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SHORT REPORT

Glyoxal-induced formation of advanced glycation end-products in type 1 collagen decreases both its strength and flexibility *in vitro*

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Keywords
Carboxymethyl arginine, Fish scales, Type 1 collagen

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ABSTRACT

The high plasma glucose induced in glucose metabolism disorders leads to the non-enzymatic glucose-dependent modification (glycation) of type 1 collagen, which is an essential component of bone tissue. The glycation of proteins induces the formation of advanced glycation end-products, such as carboxymethyl arginine, which is preferentially generated in glycated collagen. However, the effect of advanced glycation end-product formation on the characteristics of type 1 collagen remains unclear due to the lack of suitable *in vitro* experimental systems analyzing type 1 collagen. Here, we show that the glycation of type 1 collagen can be analyzed *in vitro* using a goldfish-scale bone model. Our study using these scales provides evidence that the advanced glycation end-product formation in type 1 collagen induced by glyoxal, the carboxymethyl arginine inducer, facilitates the crosslinking of type 1 collagen, decreasing both its strength and flexibility.

INTRODUCTION

Types 1 and 2 diabetes induce secondary pathologies, including bone disease[1]. In patients with type 1 diabetes, reduced bone mineral density and diabetic vascular complications have been reported to increase the risks of fracture and osteoporosis[1]. Conversely, patients with type 2 diabetes have comparable or even higher bone mineral density than healthy individuals[2]. Deranged bone turnover, as well as diabetic vascular complications, has been proposed to be related to an increased risk of fracture and osteoporosis[3]. However, the mechanism underlying diabetes-induced fracture and osteoporosis has not been fully understood[4].

Advanced glycation end-products (AGEs) are compounds that are physiologically formed through the non-enzymatic glucose-dependent modification (glycation) of proteins[5]. In addition to glucose, carbonyl compounds, such as methylglyoxal, glycolaldehyde and glyoxal, generate various types of AGEs[6]. For example, methylglyoxal reacts with proteins to form carboxyethyl lysine[7], whereas the reaction of glycolaldehyde with proteins generates glyceraldehyde-derived pyridinium[8]. Furthermore, glyoxal induces the formation of carboxymethyl lysine and carboxymethyl arginine (CMA). Carboxymethyl lysine is one of the major antigenic AGE structures accumulating in various proteins[9], whereas CMA is produced by oxidation in glycated collagen and, thus, is the AGE specific to collagen[10].

The glycation of type 1 collagen is stimulated by a high plasma glucose concentration observed in patients with diabetes, and AGEs have been confirmed as a vital risk factor for developing diabetic complications[11,12]. With respect to the quality of the bone, the possibility of altering bone matrix collagen properties through non-enzymatic glycation was reported in type 2 diabetes patients[13]. In addition, a study reported that serum levels of pentosidine, an AGE product, and poor glycemic control were associated with prevalent fractures in type 1 diabetes patients independent of bone mineral density[14]. In line with these findings, elucidation of the effect of glyoxal, the inducer of collagen-specific CMA, on the characteristics of

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type 1 collagen is expected to give insight into the mechanism underlying diabetes-induced bone diseases.

Similar to mammalian bones, fish scales are composed of osteoblasts, osteoclasts and calcified bone matrixes, including type 1 collagen[15], and they use a parathyroid hormone and calcitonin to regulate calcium metabolism in osteoblasts and osteoclasts, and help maintain blood calcium levels[16,17]. In addition, fish scales are small and are present on the body surface, facilitating the extraction of bone components and the analysis of bone metabolism using low molecular compounds. The present study utilized fish scales to analyze the formation of AGEs in type 1 collagen and the effect of glyoxal on the characteristics of type 1 collagen *in vitro*.

MATERIALS AND METHODS

Evaluation of the effects of carbonyl compounds on type 1 collagen

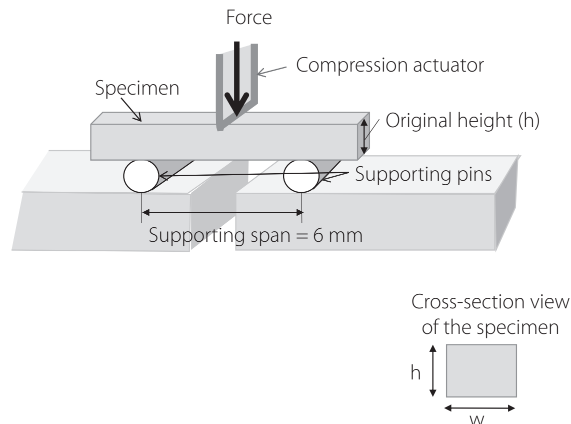
Type 1 collagen was isolated from the regenerating scales of goldfish (*Carassius auratus*), as described previously[18], and incubated with glyoxal (Wako Chemical Industries and Tokyo Chemical Industries, Tokyo, Japan), methylglyoxal (Sigma-Aldrich, St. Louis, MO, USA) or glycolaldehyde (Sigma-Aldrich) at 20°C. The samples were then fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue or analyzed through western blotting (WB) as described below.

Western blotting

WB was carried out as described previously[19]. Proteins were detected using the following primary antibodies: mouse monoclonal CMA antibody (AGE-M04, 1:500 dilution; Cosmo Bio Co., Ltd., Tokyo, Japan) and rabbit polyclonal goldfish type 1 collagen antibody (CL50171AP-G, 1:1,000 dilution; Cedarlane Labs, Burlington, ON, Canada). Secondary fluorescent IRDye conjugated anti-mouse and anti-rabbit antibodies (1:10,000 dilution; LI-COR Biosciences, Lincoln, NE, USA) were also used. Fluorescence images were acquired using an Odyssey Infrared Imager (LI-COR Biosciences).

Determination of the mechanical properties of type 1 collagen

The mechanical properties of type 1 collagen were determined through a three-point bending test using a mechanical tester (CR-500DX-SII; Sun Scientific, Tokyo, Japan; Figure 1). To prepare specimens, type 1 collagen was poured into a plastic molding die and lyophilized. A loading force was applied to the middle of the specimen, which had been placed on the supporting pins, using the loading actuator (Figure 1). The minimum loading force inducing specimen destruction, the force loading area, the height of the specimen before loading the force (original height: 2 mm) and the height of the specimen at the moment of destruction (height at destruction) were measured. These measured values were applied to equations 1 and 2, as presented in Figure 1, to calculate the bending stress (MPa) and deformation rate (%) of the specimen. Data were analyzed by using a one-way ANOVA, as compared with controls. Thereafter, we used the Dunnett's multiple comparison procedure for post-hoc analysis. Significance was set at $P < 0.05$ with IBM SPSS Statistics software (IBM Corporation, Armonk, NY, USA).



Equation 1

$$\text{Bending strength} = \frac{[\text{Maximum loading force at destruction}]}{\text{Compression area}} \ [\text{Mpa}]$$

Equation 2

$$\text{Deformation rate} = \frac{[(\text{Original height}) - (\text{Height at destruction})] \times 100}{\text{Original height}} \ [\%]$$

Figure 1 | Schematic of the three-point bending test. The type 1 collagen specimen was placed on the supporting pins. A loading force was applied to the middle of the specimen using the loading actuator. The minimum loading force inducing specimen destruction, the force loading area, the height of the specimen before loading the force (original height: 2 mm) and the height of the specimen at the moment of destruction (height at destruction) were measured. These measured values were applied to equations 1 and 2 to calculate the bending stress (MPa) and deformation rate (%) of the specimen.

RESULTS

To evaluate the effect of glyoxal on type 1 collagen *in vitro*, type 1 collagen was isolated from the scales of goldfish, incubated with glyoxal for 0, 24, 48 or 72 h, and analyzed by SDS-PAGE. Type 1 collagen molecules comprise α1 and α2 chains; they were detected as bands corresponding to approximately 116 kDa (Figure 2a). The crosslinking in two or three of the α1/α2 chains resulted in the appearance of two types of low-mobility bands: one corresponding to a β chain and the other to a γ chain (Figure 2b). Incubation for 24 h induced band shifts of the α, β and γ chains, and further increased the intensity of the γ chain signal (Figure 2b). Further incubation induced new lower-mobility bands corresponding to δ chains, and decreased the signal intensities of the α and β chains in a time-dependent manner (Figure 2b).

Next, type 1 collagen was incubated with various concentrations of glyoxal for 72 h and analyzed by WB. Signals

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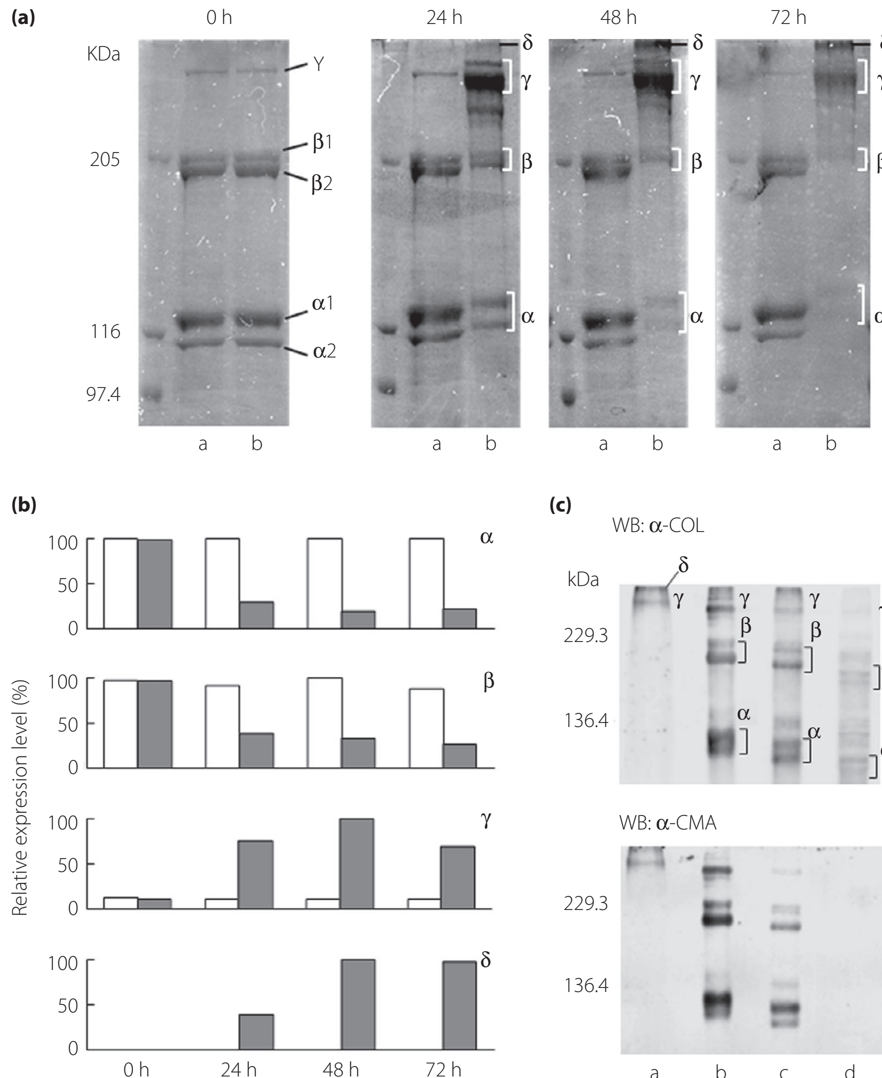

Figure 2 | Evaluation of the effect of glyoxal on the molecular mass of scale-matrix type 1 collagen and the glyoxal-induced carboxymethyl arginine (CMA) formation in type 1 collagen *in vitro*. (a) The isolated type 1 collagen was incubated with distilled water (lane a) or glyoxal (lane b) for the indicated times (0, 24, 48 and 72 h). Samples were then resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. α1, α2, β1, β2, γ and δ denote the α1, α2, β1, β2, γ and δ chain, respectively. As time passed after the addition of glyoxal (880 mmol/L) to the collagen solution, we found a band shift due to incremental changes in the molecular weight of the α and β fractions. (b) The relative intensities of the α1, β or γ chain bands in lane a and those of the α1, β or γ chain bands in lane b were densitometrically determined. Values obtained for the α1, β, γ or δ chain bands in lanes a (white bar) or b (grey bar) were normalized to those of the α1 chain bands in lane a for each given time point, and values at the time points resulting in peak signal levels were set to 100. The presented graph is from a single experiment and is representative of the three trials. (c) The type 1 collagens incubated with 880 mmol/L (lane a), 500 μmol/L (lane b) or 100 μmol/L (lane c) glyoxal or distilled water (lane d) for 72 h were analyzed by western blotting with type 1 collagen (upper panel) and CMA antibodies (lower panel). α, β, γ and δ denote the α, β, γ and δ chain, respectively.

corresponding to the α, β and γ chains were detected in the type 1 collagen that was not incubated with glyoxal (Figure 2c, upper panel). Incubation with 500 μmol/L and 100 μmol/L glyoxal caused a significant shift in the mobility of the α, β and γ chains (lanes b and c). In addition, incubation with a higher concentration of glyoxal induced new lower-mobility bands corresponding to the δ chains, and decreased the signal intensities of the α and β chains (lane a), consistent with the result in Figure 2a,b. Notably, the formation of CMA was detected in α, β, γ and δ chains in the type 1 collagens incubated with glyoxal (Figure 2c, lower panel).

The effects of glyceraldehyde and methylglyoxal on type 1 collagen were evaluated. Both were found to induce new lower-mobility bands corresponding to the β, γ or δ chains in a concentration- or time-dependent manner (Figure 3).

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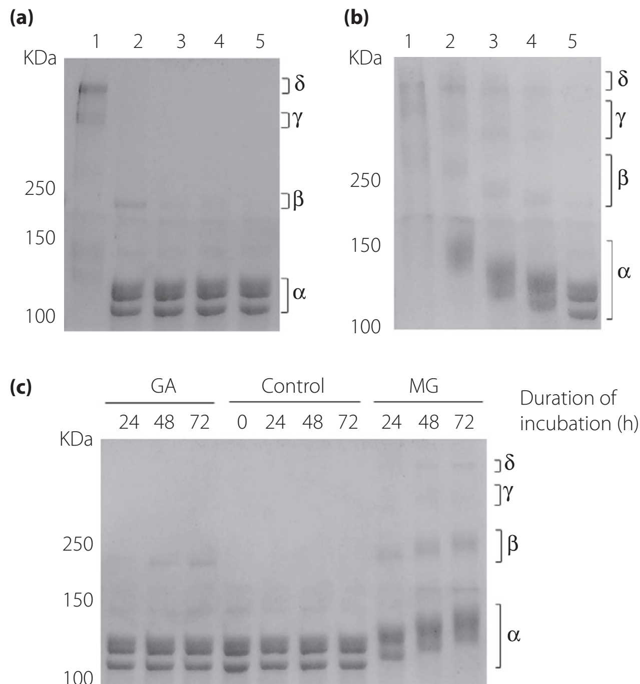

Figure 3 | Evaluation of the effects of carbonyl compounds on type 1 collagen *in vitro*. (a) The isolated type 1 collagen was incubated with 200 mmol/L (lane 1), 500 μmol/L (lane 2), 100 μmol/L (lane 3), or 25 μmol/L (lane 4) glyceraldehyde or distilled water (lane 5). Samples were then resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. (b) The isolated type 1 collagen was incubated with 500 mmol/L (lane 1), 500 μmol/L (lane 2), 100 μmol/L (lane 3) or 25 μmol/L (lane 4) methylglyoxal or distilled water (lane 5). Samples were then resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. (c) The isolated type 1 collagen was incubated with distilled water (Control), 500 μmol/L glyceraldehyde (GA) or 500 μmol/L methylglyoxal (MG) for the indicated times (0, 24, 48 and 72 h). Samples were then resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. α, β, γ and δ denote the α, β, γ and δ chain, respectively.

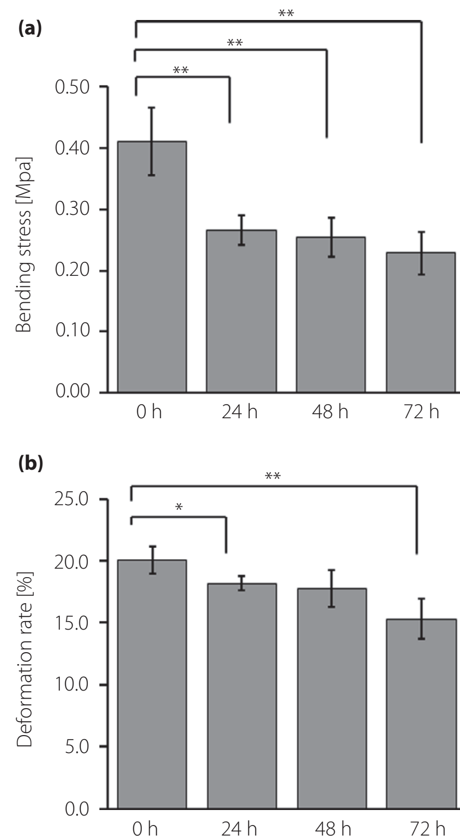

Figure 4 | Effects of non-enzymatic modification of collagen by glycation on mechanical properties using a three-point bending test. Type 1 collagen isolated from goldfish scales was incubated with glyoxal (880 mmol/L) for the indicated times. The (a) bending stress (strength) and (b) deformation rate (flexibility) were determined as described in the Methods section. Data were analyzed using a one-way ANOVA, as compared with controls. Thereafter, we used Dunnett's multiple comparison procedure for post-hoc analysis. Significance was set at $P < 0.05$ with IBM SPSS Statistics software. Error bars in each graph represent the mean ± standard deviation of 10 independent experiments (*$P < 0.05$ and **$P < 0.01$).

We carried out a three-point bending test to determine the bending stress and deformation rate of type 1 collagen, which are indicators of its strength and flexibility, respectively. For this, type 1 collagen was isolated from goldfish scales and incubated with glyoxal for 0, 24, 48 or 72 h. As shown in Figure 4, incubation with glyoxal for ≥24 h significantly decreased the bending stress (strength) and deformation rate (flexibility) of type 1 collagen.

DISCUSSION

Our previous study evaluated the bone metabolism for high-glucose concentration in goldfish scales collected from fish with alloxan-induced hyperglycemia[20]. The enzymatic activity and messenger ribonucleic acid expression of osteoblast and osteoclast markers were not significantly different between hyperglycemic and euglycemic goldfish scales. Accordingly, we proposed that hyperglycemia affects bone components other than bone cells, such as collagen and the bone matrix, leading to the deterioration of bone quality in a goldfish scale bone model. In support of this notion, the current study found that glyoxal-induced AGE formation causes decreased flexibility and strength in type 1 collagen. It should be stressed that hyperglycemia-induced glycation forms disorganized structures of collagen fibrils, which have a negative effect on the structural dynamics and stabilization of the diabetic bone matrix[21,22]. In addition, it was reported that increased pentosidine in bone collagen was inversely correlated with the bone formation rate, bone volume ratio and mineral apposition rate in dialysis patients[23]. Taken together, it is conceivable that different AGEs have distinct effects on the characteristics of bone components and induce bone defects.

Our recent study using goldfish scales in outer space uncovered the molecular mechanism underlying microgravity-induced

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osteoporosis-like loss of bone mass in astronauts[24–26]. Furthermore, the current study established *in vitro* systems for analyzing the formation of AGEs in type 1 collagen and the effects of carbonyl compounds on the characteristics of collagen, thereby significantly increasing the value of fish scales for analyzing bone diseases and their association with other pathologies, such as hyperglycemic disease.

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DISCLOSURE

The authors declare no conflict of interest.

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