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

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The objectives of this study were to determine the effect of diet restriction (DR) and the crosslinking inhibitor, aminoguanidine (AG), on PMA-induced respiratory burst, 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). No consistent effects of treatments were observed on plasma concentrations of glucose. The accumulation of Ps in AL birds increased linearly with age ($p < .001$) and was significantly retarded in all treatment groups ($p < .001$). Ps in the AL + AG group was comparable to that in the DR or DR + AG groups. PMA-induced respiratory bursts in blood leukocytes were significantly retarded in DR or AG-supplemented ($p < .0001$) groups. Although there was a marginal increase in overall mean concentrations of plasma uric acid for the DR group, no consistent differences were observed on individual time points. It is concluded that the glycosylation process may not be the primary cause of glucose-derived crosslinks and that the accumulation of Ps can be retarded by DR and AG in broiler breeder hens.

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 dramaticaly 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:

(i) 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 prodiction and, consequently, accelerated tissue damage (4–6).
(ii) 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).
(iii) 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 imidizole-[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 life span 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 (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 DR, 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). Although 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 twofold: (i) to determine the effect of DR as well as the crosslinking inhibitor (AG) on the rate of accumulation of the glycoxidation product, pentosidine, in the skin (Ps) of naturally hyperglycemic broiler breeder hens, and (ii) 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 (Cobb × Cobb) chicks (n = 450) were placed in electrically heated battery brooders and fed ad libitum until 4 weeks 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). All chicks were assigned randomly at 4 weeks of age to one of four experimental groups: AL, DR, AL and DR supplemented in feed with 400 ppm AG (Aldrich Chemical, Milwaukee, WI) (1.35 mg/kg/day) (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 weeks of age at which time, the hens were put into individual cages. Body weight was monitored weekly and recorded every 12 weeks.

Skin Samples and Pentosidine Determination

Hens (n = 5) were randomly selected from each dietary group every 12 weeks beginning at 8 weeks of age and ending at 92 weeks 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 (26) and Sell (27) and their colleagues. 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 hours. All tubes were flushed with nitrogen prior to capping for heating. Subsequent to the hydrolysis, the samples were placed into a SpeedVac centrifuge-type vacuum drier (Savant Instruments, Farmingdale, NY) until dry. Samples were then reconstituted in 250 μL H_2O and filtered using a Costar Spin-X centrifuge tube filter (Corning Costar Corp., Cambridge, MA). 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 milligram of acid-hydrolyzed skin collagen digest in 100 μL water/0.01M heptafluorobutyric acid (HFBA) was injected into a 0.46 × 25-cm Vydac 218TP104 (10 μm) C-18 column (Vydac, Hesperia CA 92345) connected to a Shimadzu HPLC (Shimadzu, Inc., Columbia, MD). 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 minutes in water and HFBA. Quantification of pentosidine was made by comparison of peak areas with a pentosidine standard (Vincent Monnier, Cleveland, OH) 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). Uric acid was determined using a kit from Sigma (procedure #685, Sigma Diagnostics, St. Louis, MO).

Leukocyte Isolation and Chemiluminescent Assay for Oxidative Stress

One milliliter of blood from 125-week-old hens (n = 5 per group except for the AL group where only two birds remained) was suspended in mono-poly resolving medium (ICN 16-980-49, Costa Mesa, CA) and leukocytes were isolated by centrifugation. The total number of leukocytes was counted using a routine hemocytometric technique. To a 3-mL luminometer tube were then added 100 μL of leukocytes, 100 μL luminol solution, 200 μL phosphate-buffered saline (PBS), and 100 μL phorbol myristate acetate (PMA). The luminometer tube was placed into a luminometer (Berthold model LB 9505C, Wilbad, Germany) with the temperature control set at 37°C. Oxidative activity was determined by measuring the luminescence generated over 30 minutes; results were reported as counts per minute (CPM). The data was analyzed by a PC running KINB software supplied with the luminometer. 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 × 2 factorial design at each age for all the parameters. Regression analysis was performed to determine the correlation between the concentration of Ps and age (30).

Results

Plasma Glucose and Body Weight

There was a significant main effect due to diet (AL vs DR) for body weight ($p < .0001$) as well as plasma glucose concentrations ($p < .004$). Hens in the DR group weighed less ($p < .0001$) at all ages monitored than those in the AL groups (Figure 1). Mean plasma glucose concentrations were higher in DR group ($p < .02$) at 144, 56, 68, and 80 weeks as compared with AL birds (Figure 2). The Diet × AG interactions were not significant for plasma glucose ($p = .36$) or body weight ($p = .23$), indicating that supplementation of AG did not significantly

affect body weight or plasma glucose concentrations over the study period. The Diet × Age interactions were significant for plasma glucose ($p < .0001$) but not for body weight ($p = .22$).

Skin Pentosidine (Ps)

There was a significant main effect due to diet ($p < .0001$) as well as supplementation with AG ($p < .0001$) on Ps. Similarly, there were significant Diet × AG ($p < .0001$) and Diet × Age ($p < .0001$) interactions. Ps accumulated in a linear fashion with age in AL-fed birds ($p < .0001$); DR significantly reduced its accumulation over the 92 weeks (Figure 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-week-old birds and 0.171 ± 0.018 pmol/mg collagen at 92 weeks 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 weeks of age, respectively. Supplementation of AG with AL lowered the rate of Ps accumulation; concentrations of Ps in the AL + AG group were comparable to those of the DR group (Figure 3). Supplementation of DR birds with AG was effective in lowering mean concentrations of Ps ($p < .001$) at 32, 44, 68, and 92 weeks of age. The AL + AG group had concentrations of Ps ranging from 0.001 ± 0.0009 pmol/mg collagen at 8 weeks to 0.049 ± 0.005 pmol/mg collagen at 92 weeks, whereas the concentrations in the DR + AG group ranged from 0.012 ± 0.002 pmol/mg collagen at 8 weeks to 0.036 ± 0.003 at pmol/mg collagen at 92 weeks.

Uric Acid and PMA-Induced Respiratory Burst

There was a significant main effect due to diet ($p < .0001$) as well as supplementation with AG ($p < .003$) on concentrations of uric acid. The Diet × Age ($p < .0001$) and AG × Age ($p < .003$) interactions were also significant. Although concentrations of plasma uric acid were higher in DR compared to AL group at 32, 44, 56, and 68 weeks of age (Figure 4), there were no consistent effects of AG on mean concentrations of uric acid at individual time points. However, a 10% and a 14% increase in overall mean plasma uric concentration in AL + AG and DR + AG as compared to AL and DR groups were observed, respectively. Similarly, there was a significant main effect due to diet ($p < .0001$) and supplementation with AG ($p < .0001$) in PMA-induced respiratory bursts. The Diet × AG interaction was also significant ($p < .0001$). PMA-induced respiratory bursts in leukocytes were significantly retarded in DR and AG-

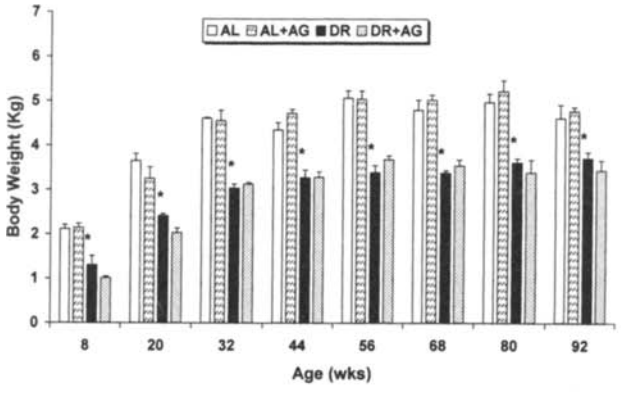

Figure 1. Effect of age, diet restriction (DR), and aminoguanidine (AG) on body weight (BW). Each point is the mean ($n = 5$) ± *SEM*. *$p < .0001$ between dietary groups (AL vs DR).

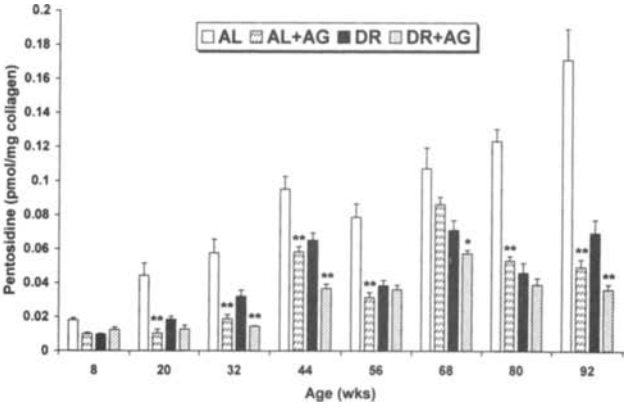

Figure 3. Effect of age, diet restriction (DR), and aminoguanidine (AG) on skin pentosidine (Ps). Each point is the mean ($n = 5$) ± *SEM*. *$p < .05$ and **$p < .001$.

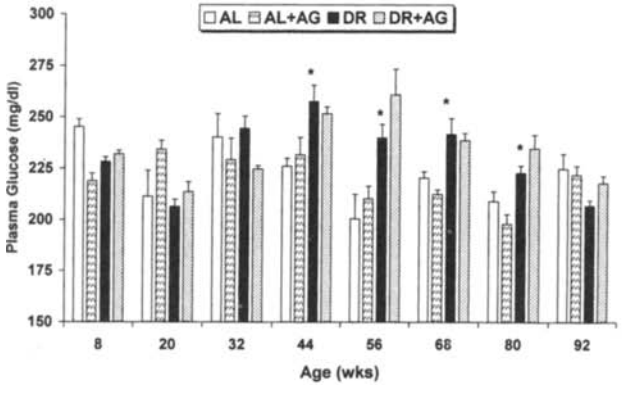

Figure 2. Effect of age, diet restriction (DR), and aminoguanidine (AG) on concentrations of plasma glucose. Each point is the mean ($n = 5$) ± *SEM*. *$p < .02$ (AL vs DR).

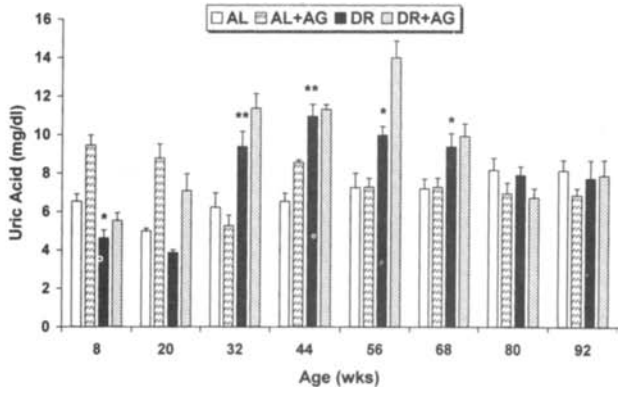

Figure 4. Effect of age, diet restriction (DR), and aminoguanidine (AG) on concentrations of plasma uric acid. Each point is the mean ($n = 5$) ± *SEM*. *$p < .05$ and **$p < .0001$ (AL vs DR).

supplemented groups ($p < .0001$) (Figure 5). DR lowered PMA-induced respiratory burst and AG further enhanced that effect (Figure 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 weeks 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-week sampling period (Figure 6), comparable to the results reported in mammals (shrew, monkey, dog, pig, cow, rat, and 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 ($p < .001$), coupled with increased average concentrations of plasma glucose ($p \leq .05$), 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 hemoglobin (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 as 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. Although 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 (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).

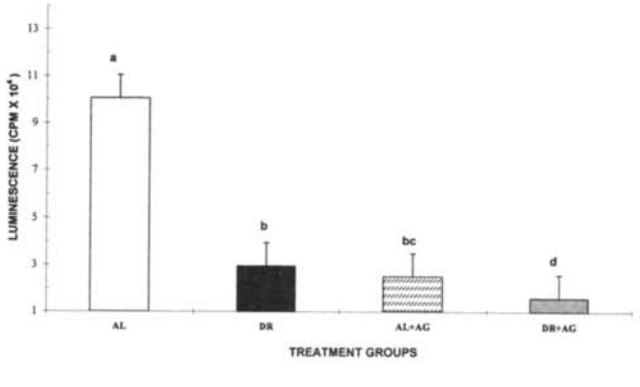

Figure 5. Effect of age, diet restriction, and AG on PMA-induced respiratory burst. Each point is the mean ($n = 5$) ± *SEM*, except AL ($n = 2$). Groups with different letters differ significantly ($p < .0001$).

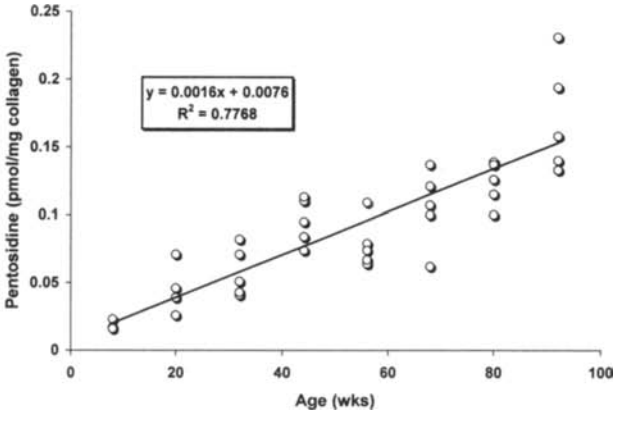

Figure 6. Correlation between age and pentosidine accumulation in the skin of ad-libitum–fed broiler breeder hens.

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 (three-fold as compared to humans) correlate with a three-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, cerebrospinal fluid (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 PMA-induced respiratory burst 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 mean 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%, whereas these 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 birds, should increase longevity as suggested by the free radical theory of aging.

It is concluded that the glycosylation process may not be the primary cause of glucose-derived crosslinks and that the rate of accumulation of Ps can be retarded by DR and AG. Birds appear to be 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 patient 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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