Anthropologist, 17(3): 929-936 (2014)
PRINT: ISSN 0972-0073 ONLINE: ISSN 2456-6802
DOI: 10.31901/24566802.2014/17.03.27

Anthropological Age Estimation with Bone Histomorphometry from the Human Clavicle

U-Young Lee[1], Go-Un Jung[2], Seung-Gyu Choi[3] and Yi-Suk Kim[2]*

[1]Department of Anatomy, Catholic Institute for Applied Anatomy, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea 137-701
[2]Department of Anatomy, Ewha Womans University School of Medicine, Seoul, Republic of Korea 158 -710
[3]Division of Forensic Medicine, National Forensic Service, Seoul, Republic of Korea 158-707

KEYWORDS Bone Histology. Compact Bone. Osteon. Grinding Method. Forensic Anthropology

ABSTRACT The purpose of present study was to apply histomorphometry to estimate the age at death using the clavicular osteonal remodeling rate and to develop an age-predicting equation. 46 clavicular samples consisting 28 males and 18 females were obtained from Korean cadavers. Two sequential 1-mm-thick sections, were prepared using a diamond wheel, and a 100 um-thick section was then made by manual grinding with polarizing filter optical microscopy. Three histomorphometric variables, including relative cortical area (RCA), osteon population density (OPD), and mean osteon area (OA), were measured. The mean values are indicated because no significant difference in any variable was noted by a paired t test between the two sequential sections. Analysis of covariance demonstrated that stratification of the statistical results of OPD and OA based on sex was unnecessary, except for RCA. Regression analysis indicated that the highest coefficient value was OPD (R^2 = 0.583), which was selected as a significant value with RCA for an age-predicting equation (R^2 = 0.628) by the multiple regression analysis stepwise method. In conclusion, a histomorphometric method using the clavicle is reliable for age estimation in physical and forensic anthropological fields.

INTRODUCTION

In forensic anthropology, biological profiles including age, sex, and populations group should be assessed to identify skeletal remains (Trotter and Gleser 1958; Han et al. 2009). To do this, it is crucial to first distinguish human bones from animal bones (Mughal et al. 2013; Formicola 1993). If the skeletal remains are confirmed to be human, procedures for assessing the biological profiles depend on individual skeletal measurements (Balseven-Odabasi et al. 2013; Absolonova et al. 2013; Buikstra and Ubelaker 1994). Among the biological profiles, the age at death provides significant information about the cause of death, irrespective of the elapsed time after death. Therefore, it is vital to determine the age at death of unidentified remains, especially in forensics. Until now, two major methods have been used for age estimation: the conventional method of visual inspection and a histomorphometric method (Kerley 1965). In visual inspection, the examiners study a specific articulation surface with the naked eye and measure the level of aging-related degeneration. However, the outcome is likely to produce a wide range and a large standard error of estimate. In addition, if the articulation surface is severely damaged, visual inspection might be difficult to satisfy the scientific demands.

In contrast, the histomorphometric method is a quantitative determination based on the measurable morphology of the osteon (Frost 1987). Microscopic examination of a cross-section of the bone is used to measure the histomorphometric features of the osteon and the measured variables are applied to confirm the regression relationship between bone and age at death. The histomorphometric method reduces subjective judgments and the level of experience needed for age estimation, and this method is available even for small fragmentary.

Currently, histomorphometric age estimation is widely performed for a number of population groups and various types of bone elements (Frost 1958; Thompson 1983). The osteonal remodeling process that contributes to bone variation

Address for correspondence:
Dr. Yi-Suk Kim
Department of Anatomy,
Ewha Womans University School of Medicine
911-1, Mok-5-dong,
Yangcheon-gu, Seoul 158-710,
Republic of Korea
Fax: 82-2-2650-5716,
E-mail: jwkiss@ewha.ac.kr

differs among population groups and bone types (Thompson and Guness-Hey 1981; Stout and Lueck 1995). In addition, age-estimating research based on diverse population groups with different materials has been expanded, especially in Korean populations. In 2007 and 2009, the sternal end of the 4^{th} rib and anterior cortex of the femur were tested for use to as materials (Kim et al. 2007; Han et al. 2009). However, previous research has not examined the extent and range for applying additional bone materials that can be assessed in diverse forensic situations. Moreover very few studies have focused on the clavicle, except in Caucasian populations (Stout and Paine 1992)

The clavicle has several clear benefits and was selected as the target material in this study. First, the size determines the relatively high ratio of compact bone to spongy bone, so there is little possibility of damage to the clavicle by a fracture. Second, the bone is likely to be found intact rather than in fragments. Third, the clavicle is not a weight-bearing bone, so few factors, including daily lifestyle, can affect the bone remodeling rate over time. Finally, the clavicle does not influence other anthropological tests (Stout and Paine 1992); the bone is not an essential elements for tests that determine utile biological profiles such as statue and sex.

The purpose of this study is to provide a new regression equation using various histomorphometric features of the clavicle for age estimation that can be applied in different forensic situations. Additionally, this study also aimed to show a positive application of the equation, which is founded on histomorphometric methods. In future studies that utilize diverse bone materials for age estimation, histomorphometric age estimation methods can be applied in the forensic field.

METHODOLOGY

Materials

Right clavicle samples were obtained from 46 dissected cadavers with a known cause of death, age, and sex (28 males and 18 females). The mean age was 47.5 ± 15.8 for men and 47.5 ± 15.8 for women (Table 1). The causes of death from chronic disease, primary bone disease, or other serious conditions that could affect the condition of the bone were excluded. Samples (3 cm long) were cut from the sternal end of the right clavicle and then two sequential slides were made by manual grinding on graded silicon carbide abrasive paper. The bone unit, including features of the osteon, was observed, measured, and calculated using a microscope and an image analysis program.

Table 1: Age distribution of specimens

Age range (years)	*Pooled sexes n (%)*	*Male n (%)*	*Female n (%)*
20-29	7 (15.2)	3 (10.7)	4 (22.2)
30-39	8 (17.4)	5 (17.9)	3 (16.7)
40-49	11 (23.9)	8 (28.6)	3 (16.7)
50-59	9 (19.6)	6 (21.4)	3 (16.7)
60-69	5 (10.9)	3 (10.7)	2 (11.1)
Over 70	6 (13.0)	3 (10.7)	3 (16.7)
Total	46 (100)	28 (100)	18 (100)

Chemical Treatments

The remaining soft tissues from the muscle, fascia, and periosteum adhering to the clavicle, were excised carefully without damaging the subperiosteal space. The remnants could produce measuring error, so pretreatment was performed at each of the following steps as needed. To prevent morphologic change from protein denaturation, the sample was fixed in 10% formalin—a mixture of formalin and distilled water—over seven days to ensure complete permeation. Fixed samples were removed from formalin and soaked in cold running water to remove formalin. After deep rinsing, the samples were transferred to another mixture of chloroform and methanol for 2 days to eliminate the remaining fat inside the bone marrow, without obstructing the examiner's field of vision during observation with the microscope. Finally, the samples were bleached in a 2% H_2O_2 solution for 1 day to maintain sterility.

Serial Sectioning

A 1-mm thick clavicular sample was cross-sectioned using a diamond wheel (IsoMet® 5000 Precision Saw, Buehler Instruments, Lake Bluff, IL USA). Then, two sequential thin sections (approximately 100-ìm thick) were obtained by manual grinding on graded silicon carbide abrasive papers. The 4-inch wheel operated at a speed of 300 rpm, and the samples were fully locked in the clamp. Despite this high speed, the samples were unexposed to frictional heat because over one-third of the wheel surface was submerged from the beginning. The paper was moistened to avoid frictional heat throughout the manual grinding work. Researchers followed the Frost technique (Ahlqvist 1969) to apply strength equally during the overall manual grinding process. Once the sample appeared transparent, with an expected thickness of approximately 100 ìm, manual grinding was stopped and the sections were immersed in distilled water using ultrasonic cleaner for approximately 2 min to loosen any tiny debris.

Slide Preparation

Dehydration was performed at room temperature using varying concentrations of alcohol, from 60% to 100%. Dehydration at each concentration took 3 min, except at the absolute (100%) concentration. With 100% alcohol, extra moisture inside the sections was eliminated in 5 min. After the dehydration procedure was complete, the specimens were soaked in xylene for 1 min to remove any alcohol. The sections were mounted in Entellan (Merck, Darmstadt, Germany) and covered with a glass cover slip.

Histomorphometric Analysis

The histomorphometric analysis performed in this study was based on captured images from a polarizing microscope (BX-51, Olympus, Tokyo, Japan) equipped with a polarizing filter (BX-POL). The total magnification was 100x, fitted with a 10 × 10 mm^2 eyepiece reticle with a grid area of 1.0 mm^2. These grids enable consistent visual measurements. Three histomorphometric variables including the ratio of the relative cortical area (RCA), osteon population density (OPD), and mean osteon area (OA), were measured microscopically and counted using an image analysis program (Image J, NIH Image, Bethesda, MD). For OA, data were obtained from 8 surface points in all directions and read in a checkerboard pattern (Fig. 1). The measured OA of the 8 points was regarded as a representative value of the entire cross-sectional OA for analysis in this study.

Statistics

SPSS 13.0 was used for data analysis (SPSS, Inc., Chicago, IL). The difference between the two serial slides was verified by a paired sampled t-test. The statistical differences were confirmed between sexes through the analysis of covariance (ANCOVA) when age was adjusted. A simple linear regression analysis showed a general association between the estimated age at death and each variable. Finally, multivariate linear regression analysis using the stepwise method provided an age estimation equation applicable to the clavicle.

RESULTS

Descriptive statistics of all histomorphometric variables for age classification and sex are recorded in Table 2. As seen in Table 2, a nega-

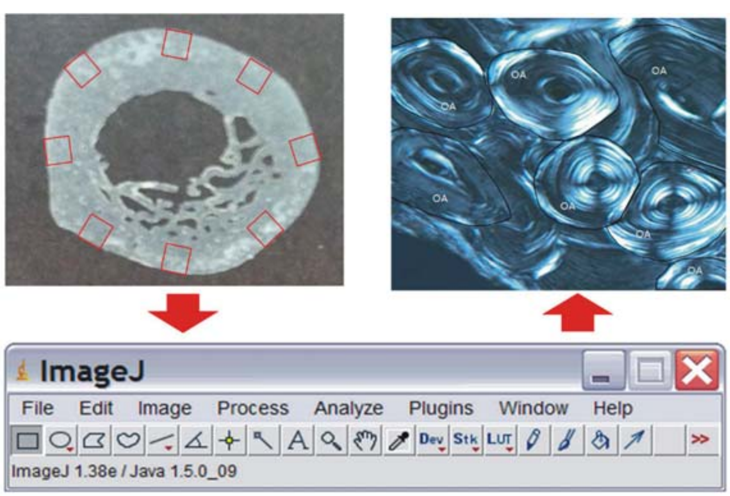

Fig. 1. Measuring osteon area (OA) by using image analysis program (image J,NIH Image, Bethesda, MD)

Table 2: Descriptive statistics of histomorphometric variables for the pooled and separate sexes (mean ± standard deviation)

	RCA (ratio)	*OPD (#D mm²)*	*OA (mm²)*
Pooled Sexes (n = 46)			
20–29 years	64.740 ± 24.559	21.714 ± 1.945	0.025 ± 0.002
30–39 years	62.734 ± 10.209	22.526 ± 3.761	0.025 ± 0.003
40–49 years	62.539 ± 9.145	23.524 ± 3.166	0.027 ± 0.002
50–59 years	55.519 ± 7.819	26.008 ± 3.072	0.026 ± 0.005
60–69 years	44.204 ± 7.678	26.911 ± 3.860	0.027 ± 0.005
Over 70 years	44.222 ± 18.618	33.216 ± 4.434	0.019 ± 0.002
Males (n = 28)			
20–29 years	84.763 ± 27.402	22.343 ± 2.999	0.025 ± 0.003
30–39 years	66.160 ± 5.789	23.222 ± 3.565	0.023 ± 0.001
40–49 years	67.035 ± 5.615	24.273 ± 2.992	0.026 ± 0.002
50–59 years	56.421 ± 6.973	26.950 ± 1.865	0.025 ± 0.004
60–69 years	47.117 ± 9.078	29.339 ± 1.632	0.027 ± 0.004
Over 70 years	61.189 ± 1.358	30.081 ± 3.113	0.020 ± 0.002
Females (n = 18)			
20–29 years	49.724 ± 2.031	21.242 ± 0.937	0.024 ± 0.002
30–39 years	57.024 ± 14.187	21.367 ± 3.985	0.028 ± 0.004
40–49 years	50.550 ± 3.365	21.525 ± 3.248	0.028 ± 0.003
50–59 years	53.716 ± 10.755	24.125 ± 4.590	0.029 ± 0.007
60–69 years	39.834 ± 2.706	23.269 ± 3.172	0.027 ± 0.007
Over 70 years	27.254 ± 1.050	36.351 ± 3.160	0.018 ± 0.002

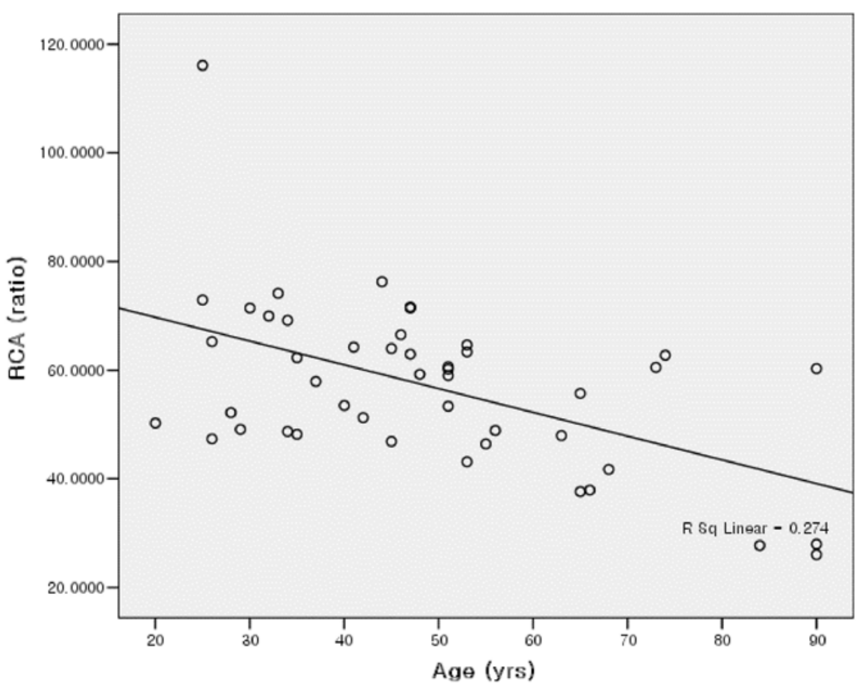

Fig. 2. Bivariate plots of histomorphological variables of relative cortical area (RCA) according to the age. The related regression results are summarized in Table 2.

tive correlation was observed between RCA and OA with age increment (Figs. 2 and 4) and a positive correlation with OPD was observed using simple linear regression analysis (Fig. 3).

No significant difference in any measurement was noted between the two serial slides (P>0.05). Therefore, a mean value of the two slides was indicated and referred to in this research (Table 3). Table 4 shows the results of the analysis of covariance with age as a covariate. No significant difference was seen in OPD and OA between sexes when age was adjusted, with the exception of RCA. Therefore only the value of pooled sexes was applied to the analysis. The results of the standard regression analysis used in this study are shown in Table 5. Among the three indicated parameters in Table 5, OPD showed the highest correlation between estimated age (R^2= 0.583). The OPD and RCA were selected for the age-predicting equation from the multiple regression analysis using the stepwise method (R^2= 0.628) (Table 6). Because OA showed the lowest correlations, it was not selected for multiple regression analysis for the age estimation equation.

DISCUSSION

Conventional methods such as visual inspection of the articular surface are common estimation techniques and that have been widely used. These methods determine the degenerative conditions and the beginning of the epiphyseal union using medical imaging analysis based on gross morphology (Balseven-Odabas 2013; Kellinghaus et al. 2010; Mughal et al. 2013). The typical articulation regions used for age estimation include the auricular surface on the ilium, the sternal end of the rib, the suture closure on the cranium, and the symphyseal surface of the pubis (Iscan 1984; Lovejoy et al. 1985; Meindl 1985; Brooks and Suchey 1990). There is currently absolute criteria for age estimation that has been establish using this technique, and thus the estimated results had a wide range with a large standard estimation error. The histomor-

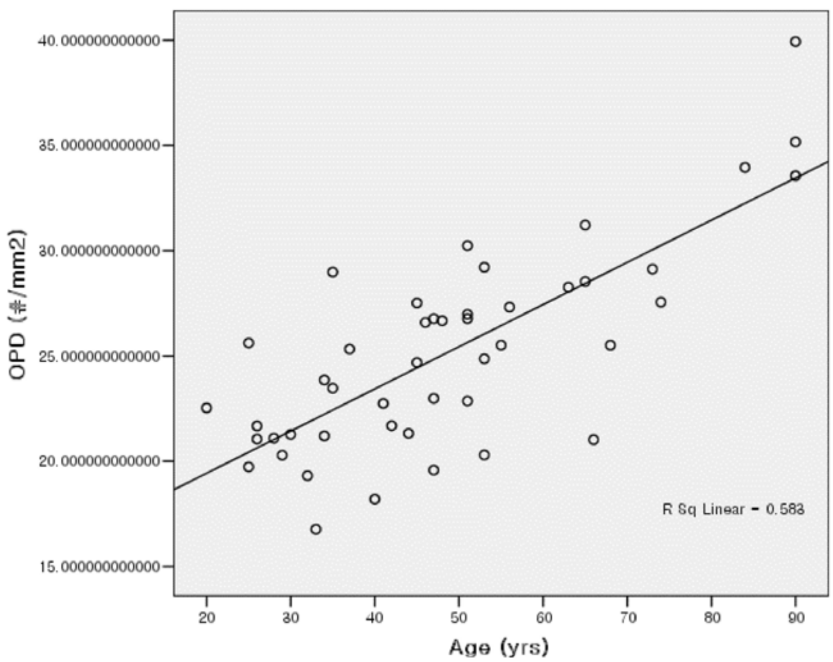

Fig. 3.Bivariate plots of histomorphological variables of osteon population density (OPD) according to the age. The related regression results are summarized in Table 2.

Table 3: Results of paired *t*-test between two serial slides

Measurements	*n*	*Mean ± SD*	*Differences*	*Significance**
RCA1-RCA2	46	0.424 ± 1.566	45	0.073
OPD1-OPD2	46	0.029 ± 1.270	45	0.877
OA1-OA2	46	0.001 ± 0.005	45	0.060

*P > 0.05.

Table 4: Results of multivariate and univariate analysis of covariance

Variable	*Parameter*	*B*	*t*	*Significance*	*Partial n²*
RCA*	Intercept	84.426	18.573	0.000	0.889
	Age	-0.427	-5.044	0.000	0.372
	Sex (F)	-16.481	-5.255	0.000	0.391
OPD*	Intercept	15.878	11.682	0.000	0.760
	Age	0.201	7.943	0.000	0.595
	Sex (F)	-1.254	-1.338	0.188	0.040
OA*	Intercept	0.028	16.450	0.000	0.863
	Age	-7E-005	-2.205	0.033	0.102
	Sex (F)	0.001	0.632	0.531	0.009

*The parameter of each dependent variable [Sex = M] was set to zero.

Table 5: Simple regression analysis between age and histomorphometric variables

Variable	*Intercept*	*Slope*	*SEE*	*R²*	*P*
RCA†	84.604	-0.627	15.746	0.274	0.000
OPD†	-24.566	2.911	11.934	0.583	0.000
OA†	84.439	-1428.534	17.538	0.100	0.033

*SEE, Standard Error of Estimate (years).
†P < 0.05.

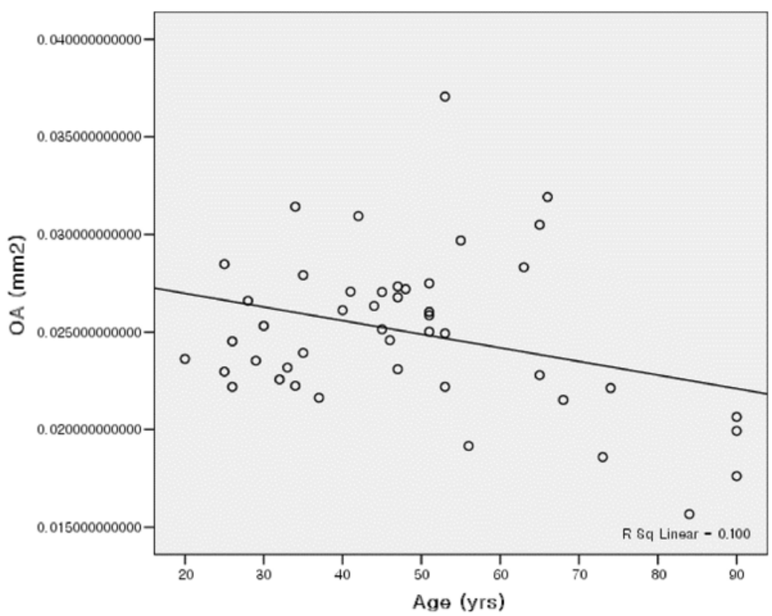

Fig. 4. Bivariate plots of histomorphological variables of osteon area (OA) according to the age. The related regression results are summarized in Table 2.

phometric method usually measures and quantifies various features of the osteon, mainly the secondary osteon with a clear cement line, to provide more absolute criteria in age estimation (Bednarek 2008). Based on this process, the histomorphometric method can be used even when the bone is discovered in fragments. Under normal circumstances, fragmented or eroded bone increases uncertainty in forensic fields, with little success in identification, and can hinder the

Table 6: Age-predicting equations conducted from stepwise regression analysis

Equation[*]	*Adjusted R^2*	*SEE*[†]
Age = 1.412 - 0.282(RCA) + 2.519 (OPD)	0.628	11.403

[*]Multicollinearity did not exist among the independent variables, and the linear regression model was verified by ANOVA at the 1% level of significance.
[†]SEE, Standard Error of Estimate.

Table 7: Comparison with previous study using clavicles

	Bone element	*Population*	*R^2*	*SEE*[*]
Present study	Clavicle	Korean (n = 46)	0.628	11.403
Stout and Paine (1992)	Clavicle	American (n = 40)	0.699	-

[*]SEE, Standard Error of Estimate.

assessment of biological profiles. Therefore, the histomorphometric method can be applied as a reliable and alternative method of age estimation.

Because the osteonal remodeling rate is different for each bone, histomorphometric studies have progressed based on the various bone elements (Frost 1958; Thompson and Gunnes-Hey 1981; Thompson 1983). Among these bones, the clavicle is mostly often found intact and has a low possibility for fragmentation. The clavicle is not a weight-bearing bone, and so is not affected by physical exercise, labor activity and life style that could influence the osteonal remodeling rate over time (Stout and Paine 1992). Despite this, only a few studies have incorporated the clavicle to estimate age at death except in Caucasian populations. Therefore, the researchers attempted to determine the reliability of the clavicle as an age indicator by confirming its regression relationship with age at after the rib and femur, especially in the Korean population.

The researchers used materials of various ages to support our conclusion. If the age groups were disproportionate, the research results would be biased, distorted, and thus less convincing (Stout et al. 1994). Therefore, this study endeavored to consider all age groups, which allows the deduced regression equation for age estimation to be applied in different age groups which are indicated in Table 1.

ANCOVA was the method used to observe the effect of covariates and age in this study. The researchers adjusted age to determine how sex affected variables thorough a general linear model (Osborne et al. 2004). When age is adjusted, only RCA was significantly different between sexes in this study (Table 4). As a result, sex should have been considered in RCA measurement. However, RCA essentially differs depending on sex (Mughal et al. 2014), and RCA is a ratio and not a directive value. In addition, considerable preceding research reported no significant relationship between bone composition and sex (Kerley 1965; Ericksen 1991). Therefore, in our final analysis, the researchers do not divide our data set into two parts (male and female) to develop an equation, although sex is considered in RCA measurement.

Table 5 shows that the OA was fairly unrelated with estimated age. However, another study showed contrasting results (Osborne et al. 2004). In this study, OA was not measured at all surfaces but at the surrounding 8 parts in all directions using the protractor on cortical bone. Until now, no clear objective criteria have been outlined to measure the average osteon area for age estimation. Accordingly, to take an objective approach for the overall distribution of the osteon area, the selected 8 parts were presented as criteria in this study. However, the results showed that OA did not adequately reflect the remodeling rate. Thus, for our additional study, the researchers proposed that the unremodeled area rather than the osteon area alone should be applied for histomorphometric age estimation.

The result of previous research regarding age estimation with bone histology from the clavicle is shown in Table 7. Stout and Paine (1992) used the clavicle as material and developed individual regression equations. The regression correlation based on the sole clavicle in the Stout and Paine study (R^2=0.699) is similar to those reported in this study (R^2=0.628). Nonetheless, comparison of descriptive statistics with previous study reveals that the osteonal remodeling rate is not in agreement with population groups. Age estimation has to be approached using multifactorial methods. Therefore, to resolve the populational differences, applying various types of bone elements measurement techniques, is suggested to achieve a more accurate estimate of the age at death. Consequently, the different bones should be collectively studied and compared with the clavicular remodeling rate to determine the age correlation between ages on histomorphometric analysis.

In conclusion, this study identified a reliable age estimation equation using repeatable and constant histomorphometric measurements of the clavicle which can be applied especially in Korean adults. In addition, age estimation using the clavicle can be applied in situations such as and international airplane crash that involves multinational victims. The results of this study could be applied and expanded if more diverse bone elements from all age groups are included and verified for accurate forensic assessment.

ACKNOWLEDGEMENTS

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0002383).

REFERENCES

Absolonova K, Veleminsky P, Dobisikova M, Beran M, Zocova J 2013. Histological estimation of age at death from the compact bone of burned and unburned human ribs. *J Forensic Sci,* 58:135-145.

Ahlqvist J, Damsten O 1969. A modification of Kerley's method for the microscopic determination of age in human bone. *J Forensic Sci*, 14: 205-212.

Balseven-Odavasi A, Yalcinozan E, Keten A, Akcan R, Tuner AR, Onaa A, Canturk N, Odavasi O, Dince AH 2013. Age and sex estimation by metric measurements and fusion of byoid bone in a Turkish population. *Journal of Forensic and Legal Medicine,* 20: 496-501.

Bednarek J 2008. Methods of age at death estimation based on compact bone histomorphometry. *Arch Med Sadowej Kryminol,* 58: 197-204.

Brooks S, Suchey JM 1990. Skeletal age determination based on the os pubis: A comparison of the Acsádi-Nemeskéri and Suchey-Brooks methods. *Human Evolution, 5*: 227-238.

Buikstra JE, Ubelaker DH 1994. Standards for Data Collection from Human Skeletal Remains. *Arkansan Archaeological Survery Report* Number 44, Fayettevill, Arkansas

Formicola V 1993. Stature reconstruction from long bones in ancient population samples: An approach to the problem of its reliability. *Am J Phys Anthropol*, 90: 351-358.

Frost HM 1958. Preparation of thin undecalcified bone sections by rapid manual method. *Stain Technol*, 33: 273-277.

Frost HM 1987. Secondary osteon populations: An algorithm for determining mean bone tissue age. *Year book of Physical Anthropology,* 30: 221-238.

Han SH, Kim SH, Ahn YW, Huh GY, Kwak DS, Park DK, Lee UY, Kim YS 2009. Microscopic age estimation from the anterior cortex of the femur in Korean adults. *J Forensic Sci,* 54: 519-522.

Iºcan MY, Loth SR, Wright RK 1984. Age estimation from the rib by phase analysis: White males. *J Forensic Sci,* 29: 1094-1104.

Kellinghaus M, Schulz R, Vieth V, Schmidt S, Schmeling A 2010. Forensic age estimation in living subjects based on the ossification status of the medial clavicular epiphysis as revealed by thin-slice multidetector computed tomography. *Int J Legal Med,* 124: 149-154.

Kerley ER 1965. The microscopic determination of age in human bone. *Am J Phys Anthropol,* 23: 149-163.

Kim YS, Kim DI, Park DK, Lee JH, Chung NE, Lee WT, Han SH 2007. Assessment of histomorphological features of the sternal end of the fourth rib for age estimation in Koreans. *J Forensic Sci*, 52: 1237-1242.

Lovejoy CO, Meindl RS, Pryzbeck TR, Mensforth RP 1985. Chronological metamorphosis of the auricular surface of the ilium: A new method for the determination of adult skeletal age at death. *Am J Phys Anthropol*, 68: 15-28.

Meindl RS, Lovejoy CO 1985. Ectocranial suture closure: A revised method for the determination of skeletal age at death based on the lateral-anterior sutures. *Am J Phys Anthropol,* 68: 57-66.

Mughal AM, Hassan N, Ahmed A 2014. Bone age assessment methods: A critical review. *Pak J Med Sci,* 30: 211-215.

Osborne DL, Simmons TL, Nawrocki SP 2004. Reconsidering the auricular surface as an indicator of age at death. *J Forensic Sci,* 49: 905-911.

Stout SD, Dietze WH, Iºcan MY, Loth SR 1994. Estimation of age at death using cortical histomorphometry of the sternal end of the fourth rib. *J Forensic Sci,* 39: 778-784.

Stout SD, Lueck R 1995. Bone remodeling rates and skeletal maturation in three archaeological skeletal populations. *Am J Phys Anthropol,* 98: 161-171.

Stout SD, Paine RR 1992. Brief communication: Histological age estimation using rib and clavicle. *Am J Phys Anthropol,* 87: 111-115.

Thompson DD, Galvin CA 1983. Estimation of age at death by tibial osteo remodeling in an autopsy series. *Forensic Sci Int,* 22: 203–211.

Thompson DD, Gunness-Hey M 1981. Bone mineral-osteon analysis of Yupik-Inupiaq skeletons. *Am J Phys Anthropol,* 55: 1-7.

Trotter M, Gleser GC 1958. A re-evaluation of estimation of stature based on measurements of stature taken during life and of long bones after death. *Am J Phys Anthropol,* 16: 79-123.