AAR (AMINO ACID RACEMIZATION) - A RELIABLE TOOL FOR AGE ESTIMATION IN FORENSIC ODONTOLOGY

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Abstract

The estimation of age is a crucial process and it should be very accurate. Various methodologies are available in the estimation of person's age in forensic odontology. The Gustafson's morpho histologic approach is most widely used method and includes six parameters to assess age. Amino Acid Racemization (AAR) is used to know the dental age using chemical method which has been reported that the amount of D-aspartic acid accumulated in enamel and dentine increased with aging. Death stops this conversion. The average age variance of the studies included in this review using Gustafson's method showed ±7.148 and that of AAR is ±1.3125. The variability was more in Gustafson's method as compared to AAR. Extensive research should be incorporated for evaluation.

Keywords- AAR, Amino acid racemization, Gustafson's method, age estimation, forensic odontology.

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1. SIGNIFICANCE OF AGE ESTIMATION

One of the important tasks of forensic science is to identify bodies of unknown identity, which is required for procedural, legal and humanitarian reasons.[1,2] This is also useful in the identifying the bodies in mass disasters and natural calamities.[3] In a country like India which is still developing, a large number of people are less educated and have scarce knowledge or records of their birth date which is required in matters like consent, rape, criminal responsibility, kidnapping, identification, employment, judicial punishment, criminal abortion, prostitution, attainment of majority by law enforcing agencies.[2] In many cases, the biological or physiological aging is not related to chronological aging. A biological marker, in this manner, independent of any environmental alteration is required to provide information about the individual's age.[4] Therefore, the assignment of age is a crucial process and estimation should be very accurate. Various methods have been performed for estimation of chronological age in forensic sciences. The apparent age can be visually assessed if the body is in a good condition**,** but histological ageing or traditional morphological techniques need to be used on bone or dental elements if the remains are degraded in any way.[5]

In the assessment of person's age various modalities are available such as skeletal and dental changes. It is an important aspect of forensic science to estimate age by dentition.[6] Hence, the field of forensic science is of supreme importance.

There are many methods of estimating age using tooth such as Gustafson's method, Amino acid racemization using tooth dentin, CpG methylation of teeth-derived DNA using real-time methylation-specific PCR,[7] Williems, Dermijian and Nolla methods,[8] Bedek's et al method,[9] by estimation of open apices,[10] etc amongst which the present work is done for comparing Gustafson's method and Amino acid racemization.

TEETH AS A CHOICE OF ORGAN (FORENSIC ODONTOLOGY)

Forensic Odontology is the popular science dealing with establishing identity of a person using teeth. It is also called as Forensic dentistry (Sengupta et al., 1999)[11]

Teeth can withstand assaults more than other parts of the body hence teeth are most durable part of the body.[3] They can often bear fire, immersion under water for longer period, burial under oil and biological agents' exposure in the natural environment. Using teeth for age estimation is very well accepted due to their ability of being resilient to change. Thus, day by day, the importance of dental identification is increasing.[2]

Dental age estimation is commonly divided into two periods in a lifetime. The first period is upto 20 years when the teeth are developing in the jaws. The second period is when all the teeth are developed fully when regressive age‑related changes can be used as the method of choice. Age can be determined in adolescent and in children by means of eruption and development of teeth in deciduous and permanent dentition upto 14 years of age. After 14 years of age, the only tooth still developing is the third molar; and hence until the age of 20 years, development of this tooth is used for dental age estimation methods. After this period, age estimation is done mainly by radiographic methods, visual examination, biochemical methods and structural changes in teeth. Scientific methods must be dependent upon attritions, secondary dentin formations and loss of periodontal attachment like regressive age changes.[3]

2. GUSTAFSONS METHOD

The determination of the chronological age using teeth can be performed by various methods in forensics. Teeth undergo structural changes after maturity, thus making age estimation possible in adults. In the dental method of identification, the Gustafson's morpho histologic approach is most widely used method and includes the following six parameters, i.e. attrition, gingival recession, thickness of secondary dentin, cementum apposition, root resorption and root dentin translucency.[12]

To evaluate dental changes, scientific methods have developed recently with Gustafson's approach, prominently used. Other methods include studying tooth degradation, analyzing tooth eruption and development and measuring trace elements and biochemical changes in dental tissues. Six variables by Gustafson's studies found that the best suited age estimation tool when used alone is dentin translucency.[2]

Six-point allotment system as per Gustafson's method [11]

Attrition (A)
A0 – No attrition
A1 – Attrition limited to enamel level
A2 – Attrition limited to dentine level
A3 – Attrition up to pulp cavity
Periodontal disease (P)
P0 – No obvious periodontal disease
P1 – Beginning of periodontal disease but no bone loss
P2 – Periodontal disease more than 1/3rd of the root
P3 – Periodontal disease more than 2/3rd of the root
Secondary dentine (S)
S0 – No secondary dentine formation
S1 – Secondary dentine up to upper part of pulp cavity
S2 – Secondary dentin up to 2/3rd of the pulp cavity
S3 – Diffuse calcification of entire pulp cavity
Root resorption (R)
R0 – No resorption
R1 – Spotted resorption
R2 – Resorption limited to cementum
R3 – Extensive resorption of cementum and dentin both
Root translucency (T)
T0 – No translucency
T1 – Beginning of translucency
T2 – Translucency more than 1/3rd of the apical root
T3 – Translucency more than 2/3rd of the apical root
Cementum apposition (C)
C0 – Normal cementum
C1 – Thickness of cementum more normal
C2 – Abnormal thickness of cementum near the apex of the root
C3 – Generalized abnormal thickness of cementum throughout the apex of the root.

Regression Formula in Gustafson's Method

The first one to note the morphological changes in the tooth structure was Gustafson (1950). Gustafson calculated age from his observation which was derived using the regression formula:

$Y = 3.52X + 8.88$ (X – total score, Y – estimated age).[11]

AMINO ACID RACEMIZATION

The characteristic of each amino acid is amino acid racemization reaction [1] It is natural and will eventually-convert optically active compounds into a racemic mixture. The L-amino acids is a result of the stereochemical specificity of enzymes which utilizes only L-enantiomers and hence it is commonly found in living systems. After the cessation of protein turnover, racemization of those amino acids takes place which is produced by living organisms. As per the recent research, at 25° C, about 100,000 years of period is required to undergo complete racemization of L-amino acid to an equilibrium mixture that is the ratio of D- to L-amino acids equals 1.0. This extent to which racemization of amino acids take place may be used to determine the ages of various fossil bones, fossil materials,

shells, including deep sea sediments, shells and coprolites.13

Aspartic acid has received popularity and attention out of various reactions of amino acid**.** It has fastest rate of racemization amongst stable amino acids. The half-life of this amino acid racemization is about 15,000 years at 20^0 C i.e. it requires 15,000 years to reach the ratio between D and L enantiomers to 0.333. It also means that 20^0C for equatorial and temperate environments, most of the racemization takes places during last 40,000 years.[13]

The belief says that the enantiomers of L-amino acids were eliminated before the existence of life and the basic components of living organisms are L-amino acids and D-amino acids. Hence, except the cell wall of microorganisms, the function and presence of of D-amino acids have not been reported. D-Asp (D-aspartic acid), recently have been reported in different human tissues like brain, lung, skin, eye lenses, teeth, erythrocytes, bone, aorta, and ligaments from individuals with old age. In Alzheimer's disease, beta-amyloid protein contains D-Ser (D-Serine).[14] Thus, in the latter half of 20^{th} Century, Asp (Aspartic acid) racemization was introduced which was considered as the most accurate method. It is a chemical method for dental age determination.[1]

Helfman and Bada in 1970s reported that D-aspartic acid gathered in tooth dentine and enamel and the amount increase with age. As per Halfman and Bada, D-aspartic acid accumulate with age (after 60 years 8% will be D-enantiomer out of total aspartic acid). This result is suggested by the rate constant in any protein with a long *in vivo* lifetime. Ohtani have also reported in detail on Asp racemization in teeth. Subsequently, Masuda *et al.* suggested that D-Aspartic acid containing in teeth might be phosphophoryn. Protein researches which are ancient where much higher investment was done into models predictive of diagenesis, is of racemization in amino acid.

A method known as palaeothermometry, which shows slow increase in isomeric forms which are non-biological forms of constituent amino acids within the protein molecules. It is also used as dating tool. Isomerization or racemization takes place in those amino acids which have one or more chiral carbon centres; whose inter- conversion rate depends upon temperature and time. According to the Good-friend in 1991, increase in D-isomer, if temperature can be used chronometrically; conversely on palaeotemperature the age is known information can be obtained. Asp (aspartic acid), is undoubtedly proved to be most popular dating tool out of all the amino acids used for racemization analysis.[15]

For racemization among the dental tissues, cementum, enamel and dentin can be used; however, dentine is considered ideal for this purpose.[5] Due to death the conversion stops because racemization rate is dependent on temperature, both in vivo and postmortem.[1] The mean values of D-aspartic acid in different type of tooth increases in the following way: lateral incisor < central incisor < canine < first premolar < second premolar < second molar < first molar.[5] High temperature enhances the rate of conversion of L-form to D-form hence burned remains shows high ratio of D/L.[4] Because dentine is formed towards root apex from the crown hence the D/L ratio should be less towards root apex and more in the crown portion [5]

Sample Strategy for AAR

The type of tooth and the part of tooth to be used for age estimation is strategized. Most of the studies show that lower central incisors, lower first molars and right lower first premolars are preferred for age identification.[16] Crown dentine is preferred over enamel as it gives better results.

Sample handling for AAR

After extracting the tooth, 10% formalin is used to disinfect it. It should be kept for 24 hours at room temperature.[17] Sample handling is vital since fixation influences racemization.[5] Sodium hypochlorite is used to remove soft tissue which is adherent on tooth. Ethanol is preferred for fixation because it does not influence racemization much. The tooth samples are washed with acetone.

During sample preparation, before demineralization, Calcified tissue is mostly pulverized which increases the quantity of organic material extracted due to pulverization which consists of collagen that is soluble. It is pulverized using mortar and pestle into powdered foarm. EDTA (ethylene diamine tetraacetic acid) is used as chelating agent for extraction of protein. Then for demineralization, HCl (mineral acid) or EDTA is used to isolate fraction of total protein from dentine. For simpler and faster demineralization, without using protease inhibitor for fragments of dentine, 0.6N HCl is used and continuously agitated at 4^0C. Higher temperature causes hydrolysis of peptide bond hence low temperature is maintained throughout the preparation. Hydrolysis temperature ranges between 100 and 110^0C and the time duration ranges between 6 and 20 hours.[5]

Chromatographic separation

For quantifying and separating D and L enantiomers, GC (Gas chromatography) will be used in dentine. This method is ideally used for forensic purpose. For quantifying and separating all the amino acids only one chromatographic run is required which is done on chiral capillary column. These amino acids are normally derived as N-trifluoroacetic acid isopropyl esters[5].

Calculation In AAR

The D/L ratio can be applied to the formula:

$\ln [(1 + D/L)/(1–D/L)] = 2k$ (Aspartic) t + constant where k=First order kinetics and t=actual age of an individual.[16]

3. COMPARISON OF RESULTS

Extensive literature search was done to compare the results of Gustafson's method and AAR. Although at present very less published literature is available on AAR but an attempt was made to compare the existing literature of AAR and Gustafson's method. An age variance of Gustafson was found to be 7 to 15 years but for AAR, it was within 3-4 years.[16]

Table 1: Comparing the results of AAR and Gustafson's method

Methods	**Various studies**	**Age variance**
AAR	**Katarzyna Wochna (2018)[17]**	**±2.6**
	Rastogi et al (2017)[18]	**±0.025**
GUSTAFSON	**Gustafson (1950)[19]**	**±3.63**
	Bang and Ramm (1970)[20]	**±10.07**
	Johanson (1971)[21]	**±8.92**
	Miles (1963)[22]	**±8.87**

	Dalitz (1962)[23]	**±8.41**
	Pillai and Bhaskar (1974)[24]	**±8.13**
	Lucy et al. (1996)[25]	**±7.0**
	Singh and Gorea (2004)[26]	**±2.16**

4. DISCUSSION

AAR can be done to know how accurate, precise and measurable it is, however the results might or might not be statistically significant.[1] It depends on quantifiable, specific research procedure and not on professional experience of examiner.[1] Among 20 amino acids, which are the constituents of protein, the aspartic acid is found to be most racemizable one. Hence, as a outcome of racemization, presence of D-Asp due to ageing in tissues has been explained.[6]

Other older methods for estimating age in adult cadavers are not accurate and highly subjective. In Gustafson's method carborendum disc is used to perform sectioning of tooth. Except sectioning all other steps are manual and hence more chances of human error. In this technological era, we cannot rely on manual methods for forensic investigation.

Amino acid racemization is most accurate and reliable method which is conversion of L-form to D-form (non enzymatic) and is age dependent but there are some limitations of this method. This technique is useful in both living and dead. Amino acids are susceptible to decomposition and oxidation in the course of time and probably are not reliable if estimating the age of those samples which are old as they may yield very high errors. Racemization is a chemical process and thus it is influenced by a number of factors such as: temperature, humidity, pH, etc so the results can vary within an error range of ±3 years.[26]

5. CONCLUSIONS

The variability in the findings was more in Gustafson's method as compared to AAR. Extensive survey is a fundamental part in evaluation. AAR shows consistent and accurate results with lesser variances.

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