An Inducible Model for Unraveling the Effects of Advanced Glycation End-Products in Collagen with Age and Diabetes

Austin G. Gouldin[1], Jennifer L. Puetzer[1, 2*]

[1] Departments of Biomedical Engineering and [2]Orthopaedic Surgery, Virginia Commonwealth University, Richmond, VA, United States

Abstract

In connective tissues there is a clear link between increasing age and degeneration. It is believed advanced glycation end-products (AGEs) play a central role in this degeneration. AGEs are sugar induced non-enzymatic crosslinks which accumulate in collagen with age and diabetes, altering tissue mechanics and cellular function. Despite ample correlative evidence linking collagen glycation to degeneration, little is known how AGEs impact cell-matrix interactions, limiting therapeutic options. One reason for this limited understanding is AGEs are typically induced *in vitro* using high concentrations of ribose which decrease cell viability and make it impossible to investigate cell-matrix interactions. The objective of this study was to develop a system to trigger AGE accumulation while maintaining cell viability. Using cell-seeded high density collagen gels, we investigated the effect of two different systems for AGE induction, ribose at low concentrations (30, 100, and 200 mM) over 15 days of culture and riboflavin (0.25 mM and 0.75mM) induced with blue light for 40 seconds. We found ribose and riboflavin with blue light are capable of producing a wide range of AGE crosslinks which match and/or exceed reported human AGE levels for various tissues, ages, and diseases, without affecting cell viability and metabolism. Interestingly, a single 40 second treatment of riboflavin and blue light produced similar levels of AGEs as 3 days of 100 mM ribose treatment and matched aged mouse tendon AGE levels. This riboflavin treatment option is an exciting means to trigger AGE crosslinks on demand *in vivo* or *in vitro* without impacting cell metabolism or viability and holds great promise for further unraveling the mechanism of AGEs in age and diabetes related tissue degeneration.

* Corresponding Author
Jennifer L. Puetzer
Address: 401 West Main Street, Engineering East Hall, Virginia Commonwealth University, Richmond, VA, 23284, United States
E-mail: jlpuetzer@vcu.edu

Introduction

Generally, with aging and/or diabetes, tissues throughout the body, such as arteries [1], lungs [2], skin [3], lens [4], cartilage [5], tendons [6], and ligaments [6] are known to become stiffer. This stiffening of the extracellular matrix leads to an increased rate of injury and degeneration of the tissues [7]. In musculoskeletal tissues alone, 75% of individuals over the age of 65 will suffer an injury [8]. While many mechanisms, such as aging stem cells, reactive oxidative species, and inflammation, contribute to this clear link between increasing age and degeneration, one of the most prominent mechanisms believed to be responsible, is progressive accumulation of advanced glycation end-products (AGEs) [7,9,10]. AGEs are sugar-induced non-enzymatic crosslinks that accumulate with age and/or diabetes in the extracellular matrix, altering tissue mechanics and cell function. AGEs were first identified in cooked food as end-products from a non-enzymatic reaction between sugars and proteins called the Maillard reaction [11]. Around 40 years ago a similar glycation process was recognized in the extracellular matrix of diabetic patients [12]. More recent studies have shown that in diabetes, protein damage due to AGE crosslinking is the major cause of disease related pathogenesis [13,14].

The first step of AGE formation is initiated by covalent attachment of reducing sugars to amino groups of proteins, lipids, or nucleic acids to produce a reversible and unstable Schiff base [11]. Then, the Schiff base may undergo Amadori rearrangement and change to a more stable Amadori product. After a certain period of time, spanning from weeks to years, the Amadori product will transition to form an AGE crosslink or adduct [7,9,15]. AGE formation is quite slow, therefore AGEs primarily form in long-lived proteins like collagen, and accumulate with age as cells decrease their turnover of the matrix [16]. AGE crosslinks are thought to alter the mechanics of the ECM, as well as increase inflammation and oxidative stress, eventually leading to chronic illnesses [12]. In fact, AGEs have been linked to many age-related diseases, such as atherosclerosis [17], chronic kidney disease [18], rheumatoid arthritis [19–21], and neurodegenerative diseases such as Alzheimer's and Parkinson's disease [22]. Further, due to the high reliance on collagen in connective tissues such as tendon, ligament, meniscus, and cartilage, AGEs have been linked to age-related changes in mechanics that lead to increased injury and degeneration [23–26]. AGE accumulation also increases with increased levels of sugars in the bloodstream, thus AGE accumulation occurs with diabetes-related hyperglycemia and is thought to play a central role in the disease progression of diabetes [13,14].

AGEs are thought to induce disease and degeneration by two main mechanisms. AGEs can either crosslink proteins, directly altering their structure and in-turn altering their properties and function, or alter binding sites on proteins changing cellular signaling [12]. Despite ample correlative evidence connecting collagen glycation to aging and disease, little is known how AGEs impact matrix mechanics and cell-matrix interactions, resulting in limited therapeutic options [9,10]. It is difficult to study the AGE mechanism *in vivo* due to differences in collagen organization, disease state, and age [9,10]. *In vitro* models are an attractive means to study AGEs because they eliminate confounding variables of disease state, lifestyle, and age. Traditionally AGE crosslinks have been induced through ribose culture in unorganized collagen gels and explanted rat tendons [7,27,28]. Ribose is typically used to induce AGEs in experiments due to its higher reactivity rate, in comparison to glucose and fructose, which ultimately shortens the timeframe needed for AGE formation to occur [29,30]. However, *in vitro* work is often performed with concentrations greater than 200 mM ribose, resulting in super-physiological levels of glycation and low cell viability [7,10,28]. Due to low cell viability in these systems it is impossible to study the cellular response to AGE protein modifications, which in

turn greatly reduces the physiological reliability of the results. More specifically, a previous experiment explored fibroblast viability in ribose treated collagen matrices and concluded anything greater than or equal to a ribose concentration of 240 mM would lead to cell death [28]. The objective of this study was to develop an inducible system to trigger native level AGEs *in vitro*, while maintaining cell viability, in order to study the effect of AGEs both on protein properties and cellular response.

One promising method for inducing AGEs instantaneously without cell viability issues is through riboflavin. Riboflavin, or B2 vitamin, is a non-toxic photosensitizer that when exposed to UV light or blue light (465 nm) results in the formation of covalent crosslinks [31]. Upon photoactivation, riboflavin will form covalent bonds with amino acids of collagen, as well as form reactive oxygen species that can further oxidize amino acids for reactions [32]. The mechanism of riboflavin is still not completely understood but the formation of covalent crosslinks between riboflavin and collagen is similar to the Milliard reaction in AGE formation [31]. In addition, the process of generating oxygen free radicals while crosslinking collagen is a trend seen in AGE formation [14]. Riboflavin is used clinically to enhance corneal strength using UV activation and *in vitro* to increase the strength of different hydrogels [31,33–36]. Specifically, it has been shown to crosslink collagen gels in a dose dependent fashion with limited viability issues, depending on the concentration of riboflavin and duration of light exposure [34]. Riboflavin holds great potential to create a truly inducible AGE model, however despite its wide use for crosslinking collagen gels, it has not been investigated as a means to induce AGEs. We hypothesize that riboflavin excited by blue light in high density collagen gels will produce native AGE levels without altering cell viability or metabolism. Additionally, in this study we evaluated traditional ribose methods of inducing AGEs, at lower concentrations typically reported in the literature, to investigate whether lower concentrations of ribose for longer periods of time can induce native levels of AGE accumulationwithout altering cell viability or metabolism.

Results

Development of Glycation Induction Systems

In this study, high density collagen gels (20 mg/ml) seeded with meniscal fibrochondrocytes were used as the construct system for AGE accumulation. Low-density collagen gels (1-5 mg/ml) have been traditionally used for AGE studies [31,36,37]; however these gels often undergo significant contraction resulting in limited culture durations. High density collagen gels (10-20 mg/ml) have collagen concentrations closer to native values, reduced contraction compared to low density gels, and have been shown to maintain cell viability over 8 weeks of culture [38–40]. Bovine meniscal fibrochondrocytes were used as a generic primary cell source, since menisci are substantially affected by AGE accumulation during aging [25,41] and we have previously shown meniscal fibrochondrocytes survive in high density collagen gels for up to 8 weeks [38,39,42]. Cells were mixed into collagen gels, cast into a sheet gels, and 6 mm biopsies were cut from the sheet gel for culture.

After construct fabrication, cell-seeded constructs were split between Dulbecco's Modified Eagle Medium (DMEM) control, riboflavin, or ribose groups and cultured for up to 15 days (**Figure 1**). DMEM control constructs were cultured in standard growth media for the duration of culture. Riboflavin treated constructs were incubated in 0.25 mM or 0.75 mM riboflavin for 1 hour after fabrication and then exposed to blue light for 40 seconds to trigger non-enzymatic glycation crosslinking [36]. Blue light was chosen as the photo-activator along with a 40 second exposure time due to previous studies showing little impact on chondrocyte cell viability with 0.25 mM and 0.75 mM riboflavin concentration [36]. After blue light exposure, the constructs were re-suspended in control DMEM media for the duration of the study. Ribose treated constructs were cultured in 30, 100, or 200 mM ribose for the duration of culture. Ribose glycation media consisted of the control DMEM media formulation plus ribose at the desired

concentrations. Media was changed every 3 days, and timepoints were taken at 0, 3, 6, 9, 12, and 15 days.

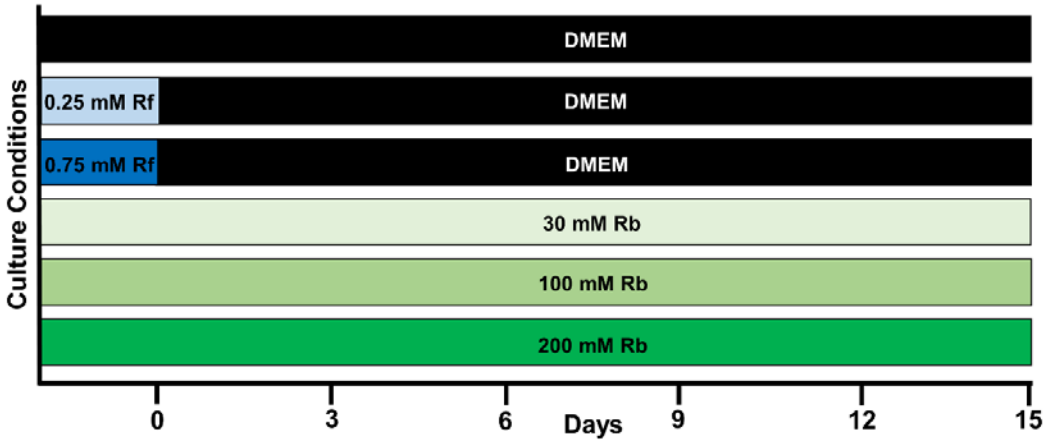

Figure 1: *Exposure timeline for all control (DMEM), Riboflavin (Rf), and Ribose (Rb) concentrations. Control constructs were kept in basic DMEM media for the full 15 days. Riboflavin constructs were soaked in riboflavin for 1 hour, stimulated with blue light for 40 seconds to activate crosslinks and then cultured in basic DMEM for 15 days. Ribose constructs were cultured in basic DMEM with ribose for the full 15 days.*

Effect of Glycation on Gross Contraction

Inspection of constructs upon removal from culture demonstrated a slight decrease in size in DMEM control constructs and low concentration riboflavin (0.25 mM) constructs by day 15, suggesting reduced cellular contraction with increased glycation (0.75 mM riboflavin, 30 mM, 100 mM, and 200 mM ribose, **Figure 2A**). However, analysis of construct percent water did not show any significant differences or trends between control and glycated (riboflavin and ribose) constructs (**Figure 2B**), indicating negligible differences in degree of contraction between groups.

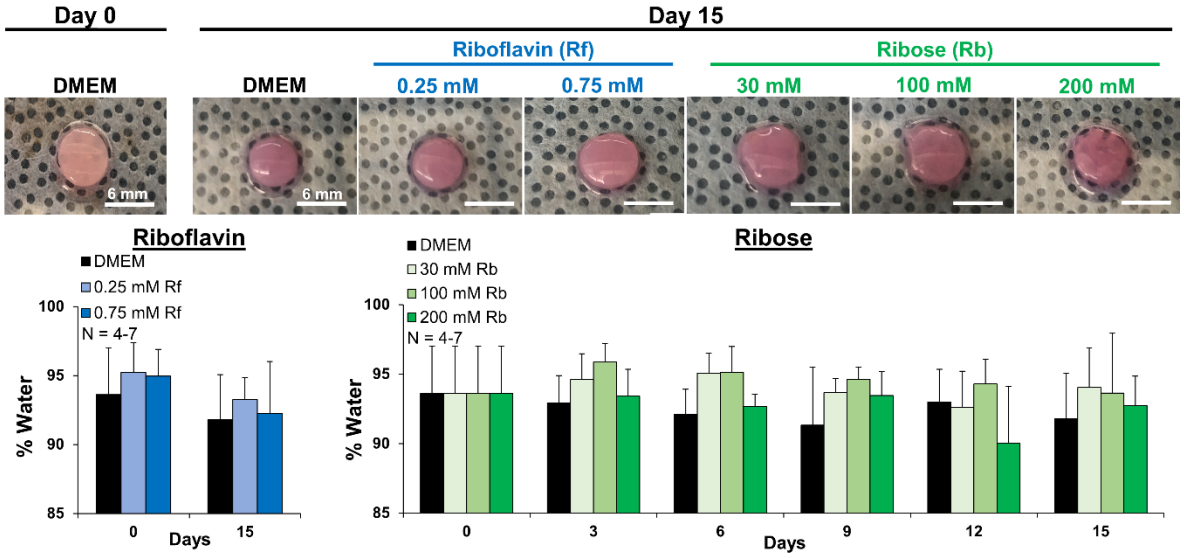

Figure 2: *Representative photos of biopsy punches at Day 0 and Day 15 and percent water concentration of constructs throughout the duration of the study. There were no significant differences in degree of contraction between groups over the 15 days.*

Effect of Glycation on Collagen Fibril Organization

Confocal reflectance analysis was used as a macroscale measure to evaluate degree of crosslinking and the effect of AGE induction on collagen organization. Evaluation of collagen organization revealed both glycation systems resulted in increased fibril formation by day 15 compared to DMEM controls (**Figure 3**). DMEM controls had no changes in organization and no clear fibril formation at the evaluated length-scale between day 0 and day 15. However, all concentrations of riboflavin and ribose treated constructs developed organized collagen fibrils by day 15, with more pronounced, elongated fibril formation in ribose treated constructs. This clear change in collagen organization suggests AGEs are actively crosslinking the collagen, resulting in fibril formation [28].

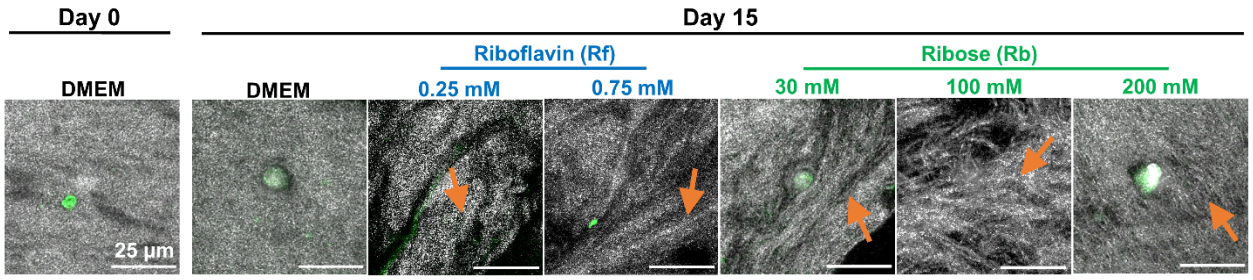

Figure 3: *Effect of glycation on collagen organization. Confocal reflectance revealed increased fibril formation (orange arrows) in all riboflavin and ribose constructs compared to DMEM control constructs suggesting increased crosslinking. Collagen = grey, auto-fluorescent cells = green, scale bar = 25 µm.*

Effects of Glycation on Cellular Viability and Metabolism

To evaluate the effect of riboflavin and ribose on cell viability and metabolism, construct DNA was measured as a representation of cell number and reduction of alamarBlue was measured as a representation of metabolic activity. DNA analysis demonstrated both concentrations of riboflavin (0.25 mM and 0.75 mM) had no change in DNA concentration compared to control at day 0, suggesting no change in cell viability with 40 seconds of blue light for photo-initiation of AGE crosslinks (**Figure 4A**). Further, both concentrations of riboflavin treated constructs maintained similar DNA to control constructs by day 15, suggesting no change in cell viability throughout culture. Similarly, both concentrations of riboflavin treated constructs reduced alamarBlue similar to control constructs at day 0 and day 15, suggesting little to no effect on cellular metabolism (**Figure 4B & C**).

In contrast, high concentrations of ribose (100 mM and 200 mM) produced significant decreases in DNA by day 9, while 30 mM ribose treatment maintained similar DNA to control constructs throughout culture (**Figure 4A**). Further, both high concentrations of ribose (100 mM and 200 mM) resulted in significantly lower percent reduction of alamarBlue, compared to control and 30 mM ribose constructs; however when normalized to DNA concentration, both high concentrations of ribose had significantly higher percent reduction of alamarBlue (**Figure 4B & C**). Collectively this suggests high concentrations of ribose reduce cell viability, while increasing cellular metabolism with time in culture.

Glycosaminoglycan (GAG) accumulation, normalized to DNA, was quantified as an additional measure of metabolism since this is a major macromolecule often produced by meniscal fibrochondrocytes (**Supplemental Figure 1**). Similar to alamarBlue analysis, both concentrations of riboflavin had no significant differences in GAG accumulation compared to control constructs throughout culture, while high concentrations of ribose (100 mM and 200 mM)

resulted in significantly higher GAG accumulation by day 15.

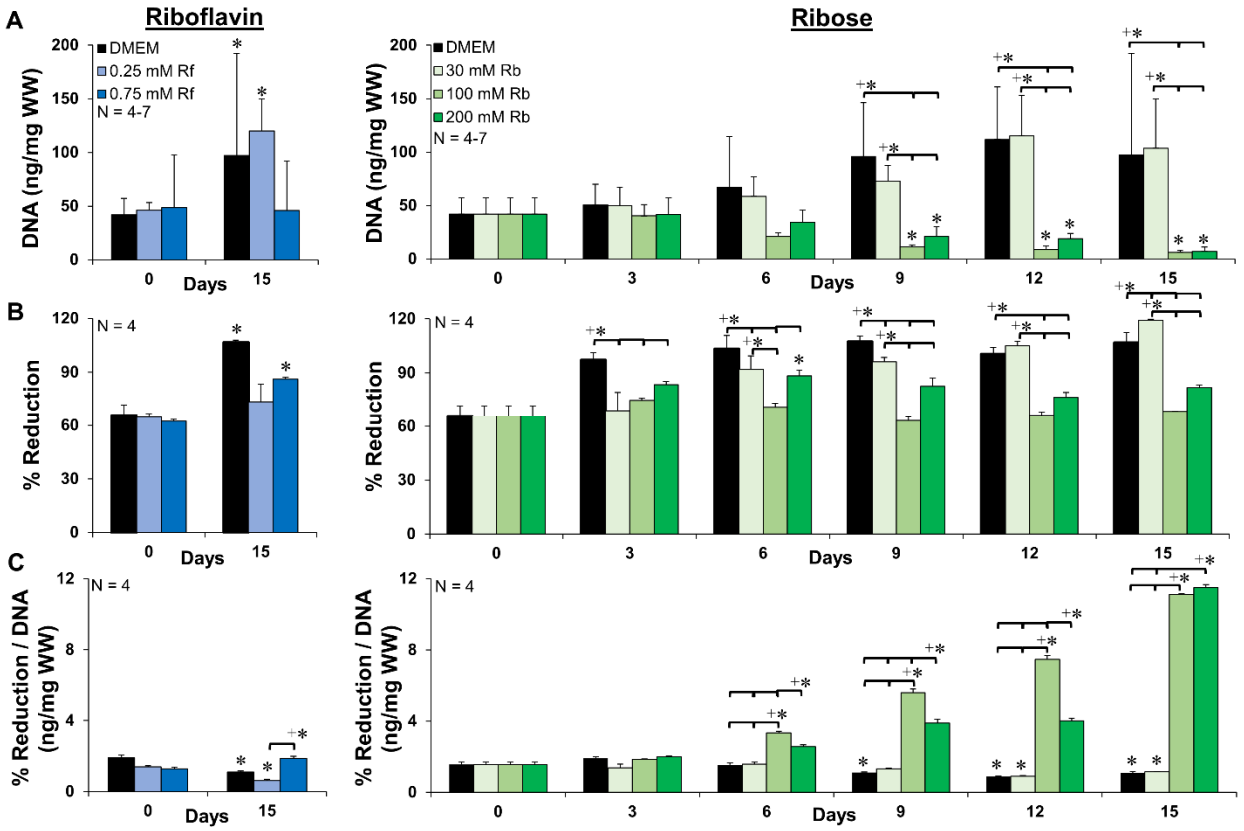

Figure 4: *Effect of glycation on cell viability and metabolism. A) DNA normalized to wet weight (WW), B) alamarBlue percent reduction, and C) alamarBlue percent reduction normalized to DNA/WW. Riboflavin had little effect on cell viability and metabolism, while higher concentrations of ribose significantly decreased cell viability starting at day 9 and significantly increased cellular metabolism by 6 days. Significance compared to *day 0 or + bracketed group (p<0.05).*

Induced AGE Crosslink Accumulation

In this study, total fluorescent AGEs and AGE specific crosslink, pentosidine, were measured to assess the ability of riboflavin and ribose to induce AGEs. Collagen concentration, represented by hydroxyproline (hypro) content, was measured to better normalize AGE crosslinks across all treatment groups. As expected, collagen content was relatively constant throughout culture with no consistent difference between DMEM control, riboflavin, and ribose groups (**Figure 5A**).

Both riboflavin and ribose induced total AGE crosslinks in a dose dependent fashion (**Figure 5**). Specifically, both concentrations of riboflavin induced significant increases in total AGE accumulation normalized to wet weight (**Supplemental Figure 2A**) and collagen content (**Figure 5B**) at day 0 and maintained a similar level of AGEs through 15 days of culture. Further, both concentrations of riboflavin treatment matched aged 21-23 month-old mouse tail tendon AGE levels after induction at day 0 (**Figure 5B,** solid line). Ribose treatment significantly increased AGE crosslinks when normalized to wet weight (**Supplemental Figure 2A**) and collagen content (**Figure 5B**) in a dose dependent fashion with time in culture. Further, all concentrations of ribose matched aged mouse tail tendon AGE levels by 3 days of culture

(**Figure 5B**).

Despite riboflavin significantly increasing total AGE crosslinks, there were no significant increases in AGE specific crosslink, pentosidine, compared to DMEM controls, when normalized to wet weight (**Supplemental Figure 2B**) or collagen content (**Figure 5C**). Further, riboflavin treatment did not match pentosidine levels of aged mouse tail tendon or aged human meniscus [25]. However, ribose treatment did significantly increase pentosidine crosslinks when normalized to wet weight (**Supplemental Figure 2B**) and collagen content (**Figure 5C**) in a dose dependent fashion, similar to total AGE accumulation. Further, 100 mM and 200 mM ribose induced pentosidine concentrations that matched aged mouse tail tendon and reported 60-70 year old human meniscus pentosidine levels by 3 days [25]. Additionally, 80-90 year old human cartilage pentosidine levels [24] were reached by day 12 with 200 mM ribose treatment, and by day 15 with 100 mM ribose treatment (**Figure 5**).

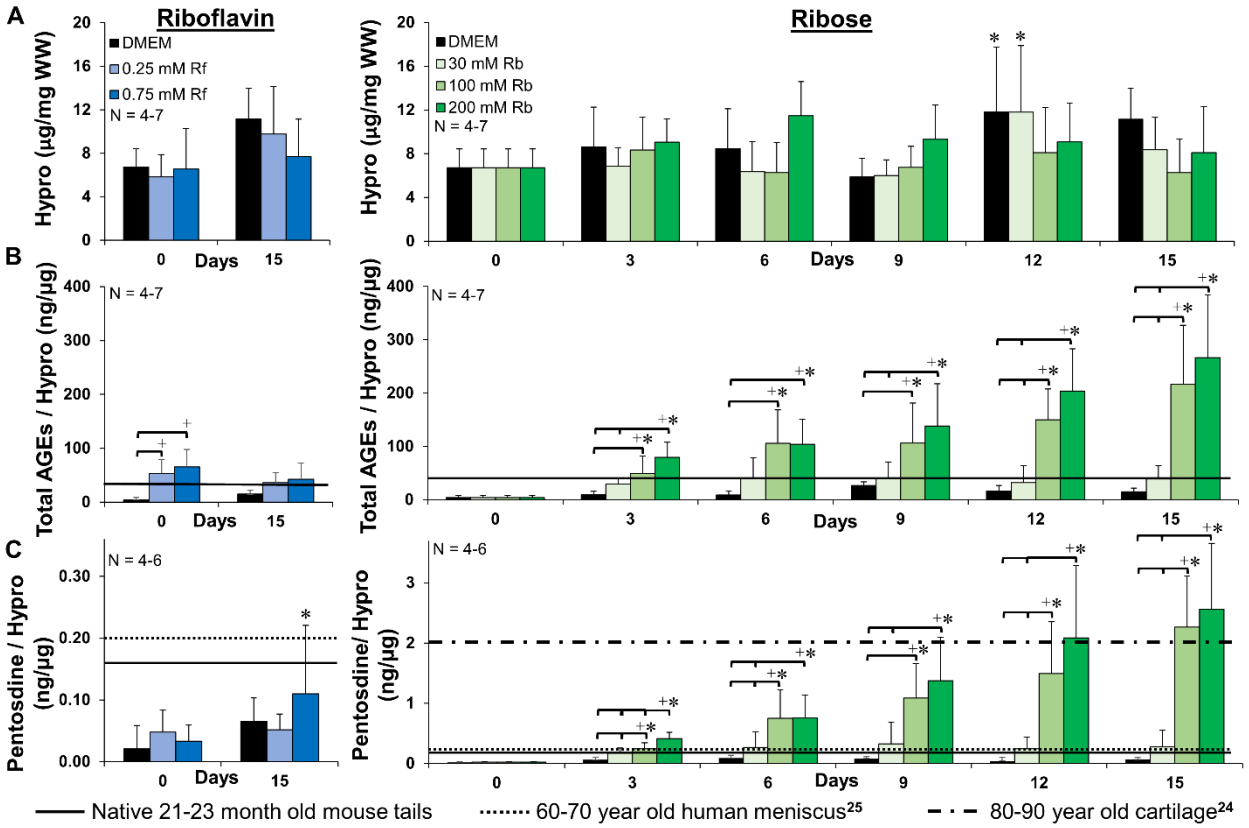

Figure 5: *Glycation significantly increases AGE formation. A) Collagen content represented by hydroxyproline (Hypro) normalized to wet weight (WW), and B) total AGE and C) AGE Specific crosslink pentosidine, normalized to hypro. Both concentrations of riboflavin induced native levels of total AGEs, but had little effect on pentosidine. Ribose induced total AGEs and pentosidine crosslinks in a dose dependent fashion, reaching aged C57BL/6 mouse and human values* [24,25] *between 3 and 15 days. Significance compared to *day 0 or + bracketed group (p<0.05).*

Synergistic Effect of Riboflavin and Ribose Treatment

In this study we also investigated whether ribose and riboflavin treatment could be combined to synergistically increase AGE crosslink formation, making it possible to reach 80-90 year old aged cartilage levels earlier in culture before the ribose induced viability issues. Combination constructs were cultured for 15 days in 100 mM ribose media and prior to removing constructs from culture at each timepoint, constructs were soaked in 0.25 mM riboflavin and induced with blue light for 40 seconds. Surprisingly, we found there was no synergistic increases in crosslinks when riboflavin and ribose were combined at any of the timepoints. The combination constructs had similar concentrations of total AGEs and pentosidine crosslinks as the 100 mM ribose treated constructs throughout culture (**Figure 6**).

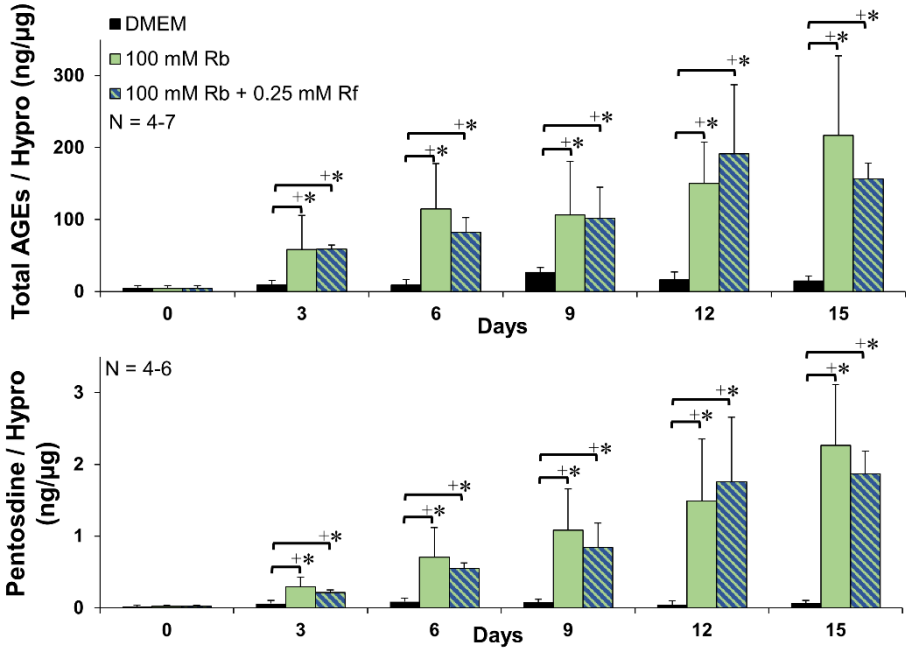

Figure 6: *Combination system does not synergistically increase total AGE or pentosidine formation. Total AGE and AGE specific crosslink, pentosidine, normalized to hydroxyproline (hypro). 100 mM ribose (Rb) alone and in combination with 0.25 mM riboflavin (Rf) induced total AGEs and pentosidine crosslinks in a similar dose dependent fashion. Significance compared to *day 0 or + bracketed group (p<0.05).*

Discussion

The population is aging at an unprecedented rate and understanding the link between aging and tissue degeneration could have major treatment and socioeconomic impacts [43]. Despite ample correlative evidence connecting AGEs to tissue injury [44,45], degeneration [23,26], and multiple chronic diseases, such as cardiovascular disease [17], chronic kidney disease [18], diabetes [46], Alzheimer's and Parkinson's disease [22], little is known how AGEs impact matrix mechanics and cell-matrix interactions, resulting in limited therapeutic options. One reason for this limited understanding is because AGES are typically induced using concentrations of ribose greater than 200 mM, which decrease cell viability and make it impossible to investigate cell-matrix interactions [7,9,10]. Here, we induced native levels of AGEs with lower concentrations of ribose and maintained cell viability through 15 days of culture. Specifically, in this study we

found ribose and riboflavin with blue light are capable of producing a wide range of AGE crosslinks which match and/or exceed reported human AGE levels for various tissues, ages, and diseases [7,23–26]. Interestingly, a single 40 second treatment of 0.25 mM or 0.75 mM riboflavin and blue light produced similar levels of AGE crosslinks as 3 days of 100 mM ribose treatment, without decreasing viability. This riboflavin treatment option is an exciting means to trigger AGE crosslinks on demand *in vivo* or *in vitro* without impacting cell metabolism with prolonged cultures in high ribose concentrations.

There are several different AGEs that occur throughout the body. The mechanism by which they individually form remains unclear, however this population of AGEs includes adducts which modify the surface of a protein and crosslinks that bridge two neighboring proteins [9,47]. In this study, we evaluated total fluorescent AGE accumulation, along with specific AGE crosslink, pentosidine. AGEs are typically categorized into four different types; fluorescent crosslinked or uncrosslinked, and non-fluorescent crosslinked or uncrosslinked [15]. Here we measured total AGE accumulation using previously established fluorescence measurements at 360 nm excitation and 460 nm emission [48–53]. Total AGE accumulation at these specific wavelengths will encompass all AGEs that naturally fluoresce, including pentosidine, pentodilysine, crossline, pyrropyridine, Vespertysine A, and Vespertysine C, as well as fluroescent AGE adducts [15]. We also quantified AGE specific crosslink, pentosidine. Pentosidine is a major type of AGE crosslink which is commonly studied due to its dominance in tissues and because it has its own fluorescence range, making it easy to analyze using fluorescence measures. More specifically, pentosidine has been found in high levels in patients with rheumatoid arthritis [20,21], plays a pivotal role in the complications of diabetes [20,54], and has even been linked to being a risk factor for patients with heart failure [55]. Pentosidine is an incredibly important AGE crosslink and being able to induce human pentosidine levels without decreasing cell viability will allow for pentosidine and its effects to be studied in a way that has not been done before in *in vitro* models.

Both the ribose and riboflavin glycation methods resulted in the development of well-organized collagen fibrils by 15 days, suggesting active AGE collagen crosslinking [28,31]. AGEs can form adducts or crosslinks, and while these glycation systems most likely induce both, similar to the *in vivo* environment, the presence of fibril formation suggests we are indeed achieving active crosslinking of the collagen molecules. AGE crosslinking of collagen will not only result in altered mechanics [7,10], but can also lead to dramatic modifications of collagen interactions with other molecules such as proteoglycans, enzymes, and cell integrins [9]. These modifications to the collagen fibril surface are known to affect cell-matrix interactions in a manner that leads to impair wound healing and increased inflammation [56]. Our high density collagen system provides a promising means to further investigate these direct effects of AGE crosslinks on collagen to better understand its role in degeneration of connective tissues throughout the body.

In this study, the ribose glycation system induced a wide range of total AGEs and pentosidine specific crosslinks, matching reported human AGE levels for various tissues, ages, and diseases [24,25]. Specifically, ribose treatment induced AGE accumulation that matched 21-23 month old mouse tail tendons (~similar to 55-65 year old human tissue [57]) by 3 days. Further, ribose induced pentosidine accumulation that matched reported human 60-70 year-old meniscus [25] by 3 days and 80-90 year-old cartilage by 12 days [24]. Previous work in the field has induced AGEs using ribose concentrations greater than 200 mM, often resulting in super-physiological levels of glycation and low cell viability [7,10,28], making it impossible to investigate cell-matrix interactions. Here, we induced native levels of AGEs with a lower concentration of ribose and maintained cell viability through 15 days of culture. As expected, we did observe decreased cell viability with higher concentrations of ribose. Specifically, 100 mM and 200 mM ribose treatments resulted in significantly lower DNA concentrations by day 9. Further, these high ribose concentrations increased cellular metabolism, as evident by

significantly higher reduction of alamarBlue, a measure of metabolic activity, by day 6 (**Figure 4**) and significantly higher accumulation of GAGs, a major macromolecular produced by meniscal fibrochondrocytes, by day 15 (**Supplemental Figure 1**). Collectively this demonstrates the limitations of inducing AGEs with high concentrations of ribose and why lower concentrations should be consider for evaluating cellular response to AGE crosslinks. In this study, we matched aged mouse tail tendon and human meniscus AGE levels by 3 days, before any changes in cell viability or metabolism.

In an attempt to overcome the cell viability and metabolic changes that occur with ribose treatment, we also investigated the potential of riboflavin to induced AGEs. Riboflavin, or B2 vitamin, is a non-toxic photosensitizer that when exposed to UV light or blue light (465 nm) results in the formation of non-enzymatic covalent crosslinks which form similar to AGEs in collagen [31]. Riboflavin is used clinically to enhance corneal strength and *in vitro* to increase the strength of different hydrogels [31,33–36]. Specifically, it has been shown to crosslink collagen gels in a dose dependent fashion with limited viability issues [34,58]. Riboflavin holds great potential to create a truly inducible AGE model, however despite its wide use for collagen crosslinking, it has not been investigated as a means to induce AGEs. In this study, we found that a single treatment of riboflavin and 40 seconds of blue light produced similar levels of AGE crosslinks as 3 days of ribose treatment, without decreasing viability. Further, this single 40 second treatment of riboflavin induced total AGE accumulation which matched aged mouse tail tendon. There was no change in cell viability or metabolism throughout 15 days of culture as well. This is the first study to our knowledge to demonstrate that riboflavin photo-initiated crosslinking results in AGE crosslink formation. This riboflavin treatment option is an exciting means to trigger AGE crosslinks on demand *in vivo* or *in vitro* without impacting cell metabolism or cell viability.

Despite matching native total AGE levels, riboflavin treatment did not appear to induce pentosidine crosslinks. This is in contrast to what was seen with the ribose glycation method. This phenomenon is most likely due to riboflavin inducing a different pool of AGE adducts and crosslink, instead of pentosidine. Possible amino acids that are vulnerable to photochemical cross-linking include tyrosine, histidine, cysteine and methionine [31]. Based off these amino acids, one possible AGE that could form would be MRX (8-hydroxy-5-methyldihy-drothiazolo(3,2-alpha) pyridinium-3-carboxylate). MRX is better known as a fluorescent AGE that is formed due to the product of cysteine and arginine interacting with glucose [59]. MRX is commonly seen to be in high levels of diabetic patient's urinary proteins and has been suggested as a potential biomarker for hyperglycemia [59]. Future work should perform high performance liquid chromatography (HPLC) analysis to confirm whether riboflavin treatment results in MRX crosslinking and further investigate the wide arrange of AGE crosslinks induced by ribose and riboflavin.

In this study we also investigated whether ribose and riboflavin treatment could be combined to have a synergistic increase in AGE crosslink formation making it possible to reach 80-90 year old aged cartilage AGE levels earlier in culture, before the ribose induced viability issues. Surprisingly, the results showed that there was no synergistic increase in crosslinks when riboflavin and ribose were combined. This suggests that while ribose and riboflavin may induce different AGE crosslinks, they may target similar amino acids in collagen. It has been well established that AGEs form at different rates with different sugars, with ribose forming AGEs quicker than fructose and glucose. Further it has been determine this rate of crosslink formation is dependent on the amount of sugar in open chain form and the rate at which the molecules transition into a Schiff base [29,60,61]. Ribose is a major component of riboflavin [60], and is present in its reduced open chain form in riboflavin, thus it is plausible riboflavin would produce AGEs quicker than ribose. Further, the photo-initiation of riboflavin with blue light might oxidize collagen molecules further enabling AGE formation [31]. Since ribose and riboflavin are both

composed of ribose they most likely target similar amino acids of collagen, thus addition of riboflavin after ribose incubation would not further increase AGE formation if most reactionary amino acids are occupied. With respect to lack of pentosidine formation with riboflavin, the flavin portion of riboflavin may restrict or slow its formation. Further the presence of flavin and the kinetics of the reaction with riboflavin may favor adduct formation over crosslink formation, as evident by less pronounced collagen fibril formation with riboflavin compared to ribose treatment (**Figure 3**). This data demonstrates that these two glycation systems can induce different types of AGE crosslinks and opens the door to exploring effects of tissue specific crosslink effects throughout the body.

A limitation of this study is that we only evaluated AGE induction in collagen since this is the main structural protein of tissues throughout the body and the most common protein evaluated for AGE accumulation. However, other long-lasting proteins in the ECM, such as elastin and fibronectin, are also susceptible to AGEs [16]. The amount of AGEs induced may vary with different concentrations of collagen gels, different degrees of collagen organization, different proteins in the ECM, or in different disease conditions. Future studies in whole tissue or other systems may have to vary concentration and duration of exposure to reach native levels of AGEs; however this work can serve as a guide for inducing native AGE and pentosidine levels with no change in cell viability or metabolism.

Conclusions

There is increasing evidence that AGEs play a central role in aging and degeneration of connective tissues; however, little is known how AGEs impact tissue mechanics and damage cell-matrix interactions. Even less is known how these factors interact to cumulatively affect local repair of matrix damage, inflammation, disease progression, and systemic health. In this study we developed and characterize two inducible AGE models, low concentration ribose and riboflavin with blue light, which produced a wide range of native AGEs without inducing cell viability and metabolic changes. These systems provide exciting new means to investigate the mechanism of AGEs in altering cell-matrix interactions and matrix mechanics *in vitro* to better mitigate the role of AGEs in tissue degeneration, injury, and chronic disease with age and diabetes.

Experimental Procedures

Cell Isolation

Bovine meniscal fibrochondrocytes were isolated from whole menisci within 24 hours of slaughter as previously reported [38,39,42]. Briefly, 1-6 week old calf legs were purchased from a local slaughterhouse. Menisci were aseptically isolated from the joint, diced, and digested overnight in 0.3% collagenase (Worthington). The next day cells were filtered, washed, and counted. Cells from 3 donors were combined to limit donor variability. Cells were seeded at 2800 cells/cm^2 into T-175 flasks and passaged 1-2 times to obtain enough cells. Cells were cultured in medium consisting of DMEM, 10% fetal bovine serum (FBS), 1% Antibiotic antimycotic, 0.1 mM non-essential amino acids, 50 µg/mL ascorbic acid, and 0.8 mM L- proline [38,39,42].

Construct Fabrication

Cell seeded high density collagen gels were fabricated as previously described [38–40]. Briefly type I collagen was extracted from purchased mix gender adult Sprague-Dawley rat tails (BIOIVT) and reconstituted at 30 mg/mL in 0.1% acetic acid [38,39]. The stock collagen solution was mixed with appropriate volumes of 1 N NaOH, 10x phosphate buffer solution (PBS), and 1x PBS to begin the gelling process and return the collagen solution to pH 7 and 300 mOsm

osmolarity [40,62]. The collagen solution was immediately mixed with meniscal fibrochondrocytes, injected between glass sheets 1.5 mm apart, and gelled at 37°C for 1 hour to obtain 20 mg/mL collagen sheet gels at $5x10^6$ cells/mL. Six mm biopsy punches were cut from sheet gels, split between experimental groups, and cultured for up to 15 days.

Glycation Culture Conditions

After construct fabrication, cell-seeded constructs were split between DMEM control, riboflavin, or ribose groups and cultured for up to 15 days (**Figure 1**). Control constructs were cultured in basic DMEM media consisting of DMEM, 10% FBS, 1% Antibiotic antimycotic, 0.1 mM non-essential amino acids, 50 µg/mL ascorbic acid, and 0.8 mM L- proline. Media was changed every 3 days and constructs were collected for time points at day 0, 3, 6, 9, 12, and 15.

Riboflavin treated constructs were incubated in 0.25 mM or 0.75 mM riboflavin (Sigma-Aldrich) in PBS for 1 hour after fabrication and then exposed to blue light (BoNew, USA, Dental 10W Cordless LED Light Lamp 2000mw) for 40 seconds [36]. After blue light exposure, the constructs were resuspended in control DMEM media for the duration of the study. Media was changed every 3 days and constructs were collected for timepoints directly after blue light exposure (day 0) or after 15 days of culture.

Ribose treated constructs were cultured in 30, 100, or 200 mM ribose (Sigma-Aldrich) for the duration of culture. Ribose glycation media consisted of the control DMEM media formulation plus ribose at the desired concentrations. Media was changed every 3 days, and timepoints were taken at 0, 3, 6, 9, 12, and 15 days.

Alamarblue Analysis

AlamarBlue (Bio-Rad) analysis was performed on days 0, 3, 6, 9, 12, and 15 for control and ribose treated constructs and day 0 and 15 for riboflavin treated constructs (N = 4) to directly assess metabolism and indirectly assess viability of cells throughout culture. Constructs were placed in 10% alamarBlue and 90% of their respective media formulation. Each media formulation, without constructs, was additionally assessed as controls at each timepoint. The constructs and media controls were kept in the incubator for 7 hours and then media was removed and read using a Biotek Synergy HTX Multi-Mode Reader at wavelengths of 570 nm and 600 nm. Percent reduction of the alamarBlue, indicating active metabolism, was determined from the absorbance readings using the standard manufacturer protocol. To account for changes in cell number throughout culture, percent reduction of alamarBlue was also normalized to average DNA/wet weight at each timepoint.

Post Culture Analysis

A separate set of constructs, not exposed to alamarBlue, were cultured for up to 15 days (N = 4-7 for each culture condition). At the completion of culture, constructs were photographed to evaluate contraction, weighted, and sectioned into pieces that were fixed or frozen for analysis of collagen organization, cellular concentration, collagen content, and AGE crosslinks. Additionally, tendons from 21-23 month-old C57BL/6 mouse tails (N=3) were obtained (Jackson Laboratory) for biochemical and AGE crosslink analysis to serve as a native controls.

Confocal Image Analysis

Cross-sections of constructs at day 0 and day 15 were fixed in 10% formalin, stored in 70% ethanol, and imaged with confocal reflectance microscopy to analyze collagen fiber organization as previously described [38,39,42,62]. Briefly, confocal reflectance imaging was performed in conjunction with fluorescence imaging by splitting a 488 nm laser on a Zeiss LSM 710 Laser Scanning Microscope using a Plan-Apochromat 63x/1.4 Oil DIC M27 (FWD=0.19mm) objective. Confocal reflectance was captured at 400-465 nm by collecting backscattered light reflected by collagen fibers through a 29 µm pinhole and a pixel dwell time of 0.79 µs. Auto-fluorescent cells

were captured at 509-577 nm. Noise was reduced by collecting images at 1024 x 1024 pixels with a line average of 4.

Biochemical Analysis

Biochemical analysis for DNA, GAG, and collagen content was performed as previously reported [38,39,42]. Briefly, half of each construct and mouse tendons was weighted wet (WW), frozen, lyophilized, and weighed dry (DW). The samples were then digested at 60°C in 1.25 mg/mL papain solution for 16 hours and analyzed for DNA, GAG, and collagen content via a QuantiFlour dsDNA assay kit (Promega), a modified 1,9-dimethylmethylene blue assay at pH 1.5 [63], and a hydroxyproline (hypro) assay [64], respectively. Additionally, construct hydration (percent water) was calculated by determining the difference between the wet weight and dry weight of the sample, and normalizing to the wet weight of the sample.

AGE Crosslink Quantification

The same samples used for biochemical analysis were also analyzed for AGE crosslinks after papain digestion. Fluorometric assays were carried out to observe and assess the effects of ribose and riboflavin on the formation of AGEs and AGE specific crosslink, pentosidine, as previously described [28,48–53]. Briefly, crosslinks were quantified by fluorescence using a Biotek Synergy HTX Multi-Mode Reader at two different ranges of wavelengths. General AGE crosslinks were quantified by reading fluorescence at 360 nm excitation,460 nm emission and compared to quinine standards (Sigma-Aldrich) [28,65]. Pentosidine specific crosslinks were quantified by reading fluorescence at 328 nm excitation, 378 nm emission [7] and compared to pentosidine standards (Caymen Chemical). Total AGEs and pentosidine crosslinks were normalized to wet weight and hydroxyproline content. Quantity of total AGEs and pentosidine crosslinks in constructs were further compared to aged-mouse tail tendons processed similar to constructs and to reported human pentosidine levels in 60-70 year old menisci [25] and 80-90 year old cartilage [24].

Evaluation of Synergistic Effect of Riboflavin and Ribose

We also investigated whether ribose and riboflavin treatment could be combined to synergistically increase AGE crosslink formation. Combination constructs were cultured for 15 days in the 100 mM ribose media with timepoints at day 0, 3, 6, 9, 12, and 15. Prior to removing constructs from culture at each timepoint, constructs were soaked in 0.25 mM riboflavin for 1 hour and induced with blue light for 40 seconds. Each construct was only induced with riboflavin treatment once, right before being removed from culture. Constructs were analyzed with the same post-culture analysis described above, specifically evaluating collagen content, total AGE accumulation, and pentosidine accumulation.

Statistics

SPSS was used to confirm normality of data within each group and detect outliers using Shapiro-Wilk tests and Q-Q plots. After confirming normality, all data was analyzed by 2-way ANOVA with Tukey's post hoc analysis (SigmaPlot 14). For all tests, $p<0.05$ was threshold for statistical significance. All data are expressed as mean ±-standard deviation.

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Conflicts of Interest
The authors declare no conflicts of interest.

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