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Deoxypyridinoline crosslinks in human dentin and estimation of age

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Abstract A new biochemical method for estimation of dental age from human dentin is presented. An adaptation and modification of a dentin protein extraction protocol was developed by which deoxypyridinoline crosslinks were measured using an enzyme immunoassay technique. Deoxypyridinoline, a non-reducible collagen crosslink, was measured in human dentin samples from permanent individual molars. The measurement of this protein in dentin samples from 22 patients with ages ranging from 15 to 73 years old was carried out to obtain an estimate of age at death. By linear regression between deoxypyridinoline values and age, equations for age calculations were computed. The error between the estimated age and the known age at extraction was calculated. Our results show that this method produces an expected associated error on calibration averaging 14.9 years about the mean estimated values, at a 65% level of confidence. Values of sensitivities and specificities were calculated for deoxypyridinoline at different ages. We conclude that determination of deoxypyridinoline in human dentin extracts is a new and reliable procedure, potentially suitable for most forensic laboratories and which could be a useful tool to estimate human dental age. This method could be considered as an alternative method to calculate dental age, contributing to a more accurate estimation of chronological age when used in addition to other morphological or biochemical dental methods.

Key words Age estimation · Human dentin · Collagen crosslinks · Deoxypyridinoline

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Introduction

The assessment of chronological age is of great importance in forensic science. Biochemical studies performed on mineralized tissues (bone and teeth) have been used as a diagnostic tool in estimating the age of unknown human remains [1–15]. The conversion of the L to the D form of aspartic acid with age has been studied in different tissues, showing an almost linear increase with age [7, 10, 11, 13, 15]; especially promising is the determination of aspartic acid racemization in dentin [1–6, 8, 9, 12, 14]. Moreover, dentin seems particularly suitable for the study of biochemical changes in teeth with aging because it is composed of approximately 20% organic matrix (principally collagen) and is thought to have minimal collagen turnover. Dentin collagen constitutes, as in bone, almost 90% of the organic matrix and is primarily of type I. The central portion, about 95% of the total collagen molecule, is triple-helical in structure and, in order to stabilize the collagen network, covalent crosslinks exist between the collagen molecules [16, 17]. The crosslink patterns of bone and dentin collagen are very characteristic, being formed by two borohydride-reducible divalent crosslinks (dehydro-dihydroxylysinonorleucine and dehydro-hydroxylysinonorleucine), and two non-reducible trifunctional crosslinks, hydroxypyridinium crosslinks: pyridinoline and deoxypyridinoline (DPD) [18, 19]. Furthermore, it is known that DPD, although a minor component of the hydroxypyridinium residues in adult dentin, seems to be unique to dentin and bone [20, 21]. It has been demonstrated that the content of crosslinks changes with age [22–24]. In fact, in adult bovine dentin, hydroxypyridinium residues were found to be 6 times more concentrated than in fetal bovine dentin [22]. Similary, preliminary studies performed on human dentin suggest an increase of hydroxypyridinium crosslinks with age [22].

Therefore, the quantification of DPD crosslinks in individual human dentin at different ages could be applied as a new marker for calculating the age of a subject. The present study was designed to measure DPD in human dentin

samples from permanent individual molars from patients aged from 15 to 73 years old. It was hypothesised that measurement of this collagen protein might be useful for the estimation of chronological age through dental studies.

Materials and methods

Healthy erupted human permanent molars, extracted for valid clinical reasons (periodontal disease, malocclusion or orthodontic treatment), were obtained from the Department of Oral Surgery at the Virgen de las Nieves Hospital of Granada (Spain) and from private dental clinics. The protocol to collect samples from human subjects was approved by the Ethics Committee of the Virgen de las Nieves Hospital. Different kinds of permanent molars were taken from 22 patients (13 females and 9 males) with ages ranging from 15 to 73 years. Table 1 shows the chronological age of the individual at extraction, the sex and the type of molar.

Tissue preparation and extraction of dentin proteins

Immediately after extraction, the teeth were cleaned of blood with distilled water and any attached soft tissue was carefully removed with a scalpel. Enamel and cementum were removed from the dentin by diamond burrs under a continuous water spray. After opening the pulp-chamber, the pulp content was scraped with a dental excavator and the pulp-canal content eliminated with Gates files (on a dental air turbine handpiece under a continuous water spray) and endodontic files. All the dentin samples were about 1.2 g wet weight. Unless otherwise stated, all steps were carried out at 4 °C. Dentin tissue was crushed by pounding with a steel hammer on a cold metallic surface. To remove soft tissue remnants, the pulverized dentin was washed overnight with 2.5 M NaCl containing a proteinase inhibitor solution (PI) (2.5 mM benzamidine HCl, 50 mM ε-amino-n-caproic acid, 0.5 mM N-ethyl maleimide, and 0.3 mM phenylmethylsulfonylfluoride), by constant stirring using magnetic stirrers.

Dentin proteins were extracted by an adaptation and modification of similar techniques applied to porcine bone [25] and bovine and rat dentin [26, 27]. A flow diagram of the sequential extraction procedure is presented in Fig. 1. After removing the nonspecifically bound material by NaCl washes, the pulverized dentin was then rinsed twice with cold distilled water. To elute hydroxyapatite-associated proteins, the dentin was extracted with a 4 M guanidine HCl solution, 65 mM Tris-HCl pH 7.4, plus the PI solution under constant agitation at 4 °C. These guanidinium chloride extracts were clarified by centrifugation at 2,000 × *g* for 10 min and the supernatants were discarded. Following the guanidinium extraction, proteins within the mineralized matrix (including collagen proteins) were extracted by demineralization with 0.5 M EDTA pH 7.4 and PI solution over a total period of 12 days. The EDTA solution was changed every 3 days and clarified by centrifugation at 2,000 × *g* for 10 min. The successive EDTA extracts (E1, E2, E3 and E4) were collected separately. The material was then exhaustively dialysed in M_r 6,000 cut-off Spectrapor dialysis tubing at 4 °C against large volumes of double-distilled water plus PI, lyophylized after a final solution change in water and then frozen at –20 °C until analysis. The remaining dentin tissue was extracted with 4 M guanidine HCl solution, 65 mM Tris-HCl pH 7.4 plus PI to dissociate organic matrix associated proteins that were not mineral associated. The guanidinium chloride extracts (G-extracts) were clarified by centrifugation at 2,000 × *g* for 10 min. The supernatants were exhaustively dialysed against two changes of 50 mM Tris-HCl pH 7.4 with PI for 4 days, and then against distilled water for 1 more day. Aliquots of G-extracts were then lyophylized and immediately frozen at –20 °C until analysis. Before lyophylization, the protein content of all samples was quantified spectrophotometrically by the Lowry protein assay [28].

Solubilization of dentin collagen and deoxypyridinoline quantification

Solubilization of dentin collagen was carried out using a method modified from Kuboki et al. [29]. E3-fractions (called E-extracts) were selected from among other EDTA-extracts for this analysis.

Table 1 Characteristics of the samples and results from the calibration of DPD ratio in human dentin against chronological age

Sample no.	Age (years)	Sex[a]	Molar[b]	DPD ratio[c]	Estimated age	Associated error on calibration[d]	Real error
1	15	F	46	0.62	24	15.1	–9
2	17	F	46	0.64	24.4	15.1	–7.4
3	17	F	48	1.14	35	14.7	–1.8
4	17	M	48	1.04	32.9	14.7	–15.9
5	19	F	28	1.07	33.5	14.7	–14.5
6	20	F	48	1.83	49.7	14.8	–29.7
7	27	M	18	1.32	38.9	14.6	–11.9
8	31	M	18	1.19	36.1	14.6	–5.1
9	36	M	46	1.28	38	14.6	–2
10	36	F	48	0.85	28.9	14.9	7.1
11	37	F	38	1.36	39.7	14.6	–2.7
12	40	F	27	1.16	35.5	14.7	4.5
13	41	M	27	1	32.1	14.7	8.9
14	52	M	17	1.69	46.7	14.7	5.2
15	56	F	36	1.62	45.3	14.6	10.7
16	57	M	16	1.25	37.4	14.6	19.6
17	61	M	46	1.49	42.5	14.6	18.5
18	61	M	36	1.45	41.6	14.6	19.4
19	61	F	36	1.14	35	14.7	26
20	64	F	47	2.4	61.9	15.4	2.1
21	69	F	47	3.05	75.7	16.8	–6.7
22	73	F	27	2.81	70.6	16.2	2.4

[a] F = Female; M = Male
[b] According to FDI notation
[c] DPD ratio = (DPD/total proteins in E-extracts)/(DPD/total proteins in G-extracts)
[d] Values represent ± associated error on calibration, at a 65% level of confidence

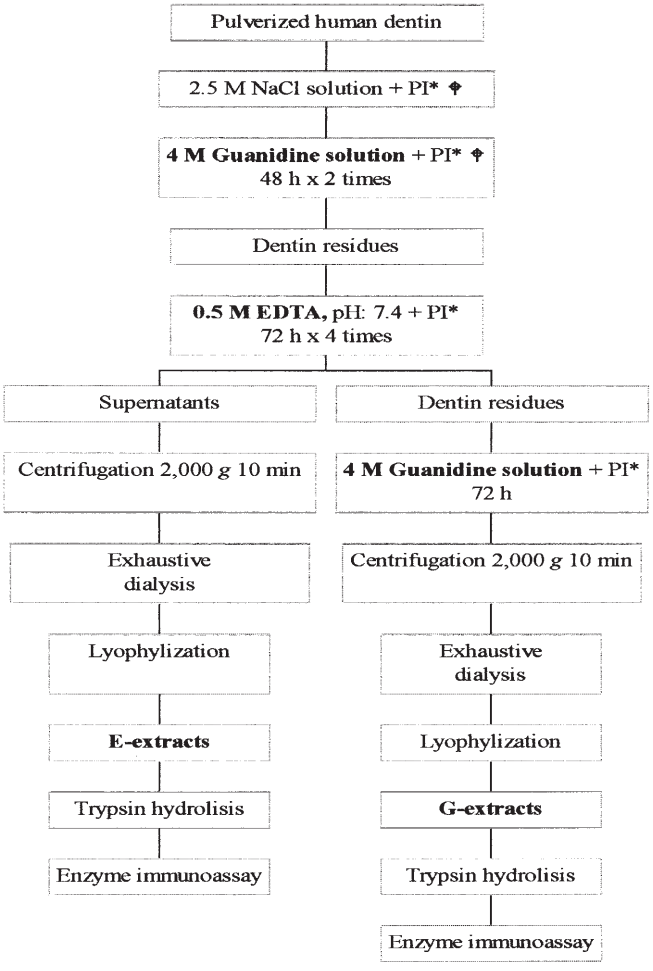

Fig. 1 Flow diagram chart from sequential extraction of human dentin proteins for deoxypyridinoline measurement. * Proteinase inhibitor solution (PI): 2.5 mM benzamidine HCl, 50 mM ε-amino-n-caproic acid, 0.5 mM N-ethyl maleimide, and 0.3 mM phenylmethylsulfonylfluoride. † Supernatant solution discarded

The lyophilized samples (E- and G-extracts) containing the insoluble collagen proteins were suspended in 50 µL 0.05 M Tris-HCl/0.01 M $CaCl_2$ pH 7.5, and then heated at 60 °C for 1 h under constant stirring. The suspension was cooled to 37 °C and hydrolyzed with trypsin (TPCK-treated, Fluka) for 15 h at 37 °C. The concentration of trypsin was adjusted to 25% final concentration in relation to the total protein content. Then the mixture was again heated to 60 °C for 15 min. The mixture was cooled to 37 °C, treated with trypsin, at the same final concentration as described previously, for 3 h and after slight centrifugation the supernatant was lyophilized. Deoxypyridinoline was measured using an adaptation of an enzyme immunoassay method (Metra Biosystems, Mountain View, Ca). Just before deoxypyridinoline quantification, the hydrolyzed and lyophilized E- and G-samples were solubilized in 50 µL of assay buffer. Amounts of DPD (µg/mL) were calculated from a standard curve obtained with adequate dilutions of deoxypyridinoline standards.

Statistical evaluation

Linear regression analysis was used to determine the presence of correlation between continuous variables. Estimated ages were assessed from the regression equation. Estimates of associated error on calibration were calculated from the equation as given by Miller and Miller [30], and Martin and Luna [31]:

$$\pm t_\alpha s \sqrt{1 + \frac{1}{n} + \frac{(x_0 - \bar{x})^2}{(xx)}}$$

where:

t_α = value of t at a given alpha level with n-2 d.f
s = estimate of the standard deviation *y* on *x*
x_0 = DPD ratio for the tooth
$\bar{x}$ = mean of DPD ratio in the reference sample
(xx) = Sum of squares of DPD ratio

One-way analysis of variance for continuous variables was used to determine statistically significant differences between age groups. The incidence of true and false positives and negatives, sensitivity and specificity values was calculated [30, 31].

Results and discussion

Determination of the degree of racemization of aspartic acid in dental tissues represents an important advance in estimating the chronological age of individuals. A series of studies have been performed in recent years [1–6, 8, 9, 12, 14], showing that dental age estimation by means of aspartic acid racemization is a valuable method for age estimation. A recent study has pointed out the limitations involved in the use of amino acid racemization in human dentin as an indicator of age at death or extraction, when accurate statistical methods are applied to the results [14]. The authors conclude that when aspartic acid racemization determination is applied in order to estimate the age of an individual, an approximately ± 15 years error at 95% level of confidence would be a safe margin. Apart from this biochemical method (racemization of aspartic acid), there has been little information from forensic odontology studies on the biochemical changes of dental tissue with age. Quantification of DPD from dentin has not yet been used as a method for dental age estimation. The fact that some preliminary changes of human dentin DPD with aging have been detected [22] suggests that such a determination could provide information of collagen crosslinks during the life of teeth. The results found in the present study are in keeping with this hypothesis.

DPD was quantified in human dentin extracts obtained during and after EDTA demineralization (E- and G-extracts, respectively). Total amounts of DPD and percentages of DPD in relation to total proteins in both extracts from individual teeth are presented in Table 2. DPD levels in human dentin expressed as total amounts were 3 times higher in G-extracts than in E-extracts. When related to total proteins, average values were approximately the same in both dentin extracts (10%) (Table 2). As an expression of the total DPD content (E- and G-extracts) in dentin for each molar, the ratio of the percentages of DPD in relation to total proteins for E- and G-extracts were calculated. Therefore, a deoxypyridinoline ratio (E/G extracts) was calculated as: DPD ratio = (DPD/total proteins in E-extracts)/(DPD/total proteins in G-extracts). A linear regression model was then constructed using the DPD ratio as *x*, against chronological age as *y*. The detected in-

Table 2 Average of total amounts and relative values of deoxypyridinoline in human dentin extracts

	DPD*			DPD/prot**		
	Mean	Range	SD	Mean	Range	SD
E-extracts	10.8	5.7–20.1	3.7	11.3	6.3–15.6	2.5
G-extracts	27.9	14.6–54.8	8.9	9.1	3.8–20.6	4.4

Values represent: * µg deoxypyridinoline/mL; ** Percentage of deoxypyridinoline in relation to total amount of proteins. SD: standard deviation; $n = 22$

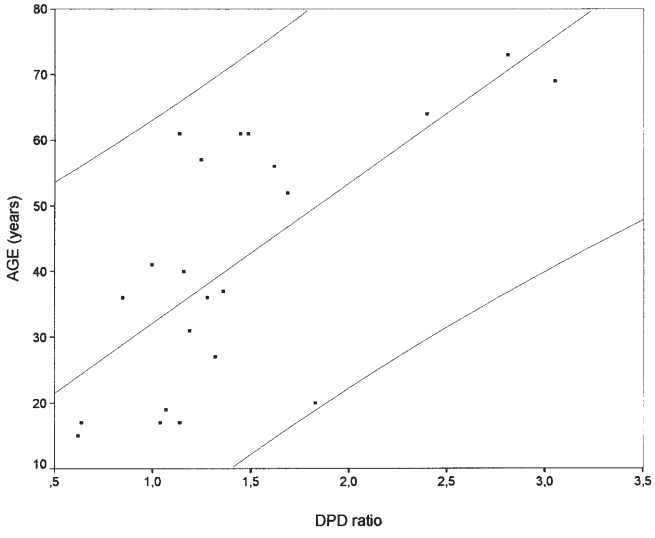

Fig. 2 Age against DPD ratio in human dentin, with 95% confidence intervals. DPD ratio was calculated as described in the results and discussion section

crease in the DPD ratio in relation to the age of the individual fits the mathematical model (Fig. 2) and therefore, this correlation could be useful for estimating the age of an individual in a particular case. Estimates of age were calculated from the regression equation: Age = 10.8 + 21.3 DPD ratio (r = 0.68; std err est = 14.34; F_{exp} = 18.0694; n=22; $p < 0.01$). Because all samples were extracted from individuals of known age, it was possible to calculate the real error between the estimated age and the chronological age of the individual at extraction (Table 1). When errors are properly taken into account through a statistically rigorous calibration on the line model, it can be seen that this method produces an expected associated error on calibration averaging 14.9 years about the mean estimated values (Table 1), at a 65% level of confidence.

Comparison of our results with previous published works is limited by the use of a different methodology. Most of the publications on biochemical dental changes relate the conversion of the L to the D form of aspartic acid with dentinal age and, therefore a correction of the actual age of the individual was estimated for each dentinal age [1, 5]. In the present study, the biochemical changes detected are correlated to the chronological age of the individual at extraction, regardless of the age of the dentin. Moreover, it is difficult to compare our results to

Table 3 Cut-off values for DPD ratio at different individual ages

Age (years)	Cut-off[a]	TP	FP	FN	TN	Sensitivity[b]	Specificity[c]
25	1.1	4	4	2	12	66%	73%
45	1.4	12	2	1	7	92%	77%
65	2	19	0	1	2	95%	100%

TP means true positive; FP means false positive; FN means false negative; TN means true negative.
[a] Cut-off values for DPD ratio. See results and discussion for rationale of cut-off values. Number of cases n = 22
[b] Sensitivity = TP/(TP+FN)
[c] Specificity = TN/(TN+FP)

previous studies on aspartic acid racemization in human dentin because the standard error estimated [5, 6, 12] is not comparable to the estimated error on calibration [14] (from our results, ± 14.9 years at a 65% level of confidence), which represents the value of the estimated error for each age calculated from the regression model.

The DPD ratio increased with aging, and differences between age groups were detected. Significant differences in the DPD ratio (F_{exp} = 4.4152; 2, 19 d.f.; $p < 0.05$) were found between individuals aged less than 20 years old (mean = 1.05; SD = 0.44; n = 6) and subjects over 55 years old (mean = 1.9; SD = 0.74; n = 8). The incidence of true and false positive and negative values was obtained and sensitivities and specificities were calculated for the DPD ratio as a possible discriminatory criterion in establishing the age of an individual (Table 3). There are many possible cut-off levels for the DPD ratio at different ages. A selection of the best possible cut-off points of the test (DPD ratio), those maximising sensitivity and specificity, for different ages of the studied population (20, 45 and 65 years) is presented in Table 3. Therefore, DPD ratio values above 1.4 are suggestive (92% sensitivity and 77% specificity) of an individual older than 45 and younger than 65 years old.

The proposal of this dentin protein extraction protocol adapted to human dentin, and the subsequent DPD quantification by the use of a commercially available enzyme immunoassay method, makes this analysis potentially suitable for most forensic laboratories. In summary, we can conclude that determination of DPD in human dentin may be a useful tool, providing forensic odontologists with an alternative method to estimate the age of an individual, especially when used in combination with other, complementary, morphological or biochemical dental methods. Further research is still needed to provide more information for the postmortem establishment of human dental age.

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