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K. Imaizumi, K. Taniguchi & Y. Ogawa

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ORIGINAL ARTICLE

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Abstract During forensic casework, it is vital to be able to obtain valuable information from burnt bone fragments to ascertain the identity of the victim. Here, we report the findings of an experimental study on burnt bovine compact bone segments. Compact bones were cut to size and heated in an electric furnace at a temperature range of 100–1,100 °C with 100 °C increments. Heat-induced alterations to the bone color, weight, volume, and density were monitored using gross morphology and micro-focus X-ray computed tomography. We found that the increase in temperature caused the color of the compact bones to change in order of yellow, brown, gray, and white. In contrast to the weight reduction that occurred immediately after burning, we measured no significant reduction in volume even at 600 °C; however, volume reduced drastically once the temperature reached 700 °C. Light microscopic histological observations of burnt bone revealed heat-induced alterations such as cracking and separation of the osteons at higher temperatures. In addition to these findings, we sought to examine the survival of DNA in the burnt bones using polymerase chain reaction of mitochondrial DNA. No amplification was found in the specimens burnt at 250 °C or higher, indicating the likely difficulty in testing the DNA of burnt bones from forensic casework. The results of this study will enable an estimation of the burning temperatures of burnt bones found in forensic cases and will provide an important framework with which to interpret data obtained during anthropological testing and DNA typing.

Keywords Forensic anthropology · Forensic DNA analysis · Burnt bone · Cremation · Computed tomography

Introduction

In forensic casework, we frequently encounter burnt bones of unknown identities [1–8]. Anthropological techniques, which include observations and morphometric analyses of gross morphology and histology, are frequently employed to estimate the species, sex, age, and time since death, among other parameters, of burnt bone specimens. Results from these anthropological tests are often confounded by alterations to the bone shape, such as cracks and bone shrinkage, which occur after exposure to harsh, high temperatures. These changes make it difficult to interpret the data obtained in tests. Furthermore, surface alterations, such as a color or texture changes to the bone surface, can also create problems in estimating time since death and cause of death. In addition to these physical changes, heat will induce chemical modifications to compact bones due to combustion and pyrolysis, such as DNA degradation, which can also crucially hinder forensic identification of the victims.

To account for these problems when performing forensic identification, it is necessary to estimate how the burnt bone specimens had been heated during burning. There are several reports regarding the relationship between burning temperatures and the physical alterations to bone, such as color, weight, and length [9–17]. In addition, the histological alterations that occur in burnt bone have been studied with both light microscopy [18, 19] and electron microscopy [20, 21], and the chemical changes, such as the heat-induced conversion of bone hydroxyapatite to β-tricalcium phosphate (β-TCP) and the decrease in collagen composition, have been examined using X-ray diffraction [10, 13, 15] and Fourier transform infrared spectroscopy [22, 23], respectively. The

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K. Imaizumi (✉) · K. Taniguchi · Y. Ogawa
Second Forensic Biology Section,
National Research Institute of Police Science,
6-3-1, Kashiwanoha, Kashiwa, Chiba 277-0882, Japan
e-mail: imaizumi@nrips.go.jp

elemental analyses of Ca, P, and Sr, among other elements, have also been undertaken on experimentally burnt compact bone using atomic absorption spectroscopy [15]. These physical, chemical, and histological properties have been summarized in detail elsewhere [14, 16, 17, 24–27], with each review illustrating a schema for the stepwise progression of changes that occur to compact bone during burning. These schemas are not only valuable in estimating the temperature to which the specimen was exposed but also provide information on how tests should be performed when examining and testing burnt bone in the forensic setting.

Unlike the studies on the physical and chemical properties of burnt bones, there is a paucity of available literature on the probability of obtaining suitable DNA from burnt bone specimens for analysis [28, 29]. Given that DNA-based applications have been crucial in the identification of bony remains in many recent forensic cases, it is becoming increasingly important to know whether it is possible to perform DNA analyses on burnt bone specimens.

The aim of this study was to explore the physical changes to bovine compact bone after exposure to burning under different experimental conditions and to ascertain the utility of polymerase chain reaction (PCR) as a tool for identifying burnt bone samples. In particular, we employed micro-focus X-ray computed tomography (CT) to accurately pinpoint the changes to bone volume that occur with burning. The results obtained in this study will be useful in forensic practice in the identification of burnt bone.

Materials and methods

Preparation of bone specimens

Bovine metacarpal bones were obtained from a slaughterhouse in Ibaraki, Japan. After removing the remaining attached soft tissues, the bone shafts were cut into approximately 1.5-cm-thick round slices using a cast cutter (810 Autopsy Saw; Stryker Corporation, Kalamazoo, MI, USA) equipped with a sterilized blade. Residual bone marrow in the marrow cavity and the outer periosteum remained were removed, and the cylindrical slices were cut radially to obtain approximately 1.5-cm cubic specimens. Each specimen was weighed with an electric scale (EK-400H; A&D Co., Ltd., Tokyo, Japan) and kept in a freezer at −80 °C until use.

Burning conditions

Specimens were experimentally burnt using an electric muffle furnace (FO 100; Yamato Co., Ltd., Tokyo, Japan). Objects placed in its chamber can be heated across a range of temperatures (100–1,100 °C), and the temperature can be kept stable for the preset time interval. The chamber is not isolated strictly from outside, so that the burning proceeds under aerobic conditions. We chose to examine the effect of burning temperatures from 100 to 1,100 °C in 100 °C increments. Three specimens were placed into the chamber on a heat-resistant tray immediately after the desired temperature was reached, and burnt for 30 min or 2 h. The specimens were removed from the furnace and then cooled at room temperature. Each burnt specimen was weighed again and photographed using a digital incident-light microscope (VHX-500; Keyence Co., Ltd., Osaka, Japan) at low magnification (×10) at a 30° tilt under constant lighting.

Volume analysis of burnt bone specimens

Micro-focus X-ray CT scanning at 120 kV and 60 μA (SMX-225CT-SV; Shimadzu Co., Ltd., Kyoto, Japan) was performed on each specimen before and after burning to analyze volume changes induced by heating. A total of 1,200 transparent X-ray images were captured as the specimen was rotated 360°, resulting in a collection of 512 slices of 512×512-pixel size. The CT data were reconstructed by a CT reconstruction software (CT Solver, Shimadzu), and three-dimensional (3D) images of the specimens were constructed using CT image analysis software (VGStudio MAX 1.2; Volume Graphics GmbH, Heidelberg, Germany). Volumetric analyses of the specimens were analyzed using the same software.

Histological observations of burnt bone specimens

Histological images of the burnt specimens were observed by X-ray microradiography [30]. Specimens were embedded in epoxide resin (SpeciFix Resin; Marumoto Struers, Co., Ltd., Tokyo, Japan) under 100-mbar vacuum pressure to immerse the resin deep inside the specimen. The embedded bone was cut into 300-μm-thick slices using a precision high-speed cutter (Accutom-50, Marumoto Struers), and slices were ground to approximately 150-μm-thick slices using an automatic sanding machine (RotoPol-15, Marumoto Struers). Microradiograms of the bone slices were taken with a soft X-ray apparatus (OMC-603; Omicron, Tokyo, Japan) at 13 kVp, 3 mA, and exposure time of 30 min, and the images were analyzed using microscopic observation. The high-speed cutter was also used to create 2-mm-thick slices of each specimen to observe the inside architecture of the burnt bone using incident-light microscopy.

DNA extraction and amplification of burnt bone specimens

In addition to studying physical changes induced by burning, we also examined the potential utility of these burnt samples for DNA analysis. These experiments were performed on samples heated over smaller (50 °C) increments, from 150 to 300 °C, and over shorter time intervals (15, 30, 45, 60, 120,

and 180 min). The burnt specimens were pulverized using a Multi-Beads Shocker (Yasui Kiki, Osaka, Japan), and the powder was decalcified with 0.5 M EDTA, pH 8.0 (Nippon Gene, Tokyo, Japan) at 56 °C overnight. Excess EDTA was removed by washing the powder twice with sterilized water and once with TNE buffer (10 mM Tris–HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA, pH 8.0). The powder was digested with 3 mL of TNE buffer containing 0.5 % sodium dodecyl sulfate (SDS, Nippon Gene) and 0.5 mg/mL of proteinase K (Wako, Osaka, Japan). DNA was isolated by phenol–chloroform extraction, and the extract was concentrated with a filter device (Centricon YM-100; Millipore, Billerica, MA, USA). The final extract was adjusted to 100 μL with TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA, pH 7.5) and kept at −80 °C until use. PCR was carried out using a thermal cycler (GeneAmp PCR system 9700; Applied Biosystems, Tokyo, Japan) at 95 °C for 9 min, followed by 33 cycles of 95 °C for 45 s, 60 °C for 30 s, and 72 °C for 2 min. Of the DNA extract (×1, ×10, ×100), 1 μL was amplified in 25 μL of the PCR reaction containing 1× PCR GOLD buffer, 200 μM dNTPs, 1.0 U of AmpliTaq GOLD DNA polymerase, 2.5 mM $MgCl_2$ (Applied Biosystems), and 0.5 μM of primers. PCR was performed for amplicons of different target lengths: a 419-bp product within the bovine mtDNA D-loop region [31] and a 128-bp product within a 16S ribosomal RNA (rRNA) coding region of bovine mtDNA [32]. The nucleotide sequences for the primers were as follows: 5′-CACAGAATTTGCACCCTAAC-3′ and 5′-CCCTGAAGAAAGAACCAGAT-3′ (D-loop) and 5′-AACCATTAAGGAATAACAACAA-3′ and 5′-CAATGAGCGATAGAGTGATTT-3′ (16S rRNA). The PCR product was separated by electrophoresis on 2.0 % agarose gels (SeaKem ME; FMC BioProducts, Rockland, ME, USA) in 1× TAE buffer (40 mM Tris–acetate, 1 mM EDTA; Life Technology, Grand Island, NY, USA) for 20 min at 100 V. The gel was stained with ethidium bromide, and the DNA bands were visualized with a gel imaging system (Bio Image Gel Print 2000i; Genomic Solution, Ann Arbor, MI, USA).

Results

Macroscopic morphology of the burnt bone specimens

We examined the surfaces and internal aspects of the specimens after burning for 30 min or 2 h across a range of temperatures (100–1,100 °C) (Supplementary Fig. 1). The color of the specimens changed drastically with the rise in temperature. Specimens burnt for 2 h became yellow-brown at 200 °C, whereas those specimens burnt for 30 min remained yellow-white at the same temperature. No such time-dependent difference in color was discernible in the samples burnt at 300 °C or higher: the surface was a charcoal-like, dark brown at 300–400 °C, an ash-like gray at 500–600 °C, and a chalk-like white at 700 °C, which did not change significantly at higher temperatures. We also sectioned samples further to examine the internal aspects of the bones after burning. For both 30-min and 2-h burning, the color of the inside of the samples correlated well with that of the surface aspect until 600 °C. However, at temperatures higher than 700 °C, samples that had been burnt for 30 min appeared bicolored inside, with black and white regions of the bone, whereas samples burnt for 2 h demonstrated an even color distribution.

Besides these changes in color, we also observed temperature-dependent differences in the toughness of the specimens. Specimens burnt at between 300 and 600 °C, with a dark brown or gray appearance after cooling, were very fragile. However, white specimens generated after exposure to temperatures of 700 °C or higher were much harder.

Weight and volume changes after burning

We next assessed the changes in bone weight and volume after burning. These changes were indicated by the percent difference between values obtained before and after burning. Bone weight measurements were taken with an electric scale. We found that specimen weight decreased drastically between 100 and 500 °C and then reached a plateau at approximately 60 % of the before-burning weight, with no significant decreases observed at these higher temperatures (Fig. 1). Changes in

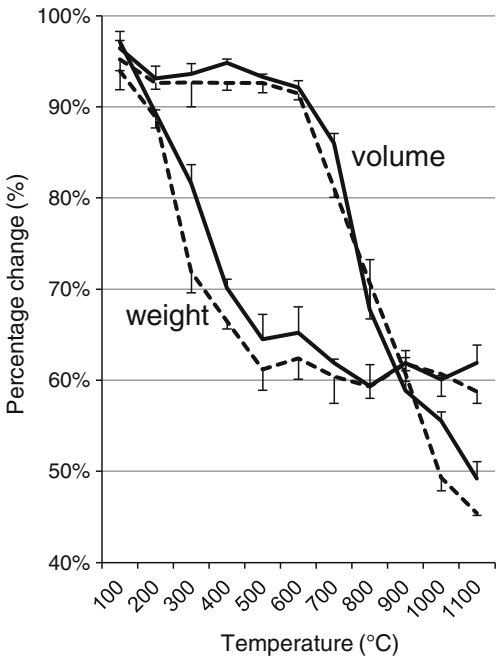

Fig. 1 Temperature-induced changes in weight and volume of burnt bone samples. Percentage change in weight and volume of the samples burnt for 30 min (*solid line*) or 2 h (*dashed line*). Sample volume and weight before burning are indicated as 100 %. *Error bars* indicate the standard deviation (n=3)

bone volume were analyzed using 3D images constructed using the micro-focus X-ray CT system, as shown in Fig. 2. We obtained detailed shapes of the specimens and identified shrinkage of the specimens burnt at 800–1,100 °C (Fig. 2b′, c′). Using these 3D images, we plotted the volume changes of the specimens (Fig. 1). In contrast to the weight changes, no significant decrease in volume was observed until 600 °C, after which the volume decreased drastically. This intense volume reduction, accompanied by the significant shrinkage in the specimens, resulted in a reduction of bone volume to less than half of its original volume at 1,100 °C.

Figure 3 shows the changes in density as a percentage of the before-burning density, calculated from the values obtained in the weight and volume analyses. We found that, after a marked drop in density from 100–500 °C, the density gradually increased to reach 130 % at 1,100 °C.

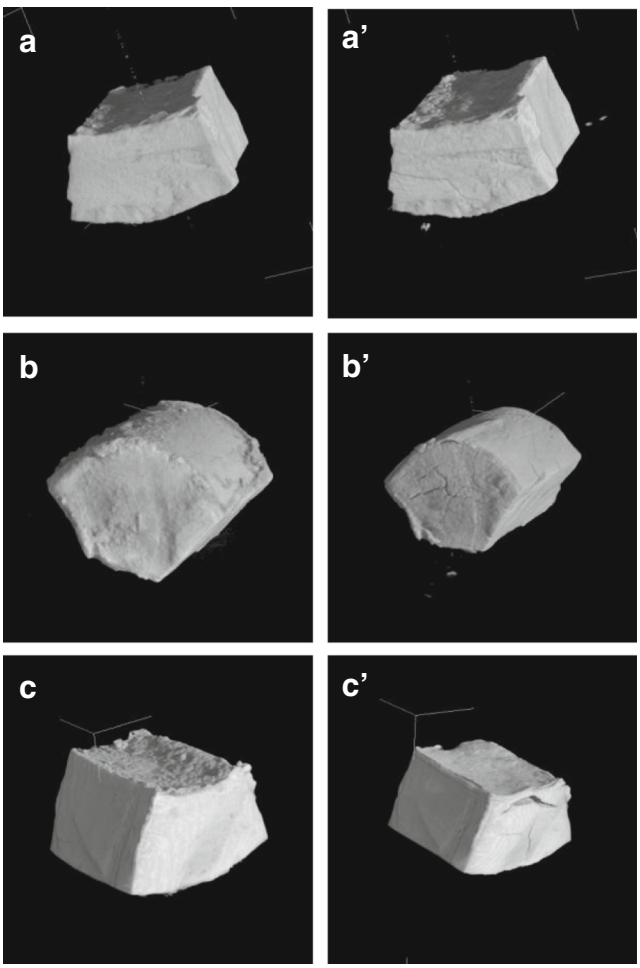

Fig. 2 Three-dimensional micro-focus X-ray CT system imaging of the bone specimens before and after burning. Scaling of the images is the same. Examples of three specimens before (**a**–**c**) and after (**a′**–**c′**) burning. Specimens were burnt at **a′** 400 °C for 30 min, **b′** 800 °C for 30 min, and **c′** 1,100 °C for 30 min. The volumes for each specimen before and after burning were **a**, **a′** 985.0 and 937.0 mm, **b**, **b′** 995.4 and 641.1 mm^3, and **c**, **c′** 998.4 and 500.1 mm^3

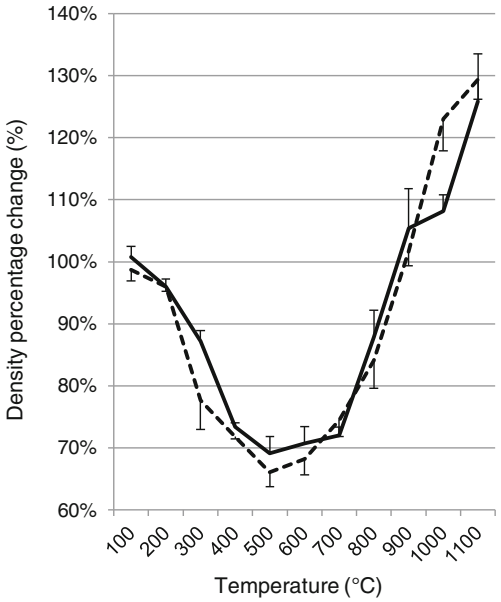

Fig. 3 Changes in density calculated from values obtained for the weight and volume analyses in Fig. 1. Data are the percent density change with respect to values before burning (indicated as 100 %). Specimens were burnt for 30 min (*solid line*) or 2 h (*dashed line*). *Error bars* indicate the standard deviation (n=3)

Histological changes to burnt bones

Figure 4 shows the X-ray microradiograms of the burnt bones. The various components of compact bone can be observed, such as osteons (Haversian canal and lamellae), osteocyte lacunae, and interstitial lamellae. Minute cracking, which tended to arise from the Haversian canal, could be observed in samples subjected to temperatures of 500 °C, with an increase in the size and number of cracks observed with higher temperatures. A unique change was observed at 1,000–1,100 °C, with imaging revealing separation of the osteon from the surrounding interstitial lamellae.

Utility of DNA analyses after burning

Finally, we sought to determine if PCR analyses could be carried out on the DNA extracted from burnt bone samples by targeting two mitochondrial DNA products of varying lengths (419- and 128-bp products). Bone specimens were burnt at a temperature range of 150–300 °C, with 50 °C increments, for various time periods. We found a complete lack of amplification for all specimens burnt at temperatures above 250 °C; therefore, here we present the results only for 150 and 200 °C (Table 1). At 150 °C, mitochondrial DNA was successfully amplified, even in samples burnt for 180 min. However, the success of amplification drastically decreased at temperatures over 200 °C, with only bone specimens burnt for 15 min showing amplification for both products. Slight

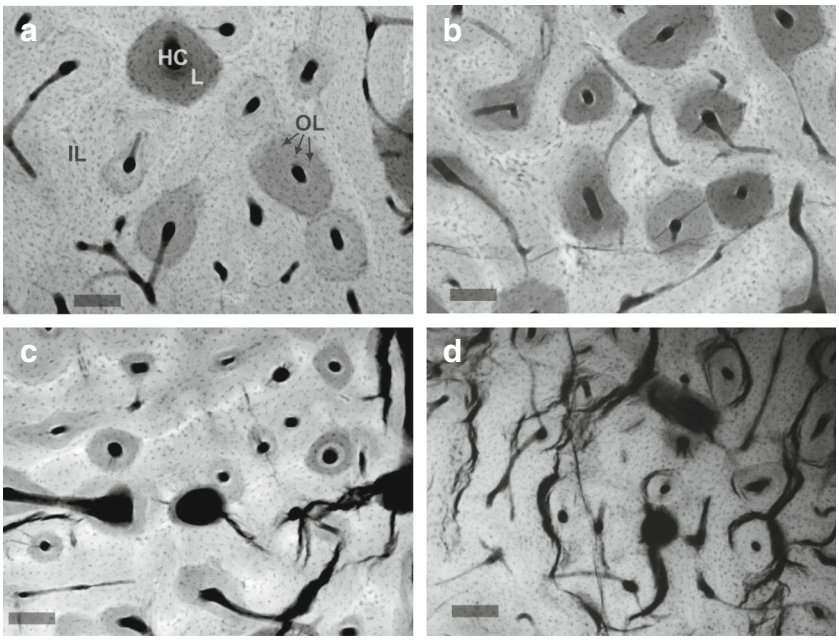

Fig. 4 X-ray microradiographs of the specimens after burning at different temperatures for 2 h: **a** 200 °C, **b** 500 °C, **c** 800 °C, and **d** 1,100 °C. *HC* Haversian canal, *L* lamellae, *OL* osteocyte lacunae, *IL* interstitial lamellae. *Bars* 100 μm

differences in amplification were found between the two target products after burning at 150 °C for 180 min and 200 °C for 30 min, with only the shorter target (128 bp) able to be amplified with DNA diluted at 1:100 and 1:1, respectively.

Discussion

To clarify the changes that occur in compact bone after burning, we experimentally burnt bovine compact bone specimens under several conditions and examined changes in the macroscopic and microscopic physical properties of the bone samples, as well as the suitability of the extracted DNA for successful PCR analysis.

Table 1 Amplification of mtDNA in burnt bones

Temperature (°C)	Time (min)	Dilution of the DNA extract		
		1:1	1:10	1:100
150	15	+/+	+/+	+/+
	30	+/+	+/+	+/+
	45	+/+	+/+	+/+
	60	+/+	+/+	+/+
	120	+/+	+/+	+/+
	180	+/+	+/+	−/+
200	15	+/+	+/+	+/+
	30	−/+	−/−	−/−
	45	−/−	−/−	−/−
	60	−/−	−/−	−/−
	120	−/−	−/−	−/−
	180	−/−	−/−	−/−

Results are shown as absent (−) or present (+) for 419- or 128-bp amplification, respectively, in agarose gel electrophoresis

The burnt bones varied in color with the variations in temperature. Roughly classified, the bone surface changed in order of yellow, brown, gray, and white with the increase in temperature. There are several reports regarding the color changes to bones as a result of burning [9, 14, 16, 17, 21–23, 25], with results differing slightly because of differences in specimen size, type of the furnace, oxygen supply, and burning time, as well as other factors. Walker et al. [16] compared the color changes to the bone over different lengths of burning time (1, 2, and 3 h) and how this differed with the surrounding material (none, diatom earth, and topsoil). The color changing patterns obtained in our experiment were almost consistent with their observations under aerobic (no surrounding material) conditions. In their study, they found that a decrease in burning time and oxygen supply resulted in a delay in color change. A similar time-dependent delay in coloration was observed at 200 °C in our experiment. We also observed coloration differences between the surface and inside of the specimens at higher temperatures. This delay in coloration on the inside of the bone is probably due to the insufficient oxygen supply at deeper layers of the tissue. In forensic casework, we frequently encounter burnt bone specimens for which the identity needs to be determined using anthropological methods and DNA typing. For these tests, it is first important to estimate how severe the bone specimen was burnt, as shrinkage to compact bones confounds anthropological measurements and heat causes DNA degradation. Thus, the color of the burnt bone could be one index with which to estimate the severity of

burning, if considerations are made for burning time and the presence of oxygen. In particular, considering that bones are usually covered with thick soft tissues during the early phase of burning, it is important to take such anaerobic conditions into account for the estimations of temperature.

The weight of the bone specimens decreased immediately after heating. This decrease was drastic until 500 °C after which it became stable at 60 % of the original weight. Kalsbeek and Richter [10] and Grupe and Hummel [15] monitored the weight loss of compact bone after experimental burning from 100 to 1,000 °C and showed results that are consistent with ours with a trend toward a decrease in weight loss. According to Ortner [33], compact bone is composed of 14 % water and 24 % organic matrix (by weight), with the remaining 62 % classified as bone mineral, consisting mainly of calcium phosphate, which is similar to hydroxyapatite. This composition strongly suggests that the weight loss observed in this study was due to the loss of water and organic material during burning, which has been described by Fairgrieve [25] and Kalsbeek [10].

In this study, we used micro-focus X-ray CT to analyze the changes in bone volume with burning. Harvig and Lynnerup [34] performed X-ray CT scanning on cremated bones extracted from urns and showed its capacity to analyze precisely the total volume of the fragmented bones. In their study, they used a medical X-ray CT system, which takes 1-mm-thick slices. In contrast, our X-ray CT system is optimized for small materials and can provide a detailed shape of the burnt specimen, including any minute cracks; this allows for a very precise analysis of volume. We measured a decrease in volume starting at temperatures over 700 °C, which was the opposite of that which occurred for weight loss. After 700 °C, specimen shrinkage was linear and seemed to continue even at the temperatures higher than 1,100 °C. Grupe and Hummel [15] found that bone apatite converted to β-tricalcium phosphate at temperatures above 800 °C, and this change resulted in a volume reduction caused by recrystallization and fusion. It is supposed that the shrinkage observed in this study could be attributed to the combined effect of this physiochemical alteration and the loss of organic material. Holden et al. [21] revealed that the structural features of the compact bones were completely destroyed at temperatures of 1,600 °C. It is conceivable that this would result in further volume reduction as a result of fusion of the spatial structures, such as the Haversian canals and osteocyte lacunae, among other structures. Although our study did not examine temperatures above 1,100 °C, it can be assumed that a reduction in volume would have continued to at least 1,600 °C as a consequence of this "melting."

In our histological examination, we noted structural changes, such as cracking and osteon separation, associated with the increase in temperature. Nelson [19] performed histomorphometrical analyses on thin sections of human femora burnt at between 1,000 °F (538 °C) and 1,500 °F (816 °C) and showed that the mean osteon diameter and osteon area decreased significantly compared with those of intact specimens. The separation of the osteons during burning might be due to a directional difference in the shrinkage between each osteon and its surrounding area, considering the unique concentric-layered structure of the osteon. This difference might result in the cracking seen at the border of the osteon. Quatrehomme et al. [20] employed 3D scanning electron microscopy to observe changes such as cracking in human compact bone. They noted multiple fissures in the compact bone burnt at 1,150 °C at low magnification (×10) and, at higher magnification (×100), observed "melting ice" and "stalagmite" patterns caused by the rearrangement of compact bone. The proportion of cracking and osteon separation differed at each temperature in our study such that would be possible to estimate the burning temperature of burnt bones in forensic case studies by observing the presence of these types of structural changes. It is well known that animal compact bone differs structurally to adult human compact bone. Animal bone samples tend to show lamellar patterns that are formed by primary osteons, rather than secondary osteons; this is primarily because animals, such as bovine and swine, are slaughtered at younger ages for meat [35, 36]. However, studies have illustrated that the likely sizes of secondary osteons and Haversian canals are still significantly smaller in animal bones than in human bones [28, 37–39]. In this study, we used bovine compact bone collected at the slaughterhouse; therefore, these two points need to be considered when extrapolating our results to that of human bone. Considering this, our observations were made in tissue regions primarily composed of secondary osteons. Although we still need to take into account the size differences, it is possible to say that our findings can be extrapolated to estimate the burning temperature of human bone. Holden et al. [8] demonstrated a case study of bone fragments of a fire victim, where they attempted to estimate the burning temperature of the fragments by observing the bone color and microradiographic histology. With microradiography, they estimated the burning temperature to be 1,000–1,200 °C through the use of a region of white bone tissue. We further identified osteon separation in their microradiographs and thus concur with their estimation of the proposed temperature.

Besides the minute fissures in histological observation, burnt bone exhibits visible cracks on its surface and the degree of them increases in severity along with elevation of burnt degree. These cracks raise the problem on the identification of traumatic injuries on bone surface which is crucial for estimating a cause of death. Poppa et al. [40] experimentally examined consistencies of several types of lesions on bone surface in burning by observing the artificially made lesions on swine skull in different stages of heating. They revealed that although the lesions on bone surface remained their

appearance well even at high temperature, soft tissues covering the bone surface made it less visible at particular degree of burning due to being dark gelatinous form. Such an alteration occurred in soft tissue in burning process should also be studied in future to accumulate more knowledge of body burning in early phase.

Finally, we showed that the utility of PCR is poor for specimens burnt at high temperatures, even after a burning temperature of 200 °C for 45 min. This burning condition was almost equivalent to that which causes the slight yellow-brown change in color. Cattaneo et al. [28] assessed the amplification of 120-bp products of human mitochondrial DNA region V in experimentally burnt human compact bones (800–1,200 °C, for 20 min) as well as in charred bones found in actual forensic cases. They found that none of these burnt specimens retained DNA that was able to be amplified and concluded that DNA typing cannot be used with charred bones. Our results support their conclusion. In addition to their study, Tsuchimochi et al. [41] attempted to amplify Y-STRs from the DNA extracted from dental pulps of heated teeth and failed to be amplified even in the teeth heated at 400 °C for 2 min. This severe DNA degradation in such a short period of heating also suggests the poor resistance of DNA against heat. Conversely, Schwark et al. [29] show better amplification of human STRs and mitochondrial DNA HV1 regions on burnt bones obtained from actual cases. They classified the degree of burning into five categories according to bone color. The specimens at the highest degree of burning were colored "blue-gray-white," and according to our findings, this coloration would suggest that the burning temperatures reached over 500 °C. They successfully achieved amplification of both nucleic and mitochondrial DNA in such severely burnt bones although the success rate of amplification decreased along with the increase in temperature. However, von Wurmb-Schwark et al. used a self-made multiplex PCR system optimized for amplifying highly degraded DNA by shortening the target length of products [42] and used the AmpF*l*STR Identifiler kit (Applied Biosystems) with a higher number of PCR cycles (32–34 cycles). In contrast, our PCRs tested were for the commonly encountered specimens containing relatively better DNA in quality so that the sensitivities are limited to moderate. In addition, differences in the DNA extraction protocol and experimental designs might contribute to the disparity in the results.

In conclusion, this study examined several changes that occur in compact bones during burning, examining both the physical changes that occur and the capacity to employ DNA analyses on severely burnt bone specimens. The results obtained will enable us to estimate the burning temperatures of forensic burnt bone specimens and will also be useful in supporting the appropriate interpretation of data obtained during anthropological (especially anthropometrical) testing and DNA analysis.

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