Relationship Between Mechanical Properties and Pentosidine in Tendon: Effects of Age, Diet Restriction, and Aminoguanidine in Broiler Breeder Hens[1]

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ABSTRACT Nonenzymatic glycosylation contributes to the formation of crosslinks, which leads to the structural and functional deterioration of tissue protein. The accumulation of these crosslinks in tissue proteins has been implicated in the alteration of biomechanical properties of connective tissues. The objective of this study was to determine whether tendon breaking time (TBT) and tendon breaking strength (TBS) of the flexor perforans et perforatus digiti iii tendon were related to 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 a 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-fed (AL); diet-restricted (DR; 60% of AL); and AL and DR groups supplemented with 1.35 mg/kg BW per day AG in the feed (AL+AG and DR+AG, respectively). In AL hens, P_t increased with increasing age ($P \leq 0.0001$). Concurrently, an age-related parallel increase was found for TBS ($P \leq 0.0001$) and TBT ($P \leq 0.0001$). Rate of P_t accumulation was lower in DR ($P \leq 0.001$), TBS ($P \leq 0.01$), and TBT ($P \leq 0.02$) hens compared with AL hens. Concentration of P_t in the AL + AG group was lower ($P \leq 0.0002$) than in the AL group; TBS and TBT ($P \leq 0.01$) followed a similar pattern. Supplementation of DR with AG did not affect P_t, TBS, or TBT. The age-related increase in P_t and loss of elasticity in the tendon was retarded by diet restriction and AG.

(*Key words*: pentosidine, tendon breaking time, tendon breaking strength, diet restriction, broiler breeder)

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INTRODUCTION

Diabetic patients incur tissue damage due to chronic hyperglycemia. The cause of these complications appears to be multifactorial (Oxlund and Andreassen, 1992). One of the factors may be nonenzymatic glycation with subsequent formation of browning products, resulting in irreversibly crosslinked protein (Kohn, et al., 1984; Kent et al., 1985; Monnier, 1990). 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 (1982) 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 in the rat are tendon breaking time (TBT) (Heller and McClearn, 1992) and the protein crosslink, pentosidine (Sell and Monnier, 1997). Increased biomechanical strength and crosslinking of collagens were observed in vitro after incubation of rat tail tendons with glucose (Andreassen and Oxlund, 1985; Andreassen et al., 1988; Menzel and Reihsner, 1991; Kent et al., 1995), and in vivo in animals with experimental diabetes (Galeski et al., 1977; Andreassen et al., 1981). Verzar (1963) demonstrated that resistance to thermal denaturation of rat tail tendon, measured as TBT, increased with advancing age. Further work by Everitt et al. (1981) showed that dietary restriction initiated at an early age in rats retarded aging 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 and Archer (1978, 1983) established a strong correlation between age and TBT in inbred and hybrid strains of mice.

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Abbreviation Key: AG = aminoguanidine; AL = ad libitum-fed group; DR = diet-restricted group; P_t = tendon pentosidine; TBS = tendon breaking strength; TBT = tendon breaking time; NO = nitric oxide.

Because solubility of tail tendon collagen decreases with age, it was suggested by Verzar (1963) that increased molecular crosslinking of collagen explained the age-related increase in TBT and the noted effects on collagen solubility. Since that time, the exact chemical structures of these putative markers of senescence have been sought (Calkins, 1981). One such marker is a glycoxidation product, pentosidine, originally isolated from collagen of aged humans (Sell and Monnier, 1989). Pentosidine has also been found in other mammals and in broiler breeder hens (Iqbal et al., 1997). Pentosidine is an imidazopyridinium crosslink involving a pentose sugar crosslinked with arginine and lysine residues (Sell and Monnier, 1989).

Birds are an interesting model for biogerontology (Holmes and Austad, 1995); concentrations of plasma glucose are typically 2 to 6 times mammalian norms (Holmes and Austad, 1995; Beuchat and Chong, 1997; Iqbal et al., 1999a), metabolic rates are as much as 2 to 2.5 times higher than similarly-sized mammals, and basal body temperature is about 3 C higher than in mammals (Holmes and Austad, 1995). All these factors should accelerate the formation of advanced Maillard products and, hence, the process of tissue aging compared with normal aging (Sohal and Allen, 1990; Holmes and Austad, 1995). Concentrations of pentosidine in bird skin, however, are approximately 1,000-fold lower than what is found in mammalian tissues (Iqbal et al., 1999a).

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 wk of age. A second objective was to determine the effects of diet restriction and a crosslinking inhibitor, aminoguanidine (AG), on P_t, TBT, and tendon breaking strength (TBS).

MATERIALS AND METHODS

Birds and Management

Day-old broiler breeder (Cobb × Cobb) female chicks (n = 450) were placed in electrically heated battery brooders and fed ad libitum until 4 wk of age. At this time, chicks were randomly assigned to four treatment groups: ad libitum-fed (AL); diet-restricted (DR) at 60% of AL energy intake; AL with aminoguanidine (AG)[4] at 1.35 mg/kg BW per day (AL + AG); and DR with AG at 60% of that fed the AL group (DR + AG). The AG was supplemented in the feed. Birds were fed these diets throughout the study. The DR chicks received the recommended amount of vitamins, minerals, and nutrients according to the Cobb Management Guide.[5] 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 the Biceps femoris muscle of broiler breeder hens (Klandorf et al., 1996). All birds were fed daily between 0800 and 1000 and had access ad libitum to water. The flock was reared in floor pens until 20 wk 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 wk, and then again at 125 wk of age. One of the longest tendons in birds, it is located on the caudal border of the tibia, and it extends from the proximal end of the tibia to the third digit. Hens (n = 5, except for the AL group at 125 wk, in which only two 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, vacuum-packed and stored at −80 C until physical and chemical analyses.

Measurement of Tendon Breaking Time and Strength. Prior to analysis, each tendon was immersed in Ringer's solution, pH 7.4. Determinations of TBT and TBS were made on an individual tendon mounted in an Instron Universal Mechanical Machine.™,[6] At 8 wk, linear growth of the bone and tendon (Table 1) is complete. A 10-mm section in the middle of the tendon was measured on each tendon, which ensured that a consistent anatomical region was measured. Tendons were attached between the Instron's movable cross-head and stationary base such that the identical 10-mm center section of tendon was tested for each analysis. The cross-head, attached to a 50-kg load cell,[7] moved away from the rigid base, in tension, at a speed of 50.8 mm/min. Output from an LVDT conditioner[8] for the tensile deformation of the tendon was acquired by a computer equipped with a DT 2805 data acquisition board.[9] Signals were processed with the HP-VEE software package.[10] Cross-sectional area at the mid-point of the tendon was used to normalize TBS as kg/mm^2. Cross-sectional area was calculated on unloaded tendons by measuring the thickness and diameter of each tendon with vernier calipers. Tendon breaking time (sec) was recorded as the time from initiation of the test until the tendon broke.

Tendon Pentosidine Determination

Preparation of Collagen Digest. Broken Flexor perforans et perforatus digiti iii tendons were used for collagen digest and subsequent pentosidine determination. The collagen digest was prepared according to the techniques described by Sell et al. (1992). Briefly, up to 20 mg of tendon was frozen in liquid nitrogen, minced, and placed into a 13 × 100-mm screw-capped tube, then

[4]Aldrich Chemical Co., Inc., Milwaukee, WI 53233.
[5]Cobb-Vantress, Inc., Siloam Springs, AR 72761.
[6]Model TM, Instron Corp., Canton, MA 45419.
[7]Model 152050, Daytronic, Miamisburg, OH 45342.
[8]Model 9130, Daytronic, Miamisburg, OH 45342.
[9]Data Translation, Marlboro, MA 01752.
[10]Hewlett Packard Co., Loveland, CO 80539-9929.

TABLE 1. Effect of age, dietary modulation, and aminoguanidine on the length and cross-sectional area of tendons (X ± SEM)[1]

Age (wk)	Length[2] (cm)				Cross-sectional area (mm^2)			
	AL	DR	AL + AG	DR + AG	AL	DR	AL + AG	DR + AG
8	11.33 ± 0.4	10.90 ± 0.3	11.70 ± 0.5	11.21 ± 0.4	2.28 ± 0.41	2.23 ± 0.41	2.31 ± 0.22	1.60 ± 0.36
20	10.87 ± 0.4	12.27 ± 0.4	11.35 ± 0.2	11.67 ± 0.5	2.62 ± 0.28	2.57 ± 0.28	2.42 ± 0.24	2.93 ± 0.28
32	9.85 ± 0.48	9.12 ± 0.54	8.00 ± 0.35	9.59 ± 0.59	2.49 ± 0.26	2.95 ± 0.35	3.33 ± 0.31	2.32 ± 0.19
44	9.77 ± 0.65	9.87 ± 0.51	9.01 ± 0.33	9.74 ± 0.53	2.81 ± 0.40	3.04 ± 0.40	3.66 ± 0.37	3.06 ± 0.39
56	9.56 ± 0.71	9.72 ± 0.23	9.19 ± 0.45	9.83 ± 0.35	3.38 ± 0.44	3.14 ± 0.43	3.46 ± 0.22	3.34 ± 0.46
68	9.30 ± 0.28	9.56 ± 0.40	9.98 ± 0.40	9.56 ± 0.33	3.06 ± 0.43	3.14 ± 0.33	3.46 ± 0.37	3.38 ± 0.30
80	9.32 ± 0.41	9.22 ± 0.28	9.36 ± 0.43	9.12 ± 0.36	3.57 ± 0.31	3.36 ± 0.33	3.72 ± 0.31	3.37 ± 0.27
92	9.23 ± 0.23	9.17 ± 0.40	9.67 ± 0.27	9.66 ± 0.39	3.14 ± 0.23	2.93 ± 0.25	3.26 ± 0.31	2.58 ± 0.30
125	9.25 ± 0.39	9.18 ± 0.43	9.44 ± 0.59	9.01 ± 0.56	3.33 ± 0.35	3.38 ± 0.31	3.45 ± 0.32	3.02 ± 0.30

[1]n = 5 at each point except at 125 wk (n = 2) for AL group.
[2]AL = ad libitum-fed group; DR = diet restricted group; AG = aminoguanidine supplemented either with AL or DR.

delipidated overnight in a chloroform-methanol (2:1) solution. Samples were rehydrated in 50% methanol and hydrolyzed in 5 mL of 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[11] until the HCl was evaporated. Samples were reconstituted in 250 μL H_2O containing 0.01 M heptafluorobutyric acid and filtered using a Costar® Spin-X® centrifuge tube filter.[12] 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 (Maekawa et al., 1970).

Tendon Collagen Pentosidine. The P_t was measured by a modified reversed-phase HPLC method (Iqbal et al., 1997). Samples of 50 to 200 μL volumes, equivalent to 1 mg of collagen, were injected into a 0.46 × 25-cm Vydac 218TP104 (10 μm) C-18 column[13] connected to a Waters HPLC.[14] The apparatus consisted of two pumps (Waters™ 600 Controller), an auto sampler (Waters™ 717Plus), and a scanning fluorescence detector (Waters™ 474Plus). Separations were achieved by a linear gradient of 12 to 42% acetonitrile from 0 to 25 min in water and 0.01M heptafluorobutyric acid at a flow rate of 1 ml/min. The pentosidine peak was monitored by an on-line scanning fluorescence detector at an excitation wavelength of 325 nm and an emission wavelength of 370 nm. Quantification of pentosidine was made by comparison of sample values with a standard curve generated from peak areas of various concentrations of a pentosidine standard[15] injected under identical conditions. A software package[16] was used for integration of peaks.

Statistical Analyses

Data were analyzed by the general linear models procedure using a 2 (feeding regimen; AL and DR) × 2 (AG; with or without) × 9 (time) factorial design. Correlations between P_t, age, TBS, and TBT were determined (SAS Institute, Inc., 1990).

RESULTS

There was a significant main effect due to diet (AL vs. DR) for P_t, TBS, and TBT ($P \leq 0.0001$; Figure 1). Interactions between diet and AG were significant for P_t ($P \leq 0.0001$), TBS ($P \leq 0.0001$), and TBT ($P \leq 0.04$). The DR diet lowered ($P \leq 0.0001$) the accumulation of P_t (Figure 1A) over the study period. Similarly, the DR diet retarded overall TBS ($P \leq 0.01$) and TBT ($P \leq 0.02$) compared with tendons from AL hens (Figire 1B,C). Supplementation of AL hens with AG retarded ($P \geq 0.0002$) the accumulation of P_t and decreased TBS and TBT ($P \leq 0.01$; Figure 2). Supplementation of DR-hens with AG (DR+AG group) did not affect ($P \geq 0.05$) P_t, TBS, or TBT (Figure 3). Interactions between diet and age were significant for P_t ($P \leq 0.0003$), TBS ($P \leq 0.03$), and TBT ($P \leq 0.04$). The effect of the DR diet was not consistent for each response; a greater decrease (112%) in the concentration of P_t was associated with a lower decrease in both TBS (55%) and TBT (33%). Similarly, a 130% decrease in P_t was observed in AL hens supplemented with AG, and this decrease in P_t was associated with 48 and 35% decreases in TBS and TBT, respectively. Regression analysis showed that there was a greater increase with age in P_t, TBS, and TBT ($P \leq 0.0001$) for AL than DR hens.

DISCUSSION

In the present study, age-related changes in P_t and mechanical properties of the tendon of broiler breeder hens were compared in AL and DR hens with or without AG supplementation. A higher cross-head speed (50.8 mm/min) was used to break the rigid tendon than that used in rats and mice (Andreassen and Oxlund, 1985; Everitt et al., 1981, 1983). The higher cross-head speed was required in order to ensure consistent breaking of the tendon. For the current work, and based on reported tendon breaking times (Table 1), tendons broke when stretched from 21.2 to 67.7% of their resting length. There are limited data available on the mechanical properties

[11]Savant Instruments, Farmingdale, NY 11735.
[12]Corning Costar Corp., Cambridge, MA 02140.
[13]Vydac, Hesperia, CA 92345.
[14]Waters, Milford, MA 01757.
[15]Vincent M. Monnier, Cleveland, OH 44120.
[16]Millennium 2.1, Milford, MA 01757.

of digital flexoral tendons. Recently, Rath et al. (1998) evaluated the effects of roxarsone and monensin on digital flexoral tendons in 6-wk-old broilers. In their study, they stretched the tendons at a rate of 25 mm/min until breakage occurred. Depending on the treatment, strain varied from 9.45 to 15.31%. Accounting for differences in test parameters and bird strain, our value for 8-wk-old broiler-breeders (21.2%) is reasonable.

A parallel, age-related increase in the concentration of P_t was associated with age-related increases in TBS and TBT in AL hens. These observations are similar to those of Fu et al. (1994), who found a parallel increase in pentosidine formation and collagen crosslinking when rat tail tendons were incubated with glucose. Likewise, Richard et al. (1991) showed a parallel increase in pentosidine and TBT when tail tendons were incubated with ribose. The relationship between TBT and collagen crosslinks was further confirmed by the fact that the overall pattern of changes in TBT shared similarities with that of another glycoxidation product, carboxymethyllysine (Elgawish et al., 1996). Numerous studies have shown TBT to be highly correlated with age (Verzar, 1963; Harrison et al., 1978; Fu et al., 1994), whereas little information is available about TBS in the literature.

The DR diet markedly affected the age-related changes in P_t, TBT, and TBS. In mammals, diet restriction has been found to increase mean and maximum life span while delaying many age-associated disease processes

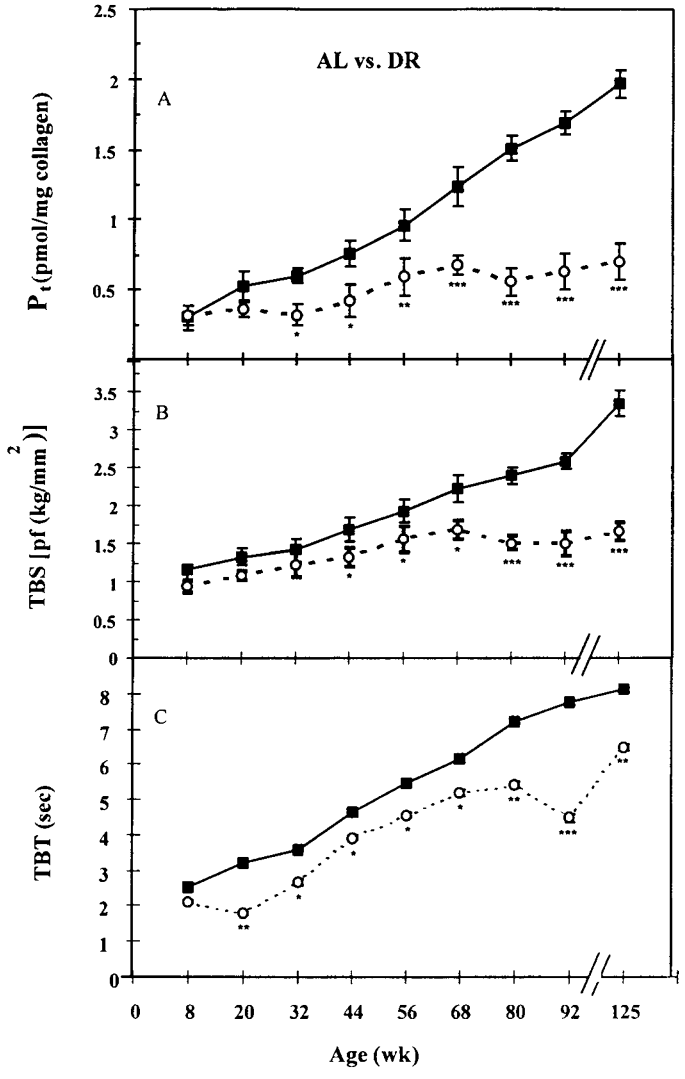

FIGURE 1. Effect of ad libitum-fed (AL) and feed-restricted (DR) diets on A) concentrations of pentosidine (P_t), B) tendon breaking strength (TBS), and C) tendon breaking time (TBT) in flexor perforans et perforatus digiti iii tendon of broiler breeder hens. Each point represents the mean [n = 5, except at 125 wk (n = 2) for AL group] ± SEM. Differences were significant (*$P \leq 0.05$, **$P \leq 0.001$, and ***$P \leq 0.0001$) between AL (closed squares) and DR (open circles) groups at those points.

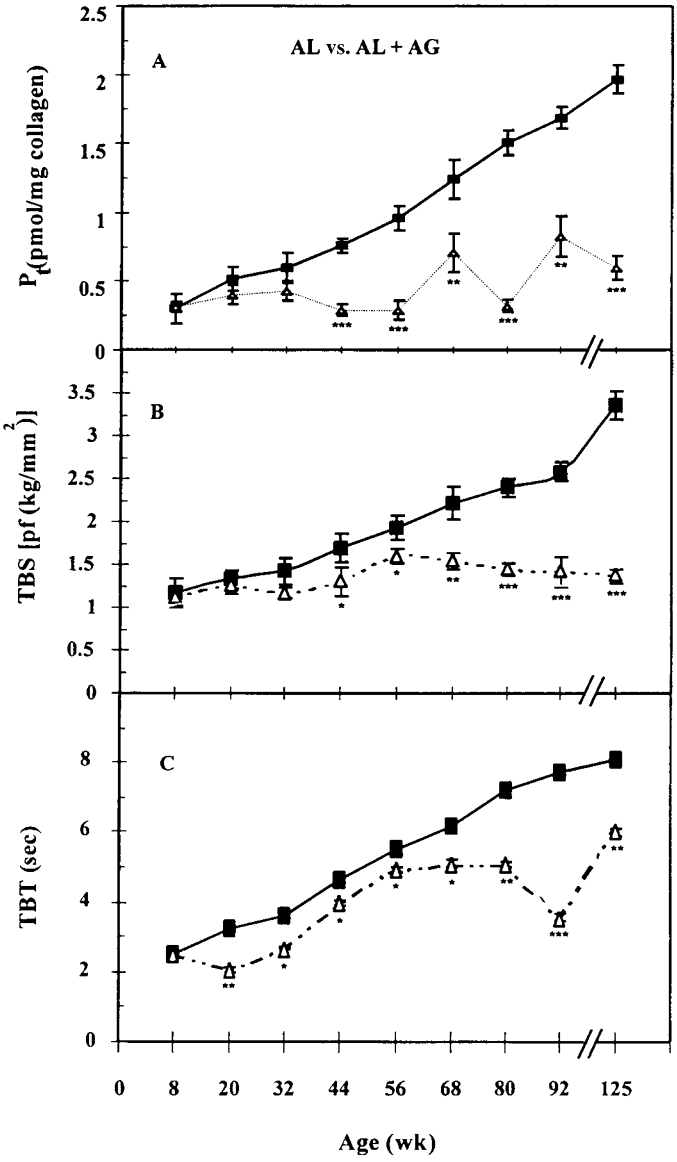

FIGURE 2. Effect of ad libitum-fed (AL) diet and aminoguanidine (AG) supplementation with AL diet (AL + AG) on: A) concentrations of pentosidine (P_t), B) tendon breaking strength (TBS), and C) tendon breaking time (TBT) in flexor perforans et perforatus digiti iii tendon of broiler breeder hens. Each point represents the mean [n = 5, except at 125 wk (n = 2) for AL group] ± SEM. Differences were significant (*$P \leq 0.05$, **$P \leq 0.001$, and ***$P \leq 0.0001$) between AL (closed squares) and AL + AG (open triangles) groups at those points.

(Weindruch and Walford, 1982; Everitt et al., 1983). Previous studies have shown that the physiological rate of collagen aging as determined by TBT is delayed by diet restriction (Everitt et al., 1981; Oxlund and Andreassen, 1992; Sell et al., 1996). Similar results were found in the present study. A significant delay of the age-related increase in TBS and TBT was observed in DR-hens compared with AL hens (Figure 1). Because diet restrction delays the aging process, this intervention would be expected to also retard the age-related increase in pentosidine. The rate of P_t formation was significantly delayed by diet restriction, and these findings are comparable to those of earlier studies in rat tail tendons (Sell and Monnier, 1997; Iqbal et al., 1999a).

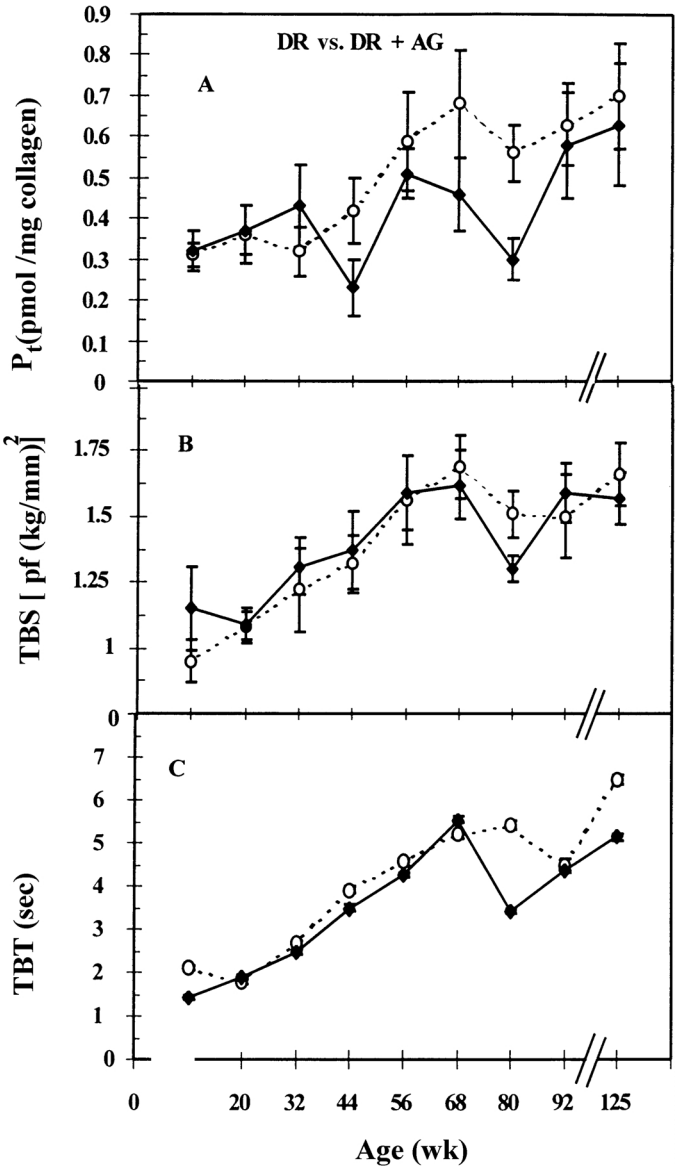

FIGURE 3. Effect of restricted diet (DR) and aminoguanidine (AG) supplementation with DR (DR + AG) on: A) concentrations of pentosidine (P_t), B) tendon breaking strength (TBS), and C) tendon breaking time (TBT) in flexor perforans et perforatus digiti iii tendon of broiler breeder hens. Each point represents the mean (n = 5) ± SEM. Supplementation of DR with AG did not affect these end-points significantly ($P \leq 0.05$) between DR (opened diamonds) and DR + AG (closed circles) groups.

The effectiveness of AG as an inhibitor of nonenzymatic glycation is controversial, and there is also disagreement over its mode of action. In the present study, accumulation of P_t in the AL + AG group was retarded (Figure 2A); whereas AG had no effect in the DR + AG group (Figure 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 the AL + AG hens was comparable to those of DR birds (Figures 1, 2). The reduction in concentration of P_t (130%) for the AL + AG birds was associated with a 48% decrease in TBS and a 35% decrease in TBT. In contrast with these observations, Oxlund and Andreassen (1992) did not find any difference between TBT of tail tendons from control rats and tail tendons from diabetic rats treated with AG for 120 d. Results suggest that the duration of treatment is a likely explanation for these differences, because differences in TBT were initially recorded in the present study beginning at 20 wk (140 d) of age. In agreement with Oxlund and Andreassen (1992), AG supplementation of the DR diet did not affect P_t, TBS, or TBT. These findings are consistent with those of Klandorf et al. (1996), who reported no effect of AG supplementation in DR birds on the advanced glycosylation end products (AGEs)-associated collagen fluorescence of Biceps femoris muscles in poultry. Similarly, in a companion study (Iqbal et al., 1999b), supplementation of the DR diet with AG did not affect the shear value of the pectoralis major muscles over a 125-wk period. These results are consistent with the view that AG is relatively ineffective in diet-restricted animals. This observation may be due to differences in total accumulation of pentosidine in a given amount of collagen and the extent of glycosylation in the body. Dyer et al. (1991) 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 (Eyre, 1980). Although diet restriction significantly reduced crosslinking, it did not reduce glycosylation (Iqbal et al., 1999a). As a consequence, diet restriction limited the effectiveness of AG supplementation in the reduction at the glycosylation process and the subsequent reduction in collagen crosslinks. Although the mode of action of AG in lowering P_t, TBS, and TBT in AL hens is not clearly understood, AG lowers phorbol myristate acetate (PMA)-induced respiratory bursts (oxidative stress) in leukocytes in broiler breeder hens (Iqbal et al., 1999a). Wu (1995) demonstrated that AG delayed the onset of diabetic complications in rats by inhibiting the inducible nitric oxide (NO) synthase that is responsible for NO synthesis from L-arginine. As a free radical, NO has been linked to the destruction of pancreatic β-cells in insulin-dependent diabetes mellitus. These studies suggest that the mode of action of AG may, in part, be to reduce either the

oxidative stress or NO synthase. More research is needed to establish this.

In summary, there was a greater age-related increase in P_t, TBS, and TBT in AL than DR hens. Diet restriction significantly retarded the rate of accumulation of P_t, TBS, and TBT, although no effect was observed when the DR diet was supplemented with AG. In conclusion, AG was effective only in AL and not in DR hens. The effects of diet restriction and AG were greater in lowering the concentration of P_t than those of TBS and TBT.

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