens. DR and supplementation of AG with AL-fed hens (AL+AG) retarded P_s ($p<0.001$; $p<0.001$) and P_t ($p<0.001$; $p<0.01$) respectively as compared to AL-fed hens. Concurrently, there was also an age-related parallel increase in TBS ($p<0.0001$; $r^2 = 0.56$), and in TBT ($p<0.0001$; $r^2 = 0.67$) in the same hens. Similarly, DR significantly retarded the overall TBS ($p<0.01$) and TBT ($p<0.02$) values as compared to AL-fed hens. Oxidative stress was lower ($p<0.0001$) and plasma uric acid higher ($p<0.0003$) in both the DR and DR+AG groups than in the AL and AL+AG groups. There was a linear age-related increase in SF ($p<0.0001$; $r^2=0.95$), cook time (CT) ($p<0.0001$; $r^2=0.95$) and breast weight (Br.W) ($p<0.0001$; $r^2=0.64$) in AL-fed hens. DR significantly retarded SF ($p<0.0001$) throughout the study. Age-related linear increase in SF correlated ($r^2 = 0.86$) with age-related increase in P_s in AL-fed hens. In the AL+AG group, SF values were similar to those of the DR group. Although, supplementation of DR with AG (DR+AG) retarded the rate of

accumulation of P_s ($p < 0.001$) over the laying cycle, the reduction in P_s did not significantly affect mean production. It is therefore concluded that P_s , P_t , TBS, TBT and the age-related increase in meat toughness can be retarded by either DR or AG. P_s can also be used as a predictor of age-related changes in meat. The same approach has the potential to be initiated in other animal production systems. Because of the linear, age-related increase in the concentrations of tissue pentosidine, it can be used as biomarker for the study of aging.

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