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Is there a correlation between age and D-aspartic acid in human knee cartilage?

G. Fisher, S. Lopez, K. Peterson, T. Goff, I. Philip, R. Gaviria, N. Lorenzo, and **M. Tsesarskaia**

Department of Chemistry, Barry University, Miami Shores, Florida, U.S.A.

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Summary. L-Aspartic acid (L-Asp) is one of the fastest racemizing amino acids such that the abnormal D-form (D-Asp) has been found in stable biological human tissues such as dentin in teeth, eye lens and brain. Earlier reports showed that there was a linear correlation between age and D-Asp in teeth. We have previously reported that significant levels of D-Asp were found in normal and osteoarthritic knee cartilage. Since cartilage is a slow regenerating tissue, we hypothesized that D-Asp should accumulate in knee cartilage and that there might be a correlation between the age of the person and the amount of D-Asp found in cartilage. Our analysis of approximately 100 samples of normal knee cartilage showed that there are detectable amounts of D-Asp (2–4% of total Asp) in knee cartilage. However, there was only a slight correlation ($r = 0.35$) between the age of the person and the amount of D-Asp (nmoles/g). Surprisingly, there was a better correlation between age and the amount of D-Asp in the male subjects ($r = 0.57$) than in the female subjects ($r = 0.21$).

Keywords: D-Aspartic acid – D-Asp – Knee cartilage

Introduction

Cartilage is a specialized connective tissue rich in extra cellular matrix. 90% of the dry weight of tissue is composed of articular cartilage that is extremely smooth and has very low friction able to lubricate weight bearing joints, allowing them to glide smoothly when flexed. Cartilage is composed principally of collagen and proteoglycans. The proteoglycan proteins are relatively stable with a slow turnover rate (Maroudas et al., 1998; Bank et al., 1998). As an individual ages, the articular cartilage wears away, causing joints to rub against each other with pain from the friction. The potential for growth and repair in cartilage decreases with age.

Aspartic acid is one of the fastest racemizing amino acids such that significant levels of D-Asp have been found in animal and human tissues (D'Aniello et al., 1998; Fujii, 2002, 2005; Furuchi and Homma, 2005). A linear correlation has been found between the age of subjects and the increased levels of D-Asp in human tooth enamel and dentine (Helfman and Bada, 1975). Other researchers found increased levels of D-Asp with age in human eye lens (Masters et al., 1977) and brain (Man et al., 1983, 1987). We have also found D-Asp in osteoarthritic knee cartilage (unpublished results). Therefore, based on the above observations, we hypothesized that D-Asp might accumulate with age in cartilage obtained from knee joints.

Materials and methods

Chemicals

D,L-Asp, *o*-phthaldialdehyde (OPA, minimum 99% pure) and *N*-acetyl-L-cysteine (NAC) were from Sigma Chemicals (St. Louis, MO, USA). All solvents used were HPLC grade. Water was distilled or deionized through a water purification system such as Millipore® or NANOpure®.

Tissue sample preparation

Normal knee cartilage samples were obtained from the United States Cooperative Human Tissue Network (Philadelphia, PA and Columbus, OH). Cartilage from each knee sample was finely minced in two ~50 mg portions of tissue and placed in small glass test tubes. Approximately 0.5 ml of doubly distilled 6 M HCl was added to each sample. The tubes were sealed under vacuum, and the samples were hydrolyzed for 6 h at 100 °C. The hydrolysates were dried in a desiccator under vacuum and redissolved in 0.1 M HCl. The hydrolysates were purified by passage through 100 mg C-18 extraction columns (Supelco), previously activated with methanol and 0.1 M HCl. After the hydrolysate was adsorbed onto the C-18, the amino acids were eluted from the column with 0.1 M HCl and then freeze-dried.

Derivatization for HPLC determination of D- and L-Asp

D- and L-enantiomers of Asp react with *o*-phthaldialdehyde (OPA) and *N*-acetyl-L-cysteine (NAC) in sodium borate buffer, pH 9.4, to form a pair

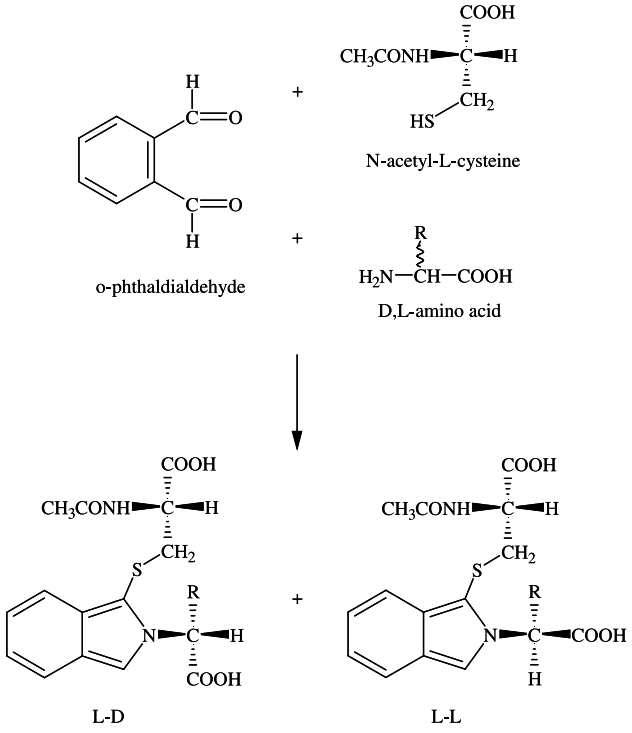


Fig. 1. Derivatization of D,L-Asp with OPA–NAC to form a pair of fluorescent diastereomeric N-alkyl-2-thioalkyl isoindole derivatives

of fluorescent diastereomeric *N*-alkyl-2-thioalkyl isoindole derivatives (Fig. 1) (Aswad, 1984; Galindo et al., 2006; Fisher et al., 2006). Each freeze-dried hydrolyzed cartilage sample was redissolved in 100 µl of 0.1 M sodium borate buffer, pH 9.3–9.4. 10 µl of this solution was derivatized

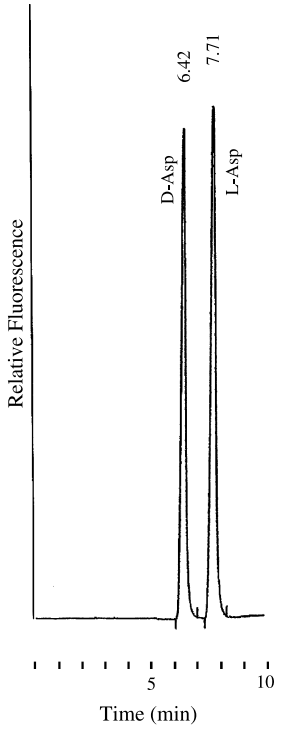


Fig. 2. HPLC chromatogram of separation of D- and L-Asp diastereomers

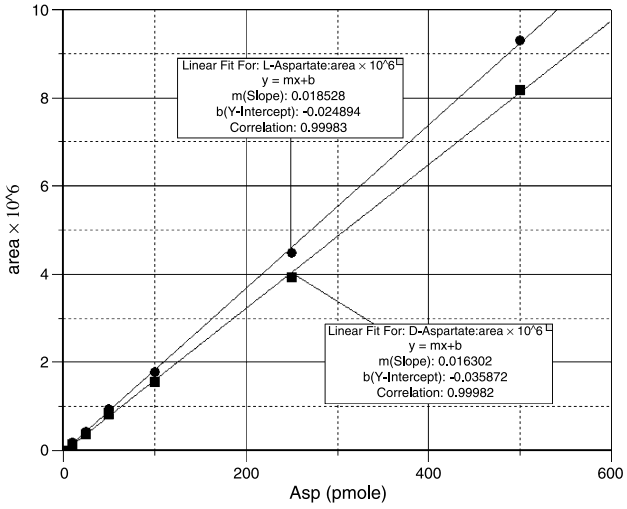


Fig. 3. Fluorescence responses of D- and L-Asp. Linear correlation calibration of peak areas with picomoles of L-Asp (●) and D-Asp (■)

with 10 µl of OPA–NAC reagent (prepared by dissolving 4 mg OPA and 5 mg NAC in 0.5 ml of methanol) and brought to a final volume of 200 µl with borate buffer. After mixing for 1 min, 10 µl of the derivatized sample was injected into a 25 × 0.46 cm Hypersil ODS-C_{18} (5µ) HPLC column. The D- and L-Asp diastereomers were separated (Fig. 2) using the following mobile phases:

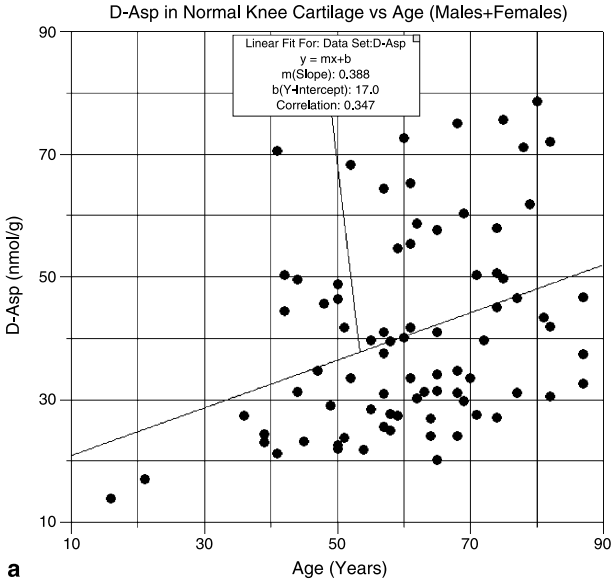
A: 30 mM sodium citrate, adjusted to pH 5.5–6.0 with solid citric acid, to which 111 ml of methanol are then added (final concentration 10% methanol)
B: 100% methanol (MeOH)
C: 100% acetonitrile (AcCN)

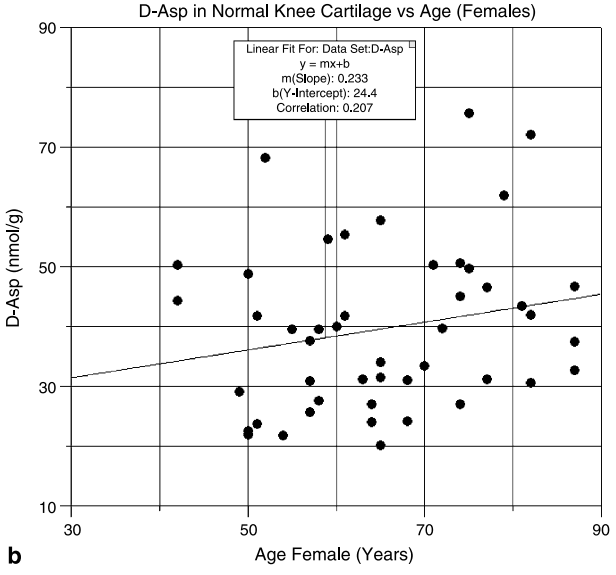
and the following gradient: 0–6 min, 100% A; 6–10 min linear gradient to 25% A, 40% B, and 35% C (to wash the column); returning to 100% A and equilibrating at 100% A over the next 5–8 min. All flow rates were 1.0 ml/min. The D- and L-Asp diastereomers were detected fluorometrically at 325 nm excitation and 415 nm emission wavelengths.

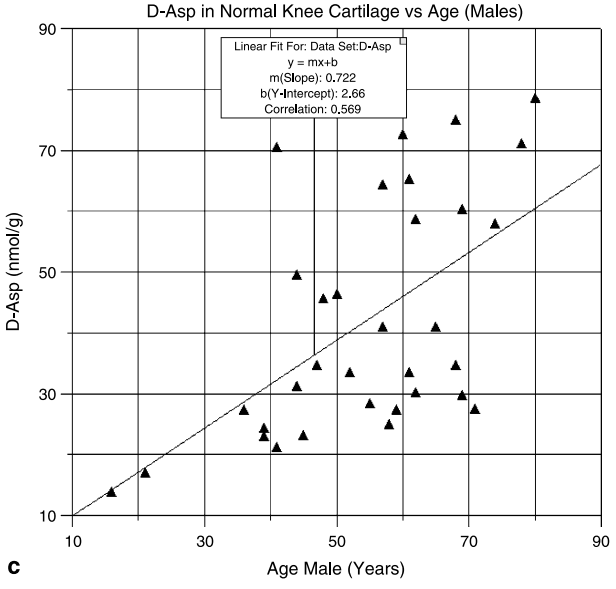
In order to quantify the amount of D- and L-Asp in the cartilage samples, a standard of 150 pmoles/µl of racemic D,L-Asp was prepared by dissolving 2 mg of D,L-Asp in water in a 100 ml volumetric flask. Appropriate amounts of the standard were derivatized with OPA–NAC and run through the HPLC. A plot of peak area vs. nmoles of Asp was made (Fig. 3), and the areas under the peaks were used to quantify the picomoles of D- and L-Asp of the standards, which in turn were used to quantify the picomoles of D- and L-Asp in cartilage samples. The picomoles of Asp in the cartilage samples were multiplied by 0.995 to account for 5% hydrolysis-induced racemization.

Results and conclusions

The D-Asp content (nmol/g) was analyzed in 71 samples of knee cartilage from females (ages 42–87) and 33 samples from males (ages 16–80). The results are shown in the plots of Fig. 4a. The D-Asp contents ranged from a low of about 16 nmol/g to a high of about 74 nmol/g in both sexes. The scatter of D-Asp content in both sexes is evident from the plots (Fig. 4a–c). The % D-Asp compared to total Asp [D/(D + L)×100] ranged from about 2 to 4%, with an average of 2.8%.







The analyses show that detectable amounts of D-Asp are present in knee cartilage. However, the results indicate very little correlation ($r = 0.35$) between the levels of D-Asp in knee cartilage and age of all subjects. Surprisingly, there was a much better correlation with age in the samples from males ($r = 0.57$) than from females ($r = 0.21$). This difference between the sexes might be a result of the fact that the age spread for the samples from males (16–80) was much wider than the age spread of the samples from females (42–87). If we had been able to analyze more samples from younger females, then there might have been a better correlation of D-Asp with age.

Accumulation of D-Asp in human knee cartilage might be a consequence of the normal aging process, that is, age-induced racemization of L-Asp to D-Asp. We had originally thought that degeneration of knee cartilage in the form of osteoarthritis might correlate with the accumulation of D-Asp. However, in related analyses of approximately 100 samples of cartilage from osteoarthritic knee joint replacements, we found no significant differences in the amount of D-Asp between the normal cartilage and "diseased" cartilage. This is not surprising, since in osteoarthritis most of the diseased cartilage has been lost due to wearing away and, thus, the remaining cartilage that was analyzed was most likely "normal" cartilage.

Acknowledgements

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Fig. 4. **a** D-Asp vs. age in knee cartilage of males plus females. **b** D-Asp vs. age in knee cartilage of females (●). **c** D-Asp vs. age in knee cartilage of males (▲)

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Authors' address: Dr. G. H. Fisher, Department of Chemistry, Barry University, 11300 NE 2nd Ave., Miami Shores, FL 33161, U.S.A.,
Fax: +1-305 899-4379, E-mail: gfisher@mail.barry.edu