



■ BONE BIOLOGY

The effect of aminoguanidine (AG) and pyridoxamine (PM) on ageing human cortical bone

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Objectives

Advanced glycation end-products (AGEs) are a post-translational modification of collagen that form spontaneously in the skeletal matrix due to the presence of reducing sugars, such as glucose. The accumulation of AGEs leads to collagen cross-linking, which adversely affects bone quality and has been shown to play a major role in fracture risk. Thus, intervening in the formation and accumulation of AGEs may be a viable means of protecting bone quality.

Methods

An *in vitro* model was used to examine the efficacy of two AGE-inhibitors, aminoguanidine (AG) and pyridoxamine (PM), on ageing human cortical bone. Mid-diaphyseal tibial cortical bone segments were obtained from female cadavers (n=20, age range: 57 years to 97 years) and randomly subjected to one of four treatments: control; glucose only; glucose and AG; or glucose and PM. Following treatment, each specimen underwent mechanical testing under physiological conditions via reference point indentation, and AGEs were quantified by fluorescence.

Results

Treatment with AG and PM showed a significant decrease in AGE content *versus* control groups, as well as a significant decrease in the change in indentation distance, a reliable parameter for analyzing bone strength, via two-way analysis of variance (ANOVA) ($p < 0.05$).

Conclusions

The data suggest that AG and PM prevent AGE formation and subsequent biomechanical degradation *in vitro*. Modulation of AGEs may help to identify novel therapeutic targets to mitigate bone quality deterioration, especially deterioration due to ageing and in AGE-susceptible populations (e.g. diabetics).

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Keywords: Bone quality, Ageing, Osteoporosis, Non-enzymatic glycation, Cortical bone

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Article focus

- The fracture resistance of bone is affected by the accumulation of advanced glycation end-products (AGEs).
- The modulation of AGEs may be a viable way to improve bone quality and fracture resistance.
- We investigated the efficacy of aminoguanidine and pyridoxamine in preventing the accumulation of AGEs and protecting bone strength.

Key messages

- The accumulation of advanced glycation end-products (AGEs) adversely affects bone biomechanical behaviour.

- The *in vitro* application of aminoguanidine and pyridoxamine prevents the accumulation of AGEs and preserves the fracture resistance of cadaveric bone.
- These changes in bone biomechanical behaviour can be effectively monitored by cyclic reference point indentation.

Strengths and limitations

- Using cadaver bone, we demonstrate that aminoguanidine and pyridoxamine can mitigate the impacts of glucose-induced accumulation of advanced glycation end-products (AGEs) to alleviate the reduction of bone fracture resistance.

- The results represent a potential therapy for increased fracture resistance due to osteoporosis.
- These *in vitro* results require further investigation with *in vivo* studies.

Introduction

Osteoporosis is a common skeletal condition that affects more than 200 million people worldwide.¹ The causes of osteoporosis are numerous and multifaceted, and include genetics, vitamin D levels, hormone levels, and lifestyle.² Skeletal fractures as a consequence of osteoporosis lead to increases in the morbidity and mortality rates among men and women.^{3,4} In particular, approximately 30% of all postmenopausal women in Europe and the United States have osteoporosis, and are therefore susceptible to incapacitating fractures.^{5,6}

Osteoporosis is marked by declining bone mineral density as well as declining bone quality.^{7,8} Advanced glycation end-products (AGEs) are among the bone quality changes that result from post-translational modifications of collagen (90% of the organic phase in bone), which form spontaneously in the skeletal matrix in the presence of reducing sugars, such as glucose. This process is called non-enzymatic glycation (NEG). Advanced glycation end-products can form cross-linkages that have detrimental effects, but the mechanism of formation and the effect of these cross-links on diabetes and bone fragility are still under extensive investigation.⁹⁻¹²

Advanced glycation end-products reduce the propensity of bone to resist fracture, and the accumulation of AGEs in bone causes stiffening of the type I collagen network.^{13,14} This increasing number of collagen cross-linkages alters the micro- and nanoscale energy dissipation mechanisms, which increases the likelihood of bone fractures.^{14,15}

Given their adverse mechanical consequences, inhibition of AGEs may be a viable strategy for the restoration of bone strength and the prevention of fractures, particularly in postmenopausal women who are susceptible to reduced oestrogen-mediated decline in bone quality and diabetic patients who are susceptible to high blood glucose levels.^{16,17} Aminoguanidine (AG) and pyridoxamine (PM) are used as AGE formation inhibitors and have been shown to decrease AGE formation in diabetic rats.¹⁸ While previous studies have confirmed that AG and PM play a significant role in the assembly process of AGEs *in vitro*, the effect of these two inhibitors on AGE accumulation and subsequent biomechanical degradation in human cortical bone remains relatively unknown.^{19,20} We therefore used an *in vitro* model to investigate the preventative effects of AG and PM on cortical bone AGE content and their subsequent effects on bone strength.

Materials and Methods

Study sample. Cortical bone segments were taken from the mid-diaphyseal tibia of 20 fresh-frozen female

cadavers (mean age 79 years, SEM 11; range 57 to 97) for this study. Female cadavers were selected to investigate AGE inhibition in the population that is susceptible to hormonal changes throughout menopause that may lead to osteoporosis.²¹ These de-identified cadavers were obtained from the Washington University Tissue Donor Program and the Anatomical Education Program of Indiana University. No live human subjects were involved in this research study (IRB Waiver, Washington University Medical Center).

We randomly assigned identical segments from each donor ($n=20$) to one of four experimental groups: negative control (C), positive control glucose (G), or two inhibitor treatment groups. A summary of the treatment groups is shown in Table I.

A base solution for glycation was prepared in Hank's buffered salt solution (Gibco 14170112; Thermo Fisher Scientific, Waltham, Massachusetts) with a final concentration of 25 mM 6-Aminocaproic acid (A2504; Sigma-Aldrich Corp., St. Louis, Missouri), 5 mM Benzamidine (12072; Sigma-Aldrich), 10 mM N-ethylmaleimide (E3876; Sigma-Aldrich), 10 mM HEPES (H3375; Sigma-Aldrich) buffer, and 100 $\mu\text{g}/\text{mL}$ Streptomycin sulfate salt (S6501; Sigma-Aldrich). One quarter of this solution was aliquot for the negative control group. Dextrose (D-(+)-Glucose) (G8270; Sigma-Aldrich) was added to the remainder of the buffer solution to create a 100 mM glucose solution.

Specimens were completely submerged in solution with five times the volume of bone at 50°C. Previous studies have shown that this temperature mimics three months of ageing per two weeks of incubation.^{22,23} The temperature was maintained at 50°C and, if necessary, the pH of the solution was adjusted and maintained between 7.2 and 7.6 using 0.5 N NaOH or 0.5 N HCl. It has been previously demonstrated that this incubation protocol does not cause loss of mineral content in bone.^{24,25}

AGE formation inhibitors. From the 100 mM glucose buffer solution, two thirds were set aside for testing the efficacy of AGE-inhibitors co-incubated with glucose. One third of the glucose solution was set aside to make a 400 μM AG hemisulfate salt (A7009; Sigma) solution (approximately 800 mg/kg of tissue), an effective AG concentration for selective prevention of AGE formation.²⁶ The remaining 100 mM glucose solution was used to make a 100 μM PM dihydrochloride (P9380; Sigma) solution (approximately 400 mg/kg of tissue), which previous studies have shown is a nontoxic and effective concentration of PM.^{18,27}

AGE analysis. Four bone segments measuring approximately 4 mm \times 4 mm \times 4 mm were cut and isolated from each donor ($n=20$) under wet conditions using a Smart Cut diamond wafering saw (UKAM Industrial, Valencia, California). Each segment was allocated to a treatment

Table 1. Outline of control and treatment groups

Negative control (C)	Positive control glucose (G)	Aminoguanidine treatment group (AG)	Pyridoxamine treatment group (PM)
Buffer solution at 50°C	100 mM glucose buffer solution at 50°C	400 μM AG and 100 mM glucose buffer solution at 50°C	100 μM PM and 100 mM glucose buffer solution at 50°C

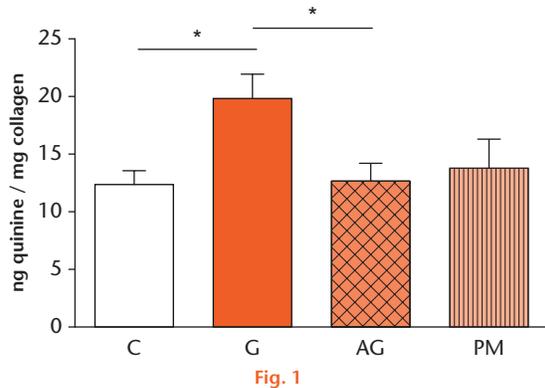


Fig. 1 Advanced glycation end-product (AGE) content (measured by ng quinine/mg collagen) across all treatment groups after seven days of incubation. White, control (C); orange, glucose only (G); cross-hatched, aminoguanidine co-incubated with glucose (AG); vertical stripes, pyridoxamine co-incubated with glucose (PM) (n=20; *p < 0.05, two-way analysis of variance (ANOVA)).

group using simple randomization, and solutions were replenished every other day for seven days. Directly following incubation for one week, each segment was decalcified using 14% ethylenediaminetetraacetic acid (EDTA) (E9884; Sigma) for four weeks. After decalcification, samples were stored in phosphate-buffered saline (PBS)-soaked gauze at -20°C until hydrolysis.

Each segment underwent acid hydrolysis in 12N HCl at 120°C for three hours. Hydrolysates were plated in triplicate, along with serially diluted quinine sulfate standards, in order to determine the content of AGEs by fluorescence in a multimode plate reader (Cytation 5; BioTek Instruments, Inc., Winooski, Vermont) using 370nm excitation and 440nm emission wavelengths. The absolute bone content was then normalized to the amount of collagen in each sample as determined by a chloramine-T colorimetric assay and standardized using serially diluted hydroxyproline via absorbance properties at a wavelength of 560nm. Protein assays used to quantify both AGEs and collagen content were conducted by the lead author (OA) blinded of treatment group.

Mechanical testing via reference point indentation. To assess the effect of AG and PM treatment on the biomechanical properties (strength) of bone, four cortical bone segments measuring approximately 4 mm × 4 mm × 4 cm were taken from the same donor cortical bone samples as the AGE analysis (n=20) and randomly assigned to an experimental group. The protocol for incubation solutions used for studying AGEs was replicated. Solutions were changed every other day and all samples were incubated at 50°C in an oven, maintained at a pH

in the range of 7.2 to 7.6. After three days, each segment was isolated from its solution and cut it in half, allowing the other half to continue incubation for a total of seven days. Three-day incubation segments with dimensions 4 mm × 4 mm × 2 cm were tested for mechanical properties using a reference point indentation (RPI) system (BioDent; Active Life Scientific, Inc., Santa Barbara, California) on the medial-anterior surface of each segment. The same mechanical testing procedure was performed on the seven-day incubation segments.

In this approach, a small reference probe rests on the surface of the bone while an indentation probe initiates a microcrack and subsequently propagates the crack through repeated indentations.^{28,29} This methodology has been shown to correlate with crack growth toughness, a known component of whole-bone fragility.^{30,31} Indentation was performed for 20 cycles with a peak force of 10N. From the force-displacement curves, the parameter indentation distance increase (IDI) was obtained, which is the difference in indentation depth between the first and 20th cycle.³¹ This parameter, which has been shown to correlate with biomechanical properties of bone such as strength and failure load, was used exclusively to assess the strength of the bone segment, where an inverse relationship exists between IDI and bone strength.³⁰ Additionally, three indentation sites were tested per segment for repeated measure, separated by a distance of 5 mm. The mean of three measurements per segment was used for statistical analysis.

Statistical analysis. The data are presented as the mean and standard error of the mean. Statistical analyses were conducted using Prism 6 (GraphPad Software Inc., La Jolla, California). Experimental groups were analyzed and compared via two-way analysis of variance (ANOVA) testing. The results were considered significant with a probability of error of 5% or lower.

Results

AGE accumulation was observed as a uniform colour change of the bone specimen from white to brownish-yellow. For the seven-day group, AGE content, defined as nanograms of quinine sulfate fluorescence per milligram of collagen, increased markedly (p < 0.05) from control to glycosylated groups (Fig. 1). Aminoguanidine treatment significantly decreased the mean AGEs concentration compared with the positive control (G) (p < 0.05).

Aminoguanidine and PM treatment both significantly inhibited AGE accumulation compared with glucose treatment alone after seven days of analysis as measured

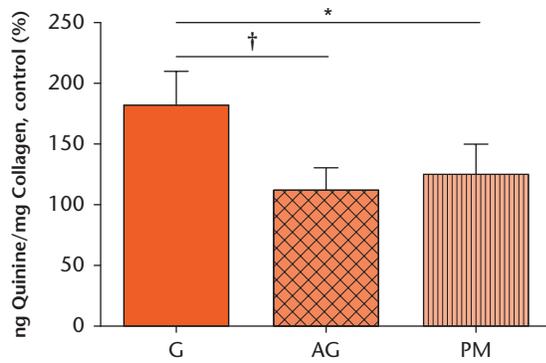


Fig. 2

Mean values of advanced glycation end-product (AGE) content were calculated and then normalized as a percentage change from the control after seven days of incubation. Treatment with aminoguanidine (AG) and pyridoxamine (PM) significantly reduced AGE content compared with glucose alone (n=20; *p<0.05, †p<0.01, two-way analysis of variance (ANOVA)). G, positive control glucose.

by the percentage change in AGE concentration from the control (Fig. 2).

Biomechanical testing via RPI strongly supports the results of the AGE analysis. After three days of glucose treatment, IDI increased significantly compared with the control group (p<0.0001) (Fig. 3). After a week of incubation, a statistically significant difference was still observed between control and glucose groups (p<0.05). These results verify the biomechanical decline in bone quality observed due to glucose treatment alone.

Two-way ANOVA with multiple comparisons revealed a significant positive effect of treatment with AG and PM on IDI after both three and seven days (Fig. 4). Aminoguanidine and PM treatment both significantly reduce IDI or improve bone strength, compared with glucose treatment alone, after three and seven days (p<0.01). There was no statistically significant difference between control, AG, and PM treatment groups after both three and seven days. These results suggest that AG and PM prevent bone mechanical degradation, likely due to NEG.

Increased AGE content corresponds with increased IDI (or reduced crack growth toughness), controlled for bone mineral density by calculating the mean of the parameter for each experimental group (Fig. 5). AG and PM mitigate increased AGE content and IDI compared with the glucose group alone.

Discussion

One of the relevant age-related changes in bone quality is the accumulation of AGEs in the organic matrix of bone.^{14,24,25,32,33} At the macro level, AGEs directly affect bone by reducing its biomechanical properties.^{14,25} Advanced glycation end-products may also affect the remodelling of bone, allowing microdamage and AGEs to accumulate at a greater rate.^{14,34-36} Here we show the ability of AG and PM to improve the quality of existing bone matrix by cleaving established AGE cross-links

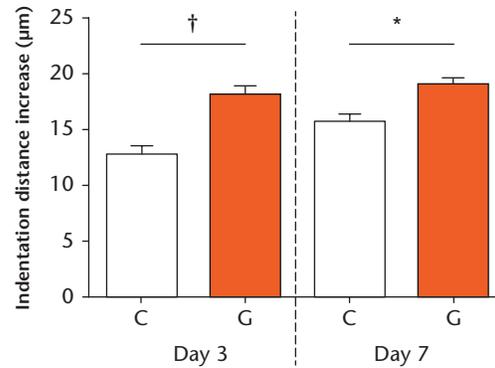


Fig. 3

Biomechanical property (indentation distance increase (IDI)) changes due to glucose treatment measured via reference point indentation. Glucose treatment (G) caused a significant increase in mean IDI after three (†p<0.0001) and seven days (*p<0.05) compared with the control (C) groups (n = 20, two-way ANOVA).

known to accumulate with ageing, disease, and the antiresorptive treatment of osteoporosis.

Properties of an effective AGE-inhibitor include the ability to trap highly reactive intermediates of AGEs formation in the glycation pathway, to arrest the formation of AGEs at the carbonyl stress stage or after formation of reactive protein adducts, and to be selective (i.e. not interfere with sugar metabolism).³⁷ Two viable treatment options are AG and PM. Both are potent AGE-inhibitors that have been shown to prevent diabetic complications including nephropathy, neuropathy, and vasculopathy.¹⁸ Aminoguanidine traps reactive dicarbonyls, impeding their conversion to AGEs, as well as preventing cross-link formation and inhibiting free radical formation.³⁷ Its limitations, however, include its short half-life (one hour) and its toxicity to humans in high concentrations. While the results show that AG is effective at preventing AGEs formation, the treatment conditions used included super-physiological concentrations of the compound. As a naturally occurring antioxidant, PM inhibits the synthesis of AGEs from glycated proteins by reducing highly reactive carbonyl intermediates in the glycation pathway.³⁷ Pyridoxamine has been shown to increase cardiovascular compliance and thus relieve hypertension.¹⁸ We observed that PM has similar effects to those of AG; it relieves AGE cross-linking and improves the mechanical properties of bone. Additionally, the concentration of PM used in this study previously has been shown to be nontoxic and effective in diabetic rats.³⁸

The mean of the control AGE content was 12.41 ng quinine/mg collagen (SEM=5.22) across all 20 donors over an age range of between 57 and 97 years (Fig. 1). Our study did not find age to be a significant predictor of AGE accumulation, which could be attributable to the fact that the donors were all above the age of 50, suggesting that significant *in vivo* AGE accumulation occurs throughout life. Previous studies have shown that age is a

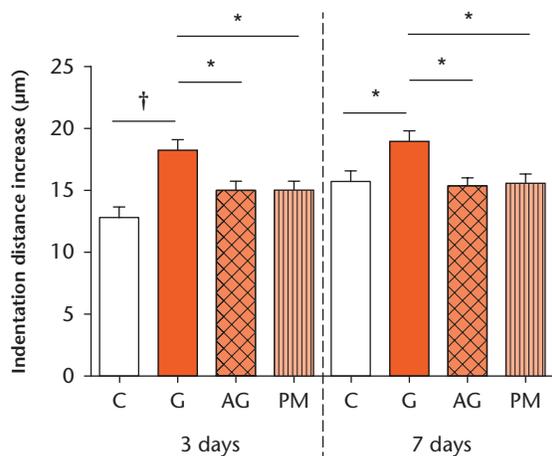


Fig. 4

Mean indentation distance increase (IDI) (in μm) across all experimental groups after three and seven days. Glucose co-incubated with aminoguanidine (AG) and pyridoxamine (PM) significantly reduced IDI (improved bone strength) compared with glucose alone after three and seven days ($n=20$; $*p < 0.01$, $\dagger p < 0.0001$, two-way analysis of variance (ANOVA)). C, control; G, glucose.

significant predictor of AGE accumulation in 60 donors over an age range of 2.5 to 103 years.³⁹

It is noteworthy that after seven days of incubation, AG inhibits mean AGEs concentration in contrast to the glucose treatment group alone (Fig. 1). Pyridoxamine shows potential as an AGE-inhibitor but statistical analysis was not significant between PM and glucose treatment alone; this could be due to the sample size for the AGEs quantification assay, where a larger sample size would produce more power that may show PM as a more promising AGE inhibitor. In Figure 2, we use the same data to receive additional empirical support that AG and PM reduce AGE content as a percentage change of the control compared with the glucose only group ($*p < 0.05$, $**p < 0.01$) after seven days. Results from bone micro-indentation testing further support these results. We focus on the parameter IDI as an indicator of bone strength, with an increase in IDI indicative of increased bone fragility or reduced crack growth toughness.^{31,40} Glucose treatment significantly increases IDI compared with the control after three days ($p < 0.0001$) and seven days ($p < 0.05$) (Fig. 3). It is particularly notable that there is no statistically significant difference between the control and inhibitor treatment groups after three and seven days, which may indicate that treatment with AG or PM mitigates the harmful effects of glucose on crack growth toughness (Fig. 4). Additionally, AG and PM treatment reduces IDI (or increased crack growth toughness) after both three and seven days compared with treatment with glucose alone (Fig. 4). These findings are valuable because they complement the AGE analysis data with biomechanical support at the bone-tissue macro level.

We are interested in the relationship between micro-level bone properties such as AGE content and macrolevel

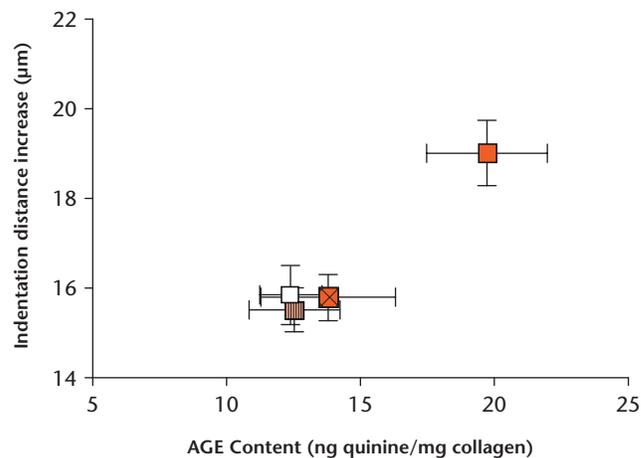


Fig. 5

Mean advanced glycation end-product (AGE) content positively correlates with mechanical properties from bone micro-indentation testing (mean indentation distance increase) controlled for bone mineral density across treatment groups ($n=20$ with standard error of the mean bars shown). White square, control; plain orange square, glucose only; cross-hatched orange square, glucose + aminoguanidine; striped orange square, glucose + pyridoxamine.

biomechanical bone properties such as IDI. Previous research has shown that AGE content correlates with IDI.¹² The data in Figure 5 suggest that, as hypothesized, an increase in AGE content corresponds with an increase in IDI (or reduced crack growth toughness), controlled for bone mineral density by calculating the mean of the parameter for each experimental group. Thus, this analysis provides support for the use of AG and PM as effective inhibitors of AGE accumulation and subsequent biomechanical degradation measured via RPI.

This study examined the preventative effects of AG and PM on AGE formation in human cortical bone. One major limitation of this work is that it was conducted *in vitro*. In order to prove feasibility for human use, *in vivo* animal and human studies need to be performed. It is noteworthy that AG administration to rats was effective in preventing diabetes-induced formation of fluorescent AGEs and cross-linking of arterial wall connective tissue protein *in vivo*.⁴¹ Additionally, another *in vivo* study showed that PM reduces oxidative stress and AGE formation in diabetic rats.³⁸ In light of these previous studies and the *in vitro* results presented in this study, we suggest that AG and PM warrant further investigation for inhibiting AGE accumulation in bone, improving bone quality, and preventing debilitating and expensive complications of diabetes and ageing.

In summary, glucose increases AGE formation in human cortical bone due to NEG. The results suggest that AG and PM prevent AGE formation and subsequent biomechanical degradation, warranting further investigation into the safety and efficacy of these treatments. Studying the accumulation of AGEs will help identify novel therapeutic targets to treat bone quality deterioration, especially in AGE-susceptible populations such as diabetics.

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Author Contributions

- O. Abar: Designing the study, Obtaining and preparing study samples, Performing all aspects of protein assays and mechanical testing, Analyzing and interpreting the data, Writing the manuscript.
- S. Dharmar: Participating in the design of the study, Assisting in performing several aspects of the protein assays and mechanical testing.
- S. Tang: Designing the study, Providing guidance in all aspects of the protein assays and mechanical testing, Analyzing and interpreting the data, Writing the manuscript.

Conflict of Interest Statement

- None declared

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