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Gender Differences in D-Aspartic Acid Content in Skull Bone

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ABSTRACT

In forensic medicine, the personal identification of cadavers is one of the most important tasks. One method of estimating age at death relies on the high correlation between racemization rates in teeth and actual age, and this method has been applied successfully in forensic odontology for several years. In this study, we attempt to facilitate the analysis of racemized amino acids and examine the determination of age at death on the basis of the extent of aspartic acid (Asp) racemization in skull bones.

The specimens were obtained from 61 human skull bones (19 females and 42 males) that underwent judicial autopsy from October 2010 to May 2012. The amount of D-Asp and L-Asp, total protein, osteocalcin, and collagen I in the skull bones was measured. Logistic regression analysis was performed for age, sex, and each measured protein.

The amount of D-Asp in the female skull bones was significantly different from that in the male skull bones ($p = 0.021$), whereas the amount of L-Asp was similar. Thus, our study indicates that the amount of D-Asp in skull bones is different between the sexes.

Key words: *Aspartic acid, Racemization, Skull bone*

In forensic medicine, the definitive and rapid personal identification of unidentified cadavers and human remains is an important task. Anthropological findings, fingerprints, odontological findings, and DNA analysis are well known as methods of personal identification. Anthropological findings lack accuracy because there are many individual variations and racial differences. Fingerprints, odontological findings, and DNA analysis have been used for accurate personal identification in judicial autopsies[15]. However, fingerprint analysis and odontological examinations require antemortem fingerprints and dental records. DNA analysis is not useful without antemortem specimens and the cooperation of close relatives. Therefore, these methods are not appropriate for personal identification of unidentified skeletal cadavers. At such a time, age estimation becomes helpful for personal identification. Helfman et al have proposed a method for the estimation of age based on the high correlation between racemization rates of amino acids in teeth and the actual age[13].

Racemization of amino acids is a natural process that eventually converts optically active compounds into a racemic mixture[13,31] (Fig.1). The L-amino acids commonly found in living systems are derived from the stereochemical specificity of enzymes that utilize only L-enantiomers[13,31]. Racemization of amino acids in the proteins produced by living organisms thus takes place only after protein turnover has ceased[13,31]. The extent of amino acid racemization may be used to estimate the age of various fossil materials, including deep-sea sediments[5], shells[30], and fossil bones[6]. In addition, humans mainly use L-amino

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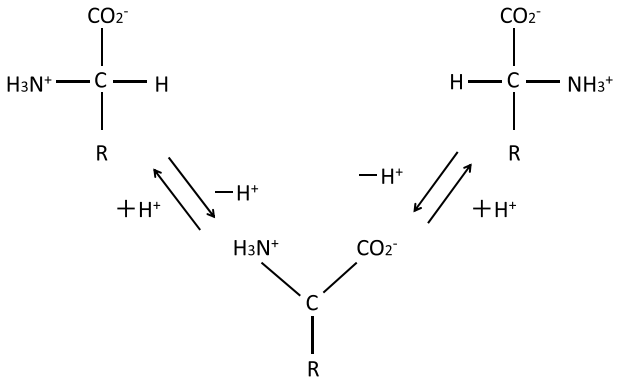

Fig. 1. Mechanism of racemization of amino acids
Although both left- and right-handed enantiomers (L-amino acids and D-amino acids, respectively) are available in nature, life is biased toward the use of L-amino acids and D-amino acids. C: a-asymmetric carbon, R: substitutional radicals.

acids for protein synthesis[25]. In permanent proteins that are synthesized early in life and not subsequently exchanged, D-amino acids can accumulate during aging[26]. In teeth, aging is closely related to the extent of aspartic acid (Asp) racemization, and this relationship provides the basis for a highly accurate method for determining the age at death that has been applied successfully in forensic odontology for several years[12]. Among various amino-acid racemization reactions, the reaction involving Asp has received the most attention because it has one of the fastest racemization rates among the stable amino acids[3].

However, few institutes have applied this method to estimate the age at death because several age-known teeth are necessary for the calibration of age estimation, and the method is difficult to apply. Moreover, it is very time-consuming. Thus, we have attempted to facilitate analysis through modification of a commercially available kit that does not require any special technique. In addition, the preparation of bones is actually much easier than the preparation of teeth. The accuracy of age determination at death based on the racemization rates of amino acids in bones has been reported to be lower than that in teeth[28]. Teeth and bones are both hard tissues; however, the relationship between actual age at death and racemization rates of amino acids are different in these tissues. Differences in the protein content and protein metabolism of these tissues may be considered reasons for this discrepancy.

The primary proteins in teeth enamel are non-collagenous, but 90% of the organic matrix in dentin is composed of collagen and phosphoprotein, which accounts for 20% of the Asp content in human dentin[10,24]. In contrast, the bone matrix mainly consists of collagen I and non-collagenous proteins, and these represent approximately 90% of the organic composition of the entire bone tissue[10,24]. The major non-collagenous protein produced in bones is osteocalcin, which plays a role in the regulation of bone formation[10,24]. The proteins in bones mainly consist of collagen I and osteocalcin.

Teeth have a lower protein metabolism because they undergo formation only once[24]. In contrast, bones are metabolized actively and undergo continuous remodeling throughout life[24]. Bone remodeling is a lifelong process wherein the mature bone tissues are removed from the skeleton and new bone tissues are formed[10,29]. Such processes also control the reshaping or replacement of bones following injuries such as fractures and micro damage, which occur during normal activity[10]. Remodeling also occurs in response to the functional demands of mechanical loading[10] and relies on complex signaling pathways to achieve proper rates of growth and differentiation[10]. In adults, remodeling proceeds at a rate of approximately 10% per year and includes the action of several hormones, including growth hormone and steroids such as estrogen[10]. Estrogen deficiency after menopause accelerates the age-related loss of bone[14]. The observed increase in the fragility of bone with age is predominantly attributable to loss of bone density, but changes in bone structure may also influence skeletal strength[14]. The transition from reproductive to non-reproductive status results from a reduction in female hormonal production by the ovaries. The typical age range for menopause is between the ages of 40 and 61 years, and the average age for the last menstrual cycle is 51 years[17].

In this study, we attempt to facilitate the analysis of racemized amino acids. We also examine the difference in the accuracy of age estimation between teeth and bones by analyzing the amount of total protein, osteocalcin, and collagen I in the skull bone between female and male groups as well as pre-menopausal and post-menopausal groups.

MATERIALS

D-Asp, L-Asp, and hydrochloric acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-Asp-d_3 was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). EZ: faast™ was purchased from Phenomenex (Torrance, CA). The Coomassie Plus (Bradford) Assay kit was purchased from Pierce (Rockford, IL), the Gla-type osteocalcin enzyme-linked immunoassay (EIA) kit was purchased from Takara Bio Inc. (Otsu, Japan), the centrifugal filter devices (Amicon® Ultra-4 30K) were purchased from Millipore (Tokyo, Japan), and the human collagen type I enzyme-linked immunosorbent assay (ELISA) kit was purchased from

Cosmo Bio Co. Ltd. (Tokyo, Japan). All other reagents and solvents purchased were of the highest quality and manufactured by Wako Pure Chemical Ind. (Osaka, Japan). All tested drugs were dissolved in hydrochloric acid (0.1 M) to obtain stock standard solutions (1.0 mg/ml) and stored at 4°C.

SUBJECTS

Skull bone specimens were obtained from 61 personally identified human skull bones (19 females, aged 0–82 years; 42 males, aged 0–81 years) that underwent judicial autopsy from October 2010 to May 2012 at Hiroshima University. There were various causes of death. Burned cadavers were excluded because racemization has been reported to be accelerated by exposure to high temperatures[13]. We measured the amount of D-Asp and L-Asp, total protein, osteocalcin, and collagen I in this study, which was performed with the approval of the Ethics Committee of Hiroshima University in accordance with the Declaration of Helsinki.

METHODS

Preparation of specimens

Specimens were collected from the skull bones after opening up the skull using a saw. Blood, soft tissues, and other contaminants were removed, and the specimens were air-dried and pulverized.

Specimen treatment for Asp assay

The powdered specimen (10 mg) was put into a glass vial containing hydrochloric acid (500 µl, 6 M) and hydrolyzed at 100°C for 6 hr. The purification and derivatization of Asp in the hydrolyzed specimen solution was performed using the amino acid clean-up kit EZ: faast™. To completely separate L-Asp and D-Asp by gas chromatography (GC), the derivatizing reagent in the kit was changed from propyl alcohol to ethanol in reagent 3 and from propyl chloroformate to ethyl chloroformate in reagent 4.

The following extraction and derivatization of Asp were slightly modified from the general instructions of the kit. The hydrolyzed solution (25 µl) and reagent 2 (50 µl) were mixed to adjust the pH of the mixture. The mixture (25 µl), reagent 1 (100 µl), and an internal standard (deuterated Asp, L-Asp-d_3, 5 µl, 6.2×10^2 µmol/liter) were put into a glass vial. The resulting solution was extracted in the sorbent of the tip, and the remaining liquid was discarded. Distilled water (200 µl) was then used to wash the sorbent. The remaining liquid in the tip was removed by passing air through it. Subsequently, elution medium (modified reagent 3, 200 µl) was added, and the sorbent was soaked in elution medium until the liquid reached the filter plug in the sorbent tip. Liquid and sorbent particles were ejected out of the tip. The extract was derivatized by adding modified reagent 4 (50 µl), and the liquid was emulsified in the vial using a vortex mixer for 10 s. At this stage, reagent 5 (100 µl) was added to the vial and mixed for a few seconds. After incubation for 1 min, the organic layer was transferred to a GC vial and used for analysis by GC.

GC-MS conditions

A 6890 GC-5973 mass-selective detector (MSD) (Agilent Technologies, Palo Alto, CA) equipped with an Rt-γDEXsa fused silica capillary column (30 m × 0.25 mm I.D., 0.25-µm film thickness, Restek, Bellefonte, PA) was used in this study. The oven temperature was set to 170°C. The temperatures of the injection port and interface were set to 250°C and 230°C, respectively, and the splitless injection mode was used. Helium was used as the carrier gas at the inlet at a flow rate of 1.0 ml/min. The MSD was operated in electron impact ionization mode at 70 eV. To identify each drug, data were obtained in the selected ion-monitoring mode with m/z 188 for L- and D-Asp, m/z 191 for L-Asp-d_3.

Evaluation of D- Asp and L-Asp

The racemization of Asp can be described as {ln[(1+D/L)/(1-D/L)]=2k(Asp.) t + constant}, where D-Asp/L-Asp represents the proportion of D-Asp to L-Asp, k(Asp.) is the first-order rate constant of the interconversion of enantiomers, and t is time[4,5,27].

The value {ln[(1+D/L)/(1-D/L)]} was calculated for each specimen. The quantity of D-Asp and L-Asp was calculated from the areas of the peaks.

Protein assay

Each powdered specimen was extracted with 20% formic acid at 4°C overnight[25], and the extracted protein was quantified using the Coomassie Plus (Bradford) Assay kit according to the manufacturer's instructions.

For the assay, specimens were diluted 5-fold. First, 50 µl of each specimen was pipetted into appropriate microplate wells. Second, 50 µl of the Coomassie Plus reagent was added to each well and mixed in a plate shaker for 30 sec, after which the plate was removed from the shaker and incubated for 10 min at room temperature (RT) to obtain the most consistent results. Third, the absorbance at 595 nm was measured using a microplate reader (Labsystem Japan, Tokyo, Japan). Bovine serum albumin was chosen as the standard protein for comparison of the test data, and the data are presented as means of triplicate determinations.

Osteocalcin assay

Each powdered specimen was extracted using 20% formic acid at 4°C overnight[25] and then diluted 3000 times in phosphate-buffered saline (PBS). The extracted osteocalcin was quantified using a Gla-type Osteocalcin EIA kit according to the manufacturer's instructions. In brief, 100 µl of the test specimen and a bovine Gla-osteocalcin standard solution were added to a microtiter plate pre-coated with a capture antibody (mouse monoclonal anti-Gla-osteocalcin) and incubated for 2 hr at RT. Following the washing stage, the detection antibody (mouse monoclonal anti-Gla-osteocalcin) conjugated to horseradish peroxidase (HRP) was applied to each well and incubated for 1 hr at RT. After washing, 100 µl of a substrate solution containing 3,3′,5,5′-tetramethylbenzine was added to each well. The plates were incubated for 15 min at RT, followed by the application of 100 µl of stop solution. After the reaction was stopped using 100 µl of 1N H_2SO_4, the absorbance at 450 nm was measured using a microplate reader (Labsystem Japan, Tokyo, Japan).

Collagen I assay

Each powdered specimen was extracted using 20% formic acid at 4°C overnight[25] and filtered using the centrifugal filter device for the efficient extraction of collagen I and removal of formic acid. The extracted collagen I was then quantified using a human collagen type I ELISA kit according to the manufacturer's instructions.

In brief, 100 µl of the test specimens and 400 µl of PBS were added to the Amicon Ultra-4 30K filter device. The mixture was then centrifuged at 7500 × *g* for approximately 15 min at 4°C. After centrifugation, 600 µl of PBS was added, and the pellet was re-suspended to remove the formic acid. This procedure was repeated 3 times. Finally, 500 µl of PBS was added, and the concentrated solution was recovered by pipetting.

A human collagen type I ELISA kit was then used to determine collagen I amounts in the specimens according to the manufacturer's instructions. Test specimens or collagen standard solution (9:1 with biotinylated anti-collagen antibody solution) was mixed. Then, 50 µl of the mixed solution was added to a collagen-coated microtiter plate and incubated for 1 hr at RT. Following the washing stage, an avidin-HRP conjugate solution was applied to each well and incubated for 1 hr at RT. After washing, 50 µl of a substrate solution containing 3,3′,5,5′-tetramethylbenzine was added to each well. The plates were incubated for 15 min at RT, followed by application of 50 µl of a stop solution. After the reaction was stopped by the addition of 50 µl of 1N H_2SO_4, the absorbance at 450 nm was measured using a microplate reader (Labsystem Japan, Tokyo, Japan).

Data analysis

All data are expressed as the mean ± standard deviation (SD). The amount of D-Asp, L-Asp, total protein, osteocalcin, and collagen I are expressed per milligram of bone. The data were compared between females and males and between pre-menopausal and post-menopausal individuals, that is, those who were below the age of 49 years and over the age of 50 years, by using a two-tailed unpaired t-test. The relationship between age and D-Asp/L-Asp proportion was evaluated using a partial correlation analysis. $p < 0.05$ was defined as statistically significant.

RESULTS

Hydrolysis of the skull bone

The reason for determining the hydrolysis condition of the teeth and skull bone is unknown, although the hydrolysis condition is reported in several articles. When protein is hydrolyzed in solution, the protein is usually dissolved in a concentrated hydrochloride solution and heated at 100°C for several hours. During the hydrolysis of protein, one concern is that the optical activity will change in the solution. Because bone has a D-Asp content of several percent, a change in the optical activity will have a large influence on the study outcome. To confirm the change in the optical activity, Asp was individually dissolved in 6 M HCl and heated at 100°C for 0.5 to 24 hr, and the change in the optical activity was monitored by GC. There was no change until 6 hr. However, D-Asp was observed when L-Asp was heated for 9 hr. The amount of D-Asp increased as the heating time increased. Therefore, a hydrolysis time of 6 hr was adopted in the subsequent experiments.

Chromatographic separation of D-Asp/L-Asp

Several chiral derivative reagents have been used for the enantiomeric separation of amino acids[7,16]. Enantiomeric excess of the derivative reagent is important for measuring the enantiomeric purity of amino acids in the specimen because 4 types of diastereomers are present; therefore, the purity can be incorrectly estimated when using a reagent with low enantiomeric purity. In this study, an achiral derivative reagent was used, and a cyclodextrin column (Rt γ-DEXsa) was used for the enantiomeric separation of D-Asp/L-Asp to prevent incorrect identification of the peaks.

In a preliminary trial, the *N*,*O*-propyloxycarbonyl (POC) propyl esters of D-Asp and L-Asp were analyzed by GC-MS as the EZ:faast kit was employed for derivatization of the amino acids with propyl chloroformate. However, the POC derivatives of D-Asp/L-Asp were not detected in the eluate because the adsorption of the derivatives and volatility were high. To improve

the volatility of the derivative, Asp was derivatized with *N*,*O*-methoxycarbonyl methyl ester or *N*,*O*-ethoxycarbonyl ethyl ester by switching the derivatization reagent in reagent 4 from propyl chloroformate to methyl chloroformate or ethyl chloroformate. The *N*,*O*-ethoxycarbonyl ester of Asp was detected by using the cyclodextrin column, although the *N*,*O*-methoxycarbonyl ester of Asp was not detected. Thus, the derivatization reagents were optimized to analyze the enantiomer of D-Asp/L-Asp by using the column. By using the achiral derivative reagent ethyl chloroformate and the cyclodextrin column (Rt γ-DEXsa), the peaks of D-Asp and L-Asp were completely separated without the influence of endogenous substances (Fig. 2).

Validation of the D-Asp/L-Asp assay

Asp-spiked specimens were prepared at 6 different concentrations in the range of 1–100 ng/ml and analyzed using the above procedure. Calibration curves were obtained by plotting the peak area ratio of D-Asp/L-Asp/L-Asp-d_3 against the respective Asp concentrations. A linear curve was observed for Asp concentrations up to 100 ng/ml. The correlation coefficients of the calibration curves were greater than 0.98. The lowest limit of quantification in the specimen was 1 ng/ml. The signal-to-noise ratio (S/N) was greater than 10 and the relative standard deviation (RSD) was less than ±15% (within 20% of the target). The detection limits in the specimen were 0.5 ng/ml (S/N = 3). The recovery rate for Asp in the specimen was 68–80%, and the RSDs of the intra- and interday reproducibility for the specimens containing 30, 45, and 90 ng/ml Asp were 2.7–4.6%. There was no possibility of specimen contamination because the extraction tip was disposable.

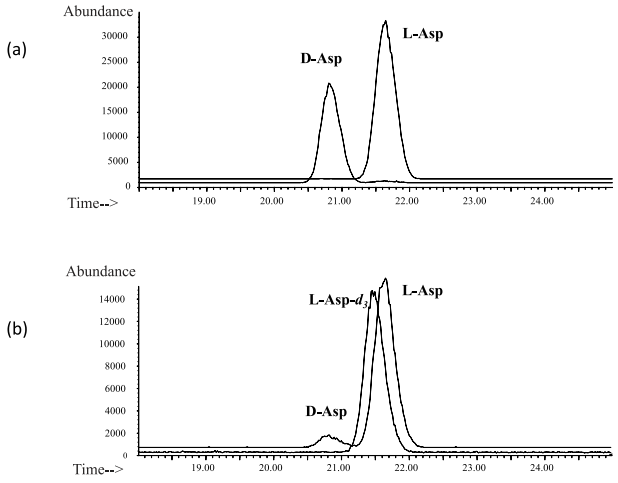

Fig. 2. Gas chromatogram of aspartic acid (Asp) in skull bone
(a) The peaks of D-Asp and L-Asp were completely separated without the influence of endogenous substances. (b) The peaks of D-Asp, L-Asp, and L-Asp-*d3* were measured.

Comparison of D-Asp amounts between females and males, and between individuals below the age of 49 years and above the age of 50 years

Figure 3 shows a comparison of D-Asp amounts between females and males, and between individuals below the age of 49 years and above the age of 50 years. The amounts of D-Asp (mean ± SD) for females and males were 3.18 ± 1.29 μg/mg bone and 4.38 ± 1.49 μg/mg bone, respectively. The amounts of D-Asp (mean ± SD) for females below the age of 49 years and above the age of 50 years were 3.93 ± 1.34 μg/mg bone and 2.29 ± 0.26 μg/mg bone, respectively. The amounts of D-Asp (mean ± SD) for males below the age of 49 years and above the age of 50 years were 3.85 ± 1.07 μg/mg bone and 5.03 ± 1.70 μg/mg bone, respectively.

The amount of D-Asp was significantly different between females and males for individuals over the age of 50 years ($p = 0.002$). In addition, the amount of D-Asp was significantly different between females and males ($p = 0.021$), and between individuals below the age of 49 years and above the age of 50 years among females ($p = 0.025$) and males ($p = 0.016$). In addition, D-Asp was detected in the body of infants.

Comparison of L-Asp amounts between females and males and between individuals below the age of 49 years and above the age of 50 years

Figure 4 shows a comparison of L-Asp amounts between females and males and between individuals below the age of 49 years and above the age of 50

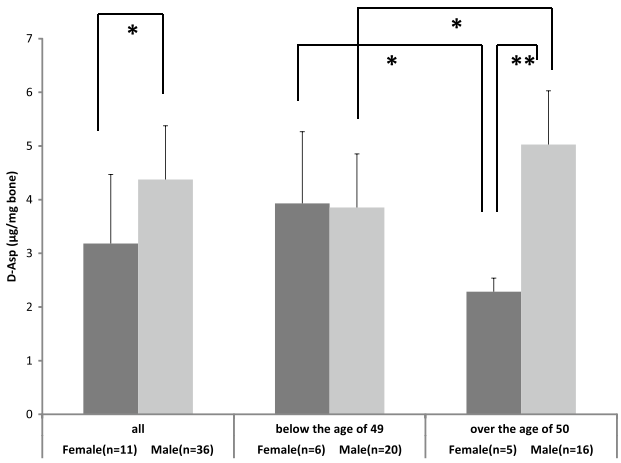

Fig. 3. Amount of D-aspartic acid (Asp) in skull bone
Values are presented as mean ± SD. Significant differences are indicated as *$p < 0.05$, **$p < 0.01$.
The amount of D-Asp (mean ± SD) for females and males was 3.18 ± 1.29 μg/mg bone and 4.38 ± 1.49 μg/mg bone, respectively. The amount of D-Asp (mean ± SD) for females below the age of 49 years and over the age of 50 years was 3.93 ± 1.34 μg/mg bone and 2.29 ± 0.26 μg/mg bone, respectively. The amount of D-Asp (mean ± SD) for males below the age of 49 years and over the age of 50 years was 3.85 ±1.07 μg/mg bone and 5.03 ± 1.70 μg/mg bone, respectively.

years. The amounts of L-Asp (mean ± SD) for females and males were 86.36 ± 14.74 μg/mg bone and 85.64 ± 22.34 μg/mg bone, respectively. The amount of L-Asp (mean ± SD) in females below the age of 49 years and above the age of 50 years was 88.12 ± 12.37 μg/mg bone and 84.23 ± 18.49 μg/mg bone, respectively. The amount of L-Asp (mean ± SD) for males below the age of 49 years and above the age of 50 years was 87.19 ± 18.49 μg/mg bone and 83.69 ± 24.61 μg/mg bone, respectively.

The amount of L-Asp was similar in each group.

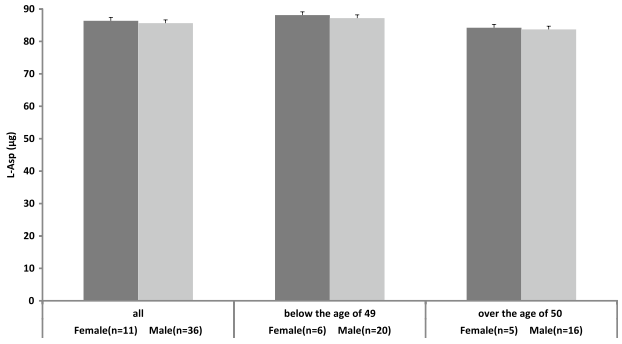

Fig. 4. Amount of L-aspartic acid (Asp) in skull bone
Values are presented as mean ± SD. Significant differences are indicated as *p < 0.05, **p < 0.01.
The amount of L-Asp (mean ± SD) for females and males was 86.36 ± 14.74 μg/mg bone and 85.64 ± 22.34 μg/mg bone, respectively. The amount of L-Asp (mean ± SD) in females below the age of 49 years and above the age of 50 years was 88.12 ± 12.37 μg/mg bone and 84.23 ± 18.49 μg/mg bone, respectively. The amount of L-Asp (mean ± SD) for males below the age of 49 years and above the age of 50 years was 87.19 ± 18.49 μg/mg bone and 83.69 ± 24.61 μg/mg bone, respectively.

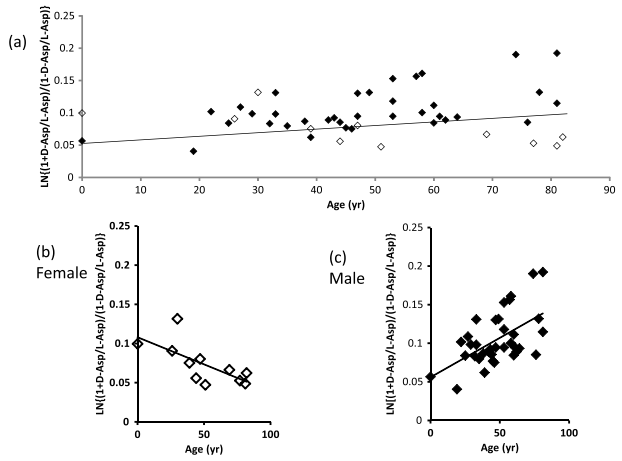

Fig. 5. Extent of aspartic acid racemization in skull bone in relation to age at death
The relationship was evaluated using a linear regression analysis.
(a) all specimens (n = 47, r = 0.22); (b) specimens from females (n = 11, r = –0.70); (c) specimens from males (n = 36, r = 0.56)

Relationship between the extent of Asp racemization in skull bone and age at death

Figure 5 presents the extent of Asp racemization in skull bone in relation to age at death. The y-axis represents the value of {ln[(1+D/L)/(1-D/L)]}, and the x-axis indicates the age at death. The r-value in the linear regression analysis was 0.22 (Fig. 5a). The r value in the linear regression analysis for females and males was –0.70 and 0.56, respectively (Fig. 5b, c). The {ln[(1+D/L)/(1-D/L)]} values in tooth extracts from females and males increased with aging, and the r^2 value in the linear regression analysis was 0.98, as shown in the graph[1]. The relationship between age and the value of {ln[(1+D/L)/(1-D/L)]} in crude acidic extracts from skull bones was much weaker than that reported for tooth extracts[26,28]. Age estimation using this result was difficult because of the high variability in the presumed age.

Comparison of protein amounts between females and males, and between individuals below the age of 49 years and above the age of 50 years

Figure 6 shows a comparison of the protein amounts between females and males and between individuals below the age of 49 years and above the age of 50 years. The amount of protein (mean ± SD) for females and males was 2.91 ± 0.90 μg/mg bone and 3.25 ± 0.93 μg/mg bone, respectively. The amount of protein (mean ± SD) for females below the age of 49 years and above the age of 50 years was 2.83 ± 0.42 μg/mg bone and 2.95 ± 1.08 μg/mg bone, respectively. The amount of protein (mean ± SD) for males below the age of 49 years

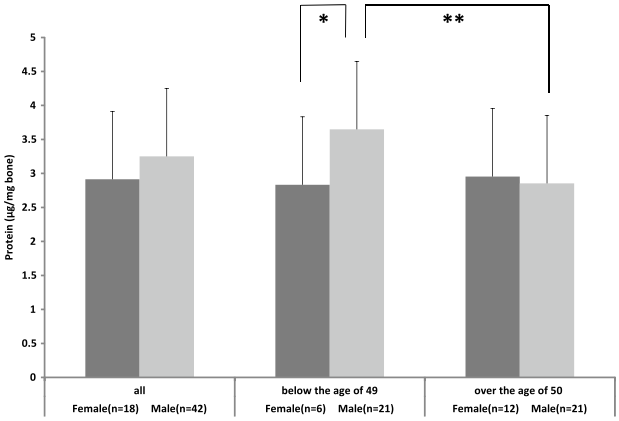

Fig. 6. Amount of protein in skull bone
Values are presented as mean ± SD. Significant differences are indicated as *p < 0.05, **p < 0.01.
The amount of protein (mean ± SD) for females and males was 2.91 ± 0.90 μg/mg bone and 3.25 ± 0.93 μg/mg bone, respectively. The amount of protein (mean ± SD) for females below the age of 49 years and above the age of 50 years was 2.83 ± 0.42 μg/mg bone and 2.95 ± 1.08 μg/mg bone, respectively. The amount of protein (mean ± SD) for males below the age of 49 years and above the age of 50 years was 3.65 ± 0.85 μg/mg bone and 2.85 ± 0.84 μg/mg bone, respectively.

and above the age of 50 years was 3.65 ± 0.85 μg/mg bone and 2.85 ± 0.84 μg/mg bone, respectively.

The protein amount was significantly different between individuals below the age of 49 years and above the age of 50 years for males ($p = 0.004$), and between females and males below the age of 49 years ($p = 0.034$). In addition, the values in the graph with the amount of protein on the y-axis and the age at death on the x-axis decreased with aging in both females and males. The r values in the linear regression analysis for females and males were −0.10 and −0.47, respectively.

Comparison of osteocalcin amounts between females and males, and between individuals below the age of 49 years and above the age of 50 years

Figure 7 shows a comparison of osteocalcin amounts between females and males, and between individuals below the age of 49 years and above the age of 50 years. The amount of osteocalcin (mean ± SD) for females and males was 0.19 ± 0.11 μg/mg bone and 0.12 ± 0.07 μg/mg bone, respectively. The amount of osteocalcin (mean ± SD) for females below the age of 49 years and over the age of 50 years was 0.21 ± 0.11 μg/mg bone and 0.18 ± 0.11 μg/mg bone, respectively. The amount of osteocalcin (mean ± SD) for males below the age of 49 years and above the age of 50 years was 0.14 ± 0.08 μg/mg bone and 0.11 ± 0.05 μg/mg bone, respectively.

The amount of osteocalcin was significantly different between females and males ($p = 0.007$). In addition, the values in the graph with the amount of osteocalcin on the y-axis and the age at death on the x-axis decreased with aging for both females and males. The r value in the linear regression analysis for females and males was −0.47 and −0.31, respectively.

Comparison of collagen I amounts between females and males, and between individuals below the age of 49 years and above the age of 50 years

Figure 8 shows a comparison of collagen I amounts between females and males, and between individuals below the age of 49 years and above the age of 50 years. The amount of collagen I (mean ± SD) for females and males was 0.19 ± 0.08 μg/mg bone and 0.21 ± 0.08 μg/mg bone, respectively. The amount of collagen (mean ± SD) for females below the age of 49 years and above the age of 50 years was 0.16 ± 0.04 μg/mg bone and 0.21 ± 0.09 μg/mg bone, respectively. The amount of collagen I (mean ± SD) for males below the age of 49 years and above the age of 50 years was 0.25 ± 0.08 μg/mg bone and 0.18 ± 0.08 μg/mg bone, respectively.

The amount of collagen I was significantly different between females below the age of 49 years and males below the age of 49 years ($p = 0.029$), and between individuals below the age of 49 years and above the age of 50 years for males ($p = 0.011$). In addition, the values in the graph with the amount of collagen I on the y-axis and the age at death on the x-axis increased with aging for females, while it decreased for males. The r value in the linear regression analysis for females and males was less than 0.02 and −0.48, respectively.

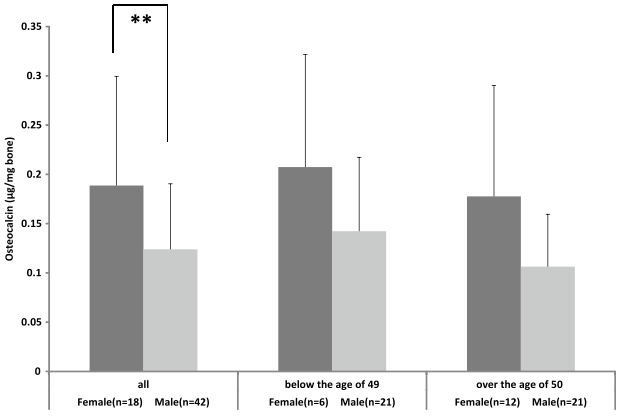

Fig. 7. Amount of osteocalcin in skull bone
Values are presented as mean ± SD. Significant differences are indicated as * $p < 0.05$, ** $p < 0.01$.
The amount of osteocalcin (mean ± SD) for females and males was 0.19 ± 0.11 μg/mg bone and 0.12 ± 0.07 μg/mg bone, respectively. The amount of osteocalcin (mean ± SD) for females below the age of 49 years and above the age of 50 years was 0.21 ± 0.11 μg/mg bone and 0.18 ± 0.11 μg/mg bone, respectively. The amount of osteocalcin (mean ± SD) for males below the age of 49 years and above the age of 50 years was 0.14 ± 0.08 μg/mg bone and 0.11 ± 0.05 μg/mg bone, respectively.

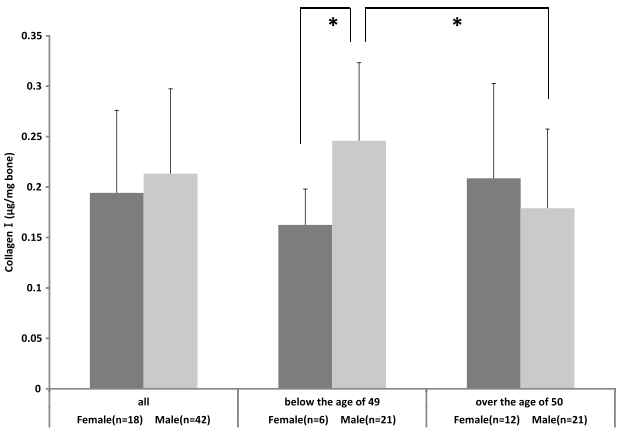

Fig. 8. Amount of collagen I in skull bone
Values are presented as mean ± SD. Significant differences are indicated as * $p < 0.05$, ** $p < 0.01$.
The amount of collagen I (mean ± SD) for females and males was 0.19 ± 0.08 μg/mg bone and 0.21 ± 0.08 μg/mg bone, respectively. The amount of collagen (mean ± SD) for females below the age of 49 years and above the age of 50 years was 0.16 ± 0.04 μg/mg bone and 0.21 ± 0.09 μg/mg bone, respectively. The amount of collagen I (mean ± SD) for males below the age of 49 years and above the age of 50 years was 0.25 ± 0.08 μg/mg bone and 0.18 ± 0.08 μg/mg bone, respectively.

DISCUSSION

In the present study, we successfully applied the modified EZ: faast™ amino acid analysis kit for the determination of D-Asp and L-Asp concentrations, in which the derivatization reagent was changed from propyl chloroformate to ethyl chloroformate for chiral separation. The proposed method is a rapid, simple, and sensitive method that possesses 2 important new features. The first feature is that it is not required to remove interfering substances such as proteins, urea, or lipids, as it uses solid-phase extraction, thereby avoiding lengthy sample preparation procedures and associated losses arising from the instability and poor recovery of some amino acids. The second feature is that the specimen preparation time is less than 30 min from start to finish, whereas half a day is required for the preparation of specimens by previously established methods[1,20-22]. The result is a dramatic reduction in the time required for the assay and a simultaneous improvement in analytical performance, that far outweighs the not-excessive cost of the equipment. Moreover, deuterated Asp (L-Asp-d_3) was used as an internal standard. This deuterated dilution method is an accurate technique that is effective in uncertain analyses where the analytical value changes for every specimen. Although a control specimen to confirm the age is required to estimate the age of the tested specimen when using previously reported methods, such a control is not required in the proposed method. The chiral separation method was established by modification of a commercially available amino acid analysis kit, and it permits the easy and rapid extraction and analysis of Asp. However, the relationship between aging and the extent of Asp racemization in skull bones was weak and, thus, is not sufficiently accurate for practical use.

A close relationship between age and the extent of Asp racemization in teeth has been reported regardless of sex[1,20-22]. The equivalent correlation in skull bones is poor, but the relationship between age and extent of Asp racemization in skull bones was different between the sexes. For males, the extent of Asp racemization in skull bones increased as in teeth, but the correlation had a lower magnitude than that in teeth. In contrast, for females, the extent of Asp racemization in skull bones decreased with aging, which was opposite to the results observed in teeth. Therefore, the amount of D-Asp was different between females and males, whereas the amount of L-Asp was similar between the groups.

Proteins in bone should be thought of as the main source of D-Asp. The bone matrix consists of approximately 70% inorganic substances and approximately 30% organic substances[10,24]. Collagen consists of approximately 90% organic substances, and the intraosseous collagen is of type I[10,24]. In addition, osteocalcin consists of approximately 25% non-collagen proteins[10,24]. There are reports that D-Asp in collagen[9] and osteocalcin in bones[18] increases with aging. Therefore, we measured the amount of total protein, osteocalcin, and collagen I in skull bones as the putative components responsible for the level of D-Asp. Asp was extracted from bone by using hydrochloric acid, whereas total protein, osteocalcin, and collagen were extracted from bone by using formic acid. Because of the differences between these extraction methods, the amount of Asp was higher than the amount of the proteins. Although it was difficult to compare these amounts directly, there were significant sex differences in the amount of osteocalcin, whereas the amounts of total protein and collagen I were similar between females and males.

Osteocalcin is one of the most abundant non-collagenous bone matrix proteins synthesized exclusively by osteoblasts[19]. Osteocalcin has 3 glutamyl residues within a molecule and exists in 2 forms: active osteocalcin (Gla-OCN) is carboxylated at all 3 glutamyl residues; all other forms are inactive osteocalcin (Glu-OCN)[18]. Most osteocalcin is Gla-OCN, is stored in bone matrix, and is often used as a marker for bone formation[19]. In contrast, Glu-OCN is released into the blood. In addition, Gla-OCN in the bone is eventually decarboxylated and transformed to Glu-OCN, which is then released into the blood in the process of bone absorption[19]. Thus, the amount of blood osteocalcin is usually used as a marker for bone absorption[19]. Yoshimura et al reported that the level of serum Glu-OCN increased with aging in females, especially in menopause when estrogen is progressively reduced[32]. In contrast, the level of serum Glu-OCN in males does not change with aging[32]. In this study, the amount of Gla-OCN in female bones was significantly less than that in male bones. Thus, bone turnover in women rapidly accelerates after menopause[32]. Ritz et al noted that the level of D-Asp in bone-matrix-associated osteocalcin (Gla-OCN) increased with age and that there was a close relationship between the D-Asp content of Gla-OCN and age[26]. In addition, it was reported that the D-Asp levels in Gla-OCN should be similar, regardless of sex. Although the amount of D-Asp contained in Gla-OCN increases with aging regardless of sex, our study showed an age-dependent decrease in the amount of D-Asp contained in skull bones of females. Thus, the amount of D-Asp in collagen I may be responsible for the decrease in the total amount of D-Asp in female skull bones. In post-menopausal female bones, bone remodeling is enhanced[32] and old molecules of collagen I are replaced with new molecules[9] without affecting the total amount of

collagen I. It is possible that the D-Asp in collagen I does not accumulate in skull bones and that the total amount of D-Asp contained in the skull bones thus decreases with aging in females.

Previous studies documented that racemization of Asp *in vivo* was a universal phenomenon in aging humans and that nearly every tissue contained long-lived aging proteins with age-dependent accumulation of D-Asp[2,12,13,31]. In particular, D-Asp levels increased with age in many tissues, including the brain, teeth, and arteries[11]. It has been reported that D-Asp is not present in infants and that racemization occurs with aging[2,12,13,31]. However, our findings indicate that D-Asp exists in young humans, even in infants. In recent years, it has been suggested that ion channels are activated by D-Asp in the nervous system of *Aplysia*[8]. Thus, although the effect of the presence of D-Asp in skull bones of young individuals, including infants, is unclear, D-Asp may have some unknown physiological function that should be addressed in future studies.

CONCLUSION

A chiral separation method for D-Asp and L-Asp was developed by modifying a commercially available amino acid analysis kit. The amounts of D-Asp and L-Asp in skull bones were measured using the newly established method. A relationship between the extent of Asp racemization in skull bones and age was observed; however, the accuracy was too low for use in forensic casework. In contrast, the amount of D-Asp in the skull bones differed between the sexes because of differences in the remodeling of the bone. Moreover, D-Asp was detected in young humans, even infants. These new findings should be further addressed in future studies.

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