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Diagnosing post-mortem treatments which inhibit DNA amplification from US MIAs buried at the Punchbowl

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Abstract

The US military is committed to recovering and identifying the remains of unknown military service members. Casualties of the Korean War were exhumed from the National Memorial Cemetery of the Pacific, or Punchbowl, and submitted to the Armed Forces DNA Identification Laboratory (AFDIL) for mtDNA sequencing. Contrary to AFDIL's experience on other samples from this era, most failed to yield amplifiable DNA. Suspicion fell on mortuary practices that may have been applied to the remains, evidenced by a white powder found with the bones, and general records suggesting the use of formaldehyde-based stablizing agents. To improve the chances of successful identification of the unknown individuals, we looked for the causes underlying this failure. We did this by examining the state of the collagen, the most abundant biomolecule in bone, by using differential scanning calorimetry (DSC) and transmission electron microscopy (TEM). The DSC analyses showed collagens with a range of different thermal stabilities. When these results were compared with the DNA amplification results, a clear correlation between elevated thermal stability and amplification failure was evident. TEM analysis revealed that fibril integrity was maintained after thermal and acid treatments in the samples which failed amplification. Together these two approaches implicate a stabilization agent as the cause of problems with DNA analysis, presumably due to excessive cross-linking. Following the initial study, the ability of DSC to rapidly identify problem samples was tested in a blind study of 14 samples, the method successfully identifying all the problematic samples from Punchbowl. Within this unusual context, DSC analysis is a useful method to assess the likelihood of successful DNA extraction and amplification.

Keywords: DNA; Cross-linking; Collagen; Bone; Mortuary practices; TEM; DSC

1. Introduction

"The Punchbowl," the National Memorial Cemetery of the Pacific in Hawaii, holds the remains of US servicemen who died in conflicts around the Pacific. This includes casualties from the 1941 attack on Pearl Harbour and remains repatriated from Korea following the 1950–1953 War. From 1999, the remains of some of the 866 "unknowns" from the Korean conflict have been exhumed for possible identification. To date bone and tooth samples from eleven individuals buried at Punchbowl have been submitted to the Armed Forces DNA Identification Laboratory (AFDIL) in Rockville, MD. AFDIL conducts high throughput mtDNA typing from bone and tooth samples from individuals missing from previous military conflicts [1]. From the Punchbowl, five individuals from the 7 December 1941 loss of the USS Oklahoma during the attack on Pearl Harbour have all yielded sequence data. Of 11 Korean exhumations, none yielded usable mitochondrial DNA despite repeated aggressive attempts. Whilst DNA was visible (on an extraction gel) this could not be amplified with human primers. AFDIL normally has good success with mtDNA analysis from remains of this era, so it was surprising that DNA could not be reproducibly amplified. The burials themselves were unusual, however; the bones had a shiny appearance and were covered

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with a white powder. All of these samples from Korea had initially been processed in Camp Kokura, a temporary mortuary facility in Japan. General records indicate that a variety of aggressive chemical mortuary treatments may have been applied to the remains in question, including: embalming with formaldehyde-based mortuary fluids (in case of relatively intact bodies), packing body cavities with formaldehyde-based ''hardening'' powders, or, for skeletonised or partly skeletonised remains, immersion in formaldehyde-based fluids.

One possible explanation for the problems with DNA amplification in the Camp Kokura samples may have been the method of processing the bodies. When originally faced with this problem (and ignorant of the details of the mortuary treatments) two alternative treatment strategies were considered, the use of strong alkali (lime) and cross-linking agents (such as aldehydes). Addition of lime (a bactericide) would enhance DNA hydrolysis, destroying the original template, thus the only remaining high molecular weight DNA would be subsequent contamination by fungi or bacteria; human DNA would then fail to amplify. Alternatively, the use of embalming agents would preserve DNA, but additional cross-linking would make it difficult to extract and amplify. Inter- and intra-strand cross-links can very plausibly be supposed to inhibit PCR by preventing denaturation of the two DNA strands, sterically hindering access of polymerase to the template strand, and because of covalent modification of the nucleotide bases, preventing proper recognition of the nucleotide bases for DNA replication.

With more than 800 Korean unknowns buried at Punchbowl and with all Kokura cases sampled giving negative results for DNA, the aim of this research was to determine what kind of taphonomic process had affected the bones and to establish a quick and simple way to screen bone samples for those likely to yield DNA.

2. Approach

In order to distinguish whether the inability to amplify DNA was the result of excessive hydrolytic fragmentation or cross-linking, this investigation used differential scanning calorimetry (DSC) and transmission electron microscopy (TEM) to target the bone collagen. Collagen is the most abundant organic component of bone and like DNA is a helical polymer. If the treatment of the bones had fragmented the DNA the collagen would very likely also be damaged (making the helix more prone to unravel to form gelatin). Conversely, modification of the DNA through an aldehyde treatment would also lead to molecular stabilization of the collagen helix (making the helix more resistant to denaturation).

Three properties of the bone collagen were investigated on a sub-set of four samples: these included: (i) sensitivity to acid swelling, (ii) hydrothermal stability and (iii) denaturation temperature. The denaturation temperature was subsequently chosen as the screening tool used to analyse a larger sample set.

2.1. Acid swelling

Bone contains long fibrils of type I collagen. Lowering the pH of bone collagen below its isoelectric point (just above neutral pH) causes these collagen fibrils to swell [2]. If the collagen is degraded it will swell more readily or completely collapse to form gelatin. However, if the collagen fibrils are highly cross-linked they will be more resistant to swelling. The extent of swelling can be seen by removing the mineral, leaving the demineralized collagen in dilute acid and then viewing the positively stained collagen fibrils under a TEM [3; see also 4].

2.2. Hydrothermal stability

Hydrothermal stability has been commonly investigated in relation to collagen tanning and other forms of cross-linking. Type I collagen is transformed from individual fibres to a gelatinous mass if heated to approximately 60–65 °C, but if treated with an aldehydic cross-linker the temperature of this transition is raised to above 70 °C [5,6]. In brief, synthetic cross-links such as aldehydes are believed to stabilize collagen in two ways: firstly by physically preventing the collapse of the structure [5,7], but also by drawing collagen molecules closer together through the elimination of water molecules [5,8]. Therefore if the Punchbowl samples were demineralized then heated to 70 °C, a resistance to thermal alteration would indicate a cross-linking agent was used whilst a lower thermal stability would suggest more degraded material (e.g., hydrolysed by lime).

2.3. Denaturation temperature

The denaturation temperature of collagen can be measured using DSC. With this technique the sample is heated at a constant rate and the enthalpy associated with denaturation is measured. DSC is regularly used within the leather industry to assess the denaturation temperature of collagen [9,10] and has been used to analyse the state of collagen preservation within archaeological bone [11]. Chemical treatments which damage the macromolecules (e.g., liming) will lower and broaden the temperature of this transition. Conversely, stabilization of the fibril (cross-linking) will elevate the transition temperature.

3. Materials and methods

3.1. Bone samples

Small quantities (approximately 2–3 g) of dense compact bone from the Punchbowl samples were supplied by the Joint POW/MIA Accounting Command-Central Identification Laboratory, with a coded identification number. These samples included bone from caskets which contained the white powder and for which DNA amplification was not possible or difficult and, as controls, other bone samples from past conflicts that were not associated with white powder or other mortuary chemical treatment. DNA extraction and mtDNA amplification from these control samples was generally successful following normal procedures [1]. Specimens also included samples that had been taken from different bones from the same individual. All of these details were withheld until after the analyses were completed.

The samples of compact bone were crushed into smaller shards $\leq$3 mm using a hammer and these smaller shards were used for the subsequent analyses.

3.2. EDTA demineralization

60 mg of bone shards from each sample were placed in 15 ml 0.1 M EDTA (GPR, BDH, Poole) and stirred continuously on a Roller Mixer SRT1 (Stuart Scientific) at 4 °C. The demineralization solution was changed after 7 days then the samples were demineralized for a further 7 days. Once demineralized, the samples were washed twice with ultra-pure water (ELGA) then stored in 2 ml pH 7 phosphate buffer saline (PBS) at 4 °C prior to analysis.

3.3. Acid swelling

60 mg of bone shards from each sample were placed in 2 ml 0.6 M HCl (Aristar sp. Gr. 1.18, BDH, Poole) and stirred continuously on a roller at 4 °C. The demineralising solution was changed after 7 days then the samples were left in the acid for a further 7 days. The samples were then washed twice with ultra pure water then stored in 2 ml pH 7 PBS at 4 °C prior to analysis.

3.4. Thermal stability

5 ml of pH 7 PBS was placed in a glass tube and heated on a heating stage at 70 °C for 10 min to ensure it had reached the desired temperature. The EDTA demineralized samples were added to the PBS and heated at 70 °C for 1 h then quenched in a bath of crushed ice.

3.5. TEM analysis

The TEM preparation of the demineralized bone collagen involved positive staining with phosphotungstic acid and uranyl acetate (for details see Ref. [4]). Analysis was conducted using a FEI Technai G^2 transmission electron microscope fitted with a CCD camera. The diameter measurements quoted in the text were taken from digital images using Technai G^2 software. Each mean diameter value is based on the average of five measurements taken from undamaged regions along a fibril and 30 fibrils were measured per sample.

3.6. DSC analysis

EDTA demineralized samples were soaked in pH 7.0 PBS for 24 h prior to analysis to ensure a neutral pH and full hydration. 10–20 mg of demineralized bone shards from each sample were then blotted dry and sealed into aluminium DSC pans. The pans were heated from 15 to 95 °C at a heating rate of 5 °C min^{-1} in a DSC822e differential scanning calorimeter (Mettler Toledo, Leicester, UK) fitted with a nitrogen gas intracooler, using a sealed empty pan as a reference. The onset, peak maximum and peak end temperatures for each endotherm were measured using Mettler STARe integration software where possible or else manually using a printout of the thermal scans.

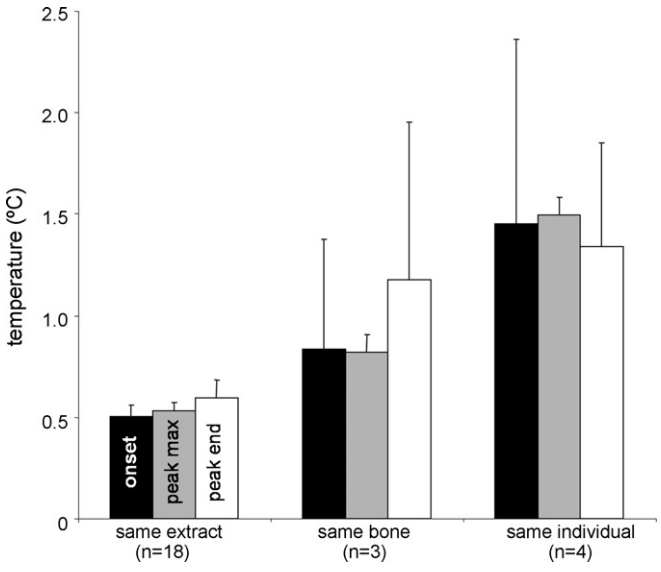

Fig. 1. A bar chart showing the reproducibility of the DSC analysis by looking at the variability in the results between the same extracts, the same bone and the same individual.

Some of the samples in the large blind study were taken from different bones from the same individuals, whilst others were the same bone already analyzed in the initial pilot study (samples A–D). Therefore it is possible to explore within bone and within individual variation in the DSC measurements, as well as multiple runs from the same collagen extract. Variation for peak max within a bone and within the same collagen extract was less than 1 °C and increased to 1.5 °C between different bones from the same individual (Fig. 1)

3.7. DSC control samples

For comparison two additional DSC traces are also presented. The scans were produced from EDTA demineralized 18-month-old modern bovine bone collagen. One sample was untreated, the other was extensively cross-linked by treatment overnight at room temperature with 1% glutaraldehyde in PBS at neutral pH.

4. Results

4.1. Acid swelling and thermal alteration visualized by TEM

Four collagen samples A–D, from bones which included Punchbowl material and non-Punchbowl bones of a similar age which had yielded DNA, were treated with both an acid swelling and a thermal treatment (see Section 3 for details). Two images representative of the state of the collagen fibrils after each treatment are presented in Fig. 2.

The collagen fibrils from samples B and D appeared to be unaffected by either the acid or thermal treatments; the fibrils had clearly defined edges and uniform diameters. In both samples the fibrils were also mainly observed on the TEM grid bundled together and rarely as individual fibrils. Sample C was markedly different, the acid swelling produced fibrils with the appearance of flat 'ribbons'. The ribbons often had ill-defined edges and the banding, characteristic of positively stained collagen, was fainter than that observed in samples B and D. The diameters of the fibrils were also much larger for sample C ($\bar{x} = 237.4$ nm, $\sigma = 57.8$, $n = 30$), compared to sample B ($\bar{x} = 56.6$, $\sigma = 7.8$, $n = 30$). Sample C had also undergone the most significant thermal alteration. The collagen was almost completely gelatinized and was observed under the TEM as electron dense masses, with no individual fibrils preserved. Sample A displayed some evidence of acid swelling and thermal alteration but to a lesser extent that sample C. The alteration was limited to discrete regions along the fibrils whilst other parts appeared unaltered.

4.2. Denaturation temperature

Untreated modern bone collagen had a denaturation temperature of 60 °C (the peak maximum value) however, that temperature was raised to 85 °C when the collagen was treated with 1% glutaraldehyde. In Fig. 3 the DSC traces from these two control samples are presented alongside those obtained from samples A to D. Both samples B and D had denaturation temperatures which were at least 10 °C higher than the untreated control sample. Sample C also appeared to

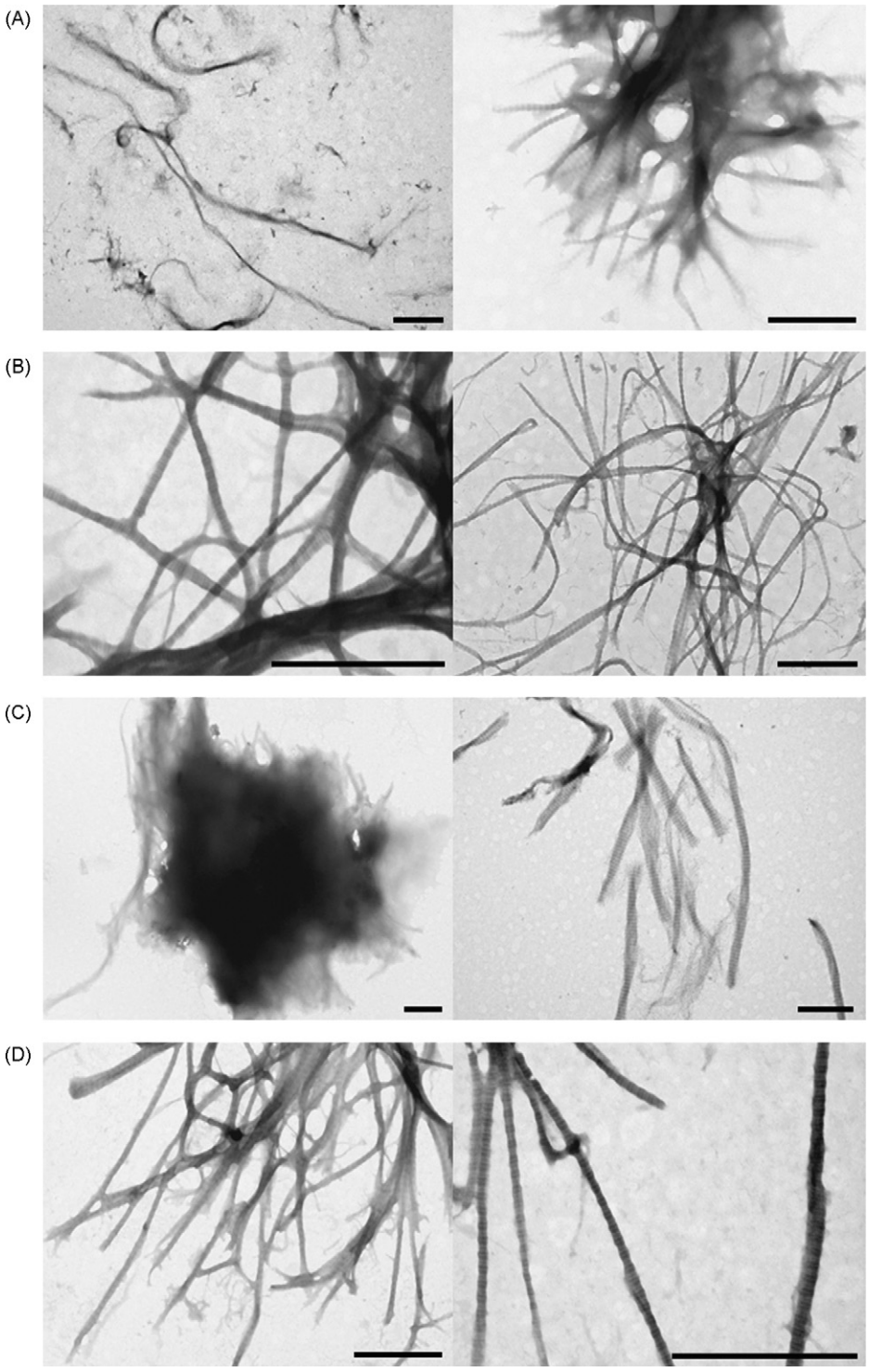

Fig. 2. TEM images taken from samples A to D showing the effects of thermal alteration (left-hand image) and of acid swelling (right-hand image) on the collagen fibrils. The scale bars are 1 μm.

have a second small high temperature transition within the same temperature region as sample D; however, the major denaturation transition gave a peak maximum of 60 °C. Sample A also showed a peak maximum within this lower temperature region, consistent with untreated collagen.

The DSC approach had advantages over TEM of speed, simplicity and quantification, which made it more appropriate as a screening technique. DSC was therefore applied to a further 14 samples which included material from individuals processed through Camp Kokura and individuals from other conflicts; including, in some cases, different bones from the same individual. In Fig. 4 the onset (the point at which the DSC trace begins to deviate from the baseline at the start of the endothermic transition), peak max (maximum of the peak)

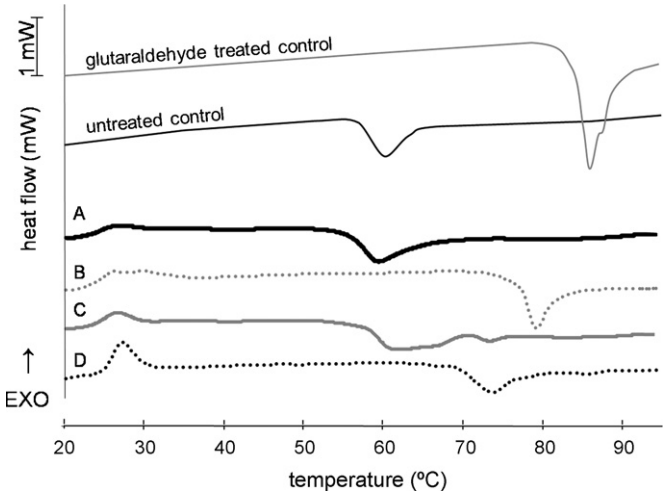

Fig. 3. A combined DSC trace showing a typical trace from bone samples A to D and two controls; untreated demineralized bovine bone collagen (thin black line) and bovine bone collagen which has been treated with 1% glutaraldehyde (thin grey line). The DSC traces are to the same scale but have been offset along the *y*-axis so that they can be viewed more easily.

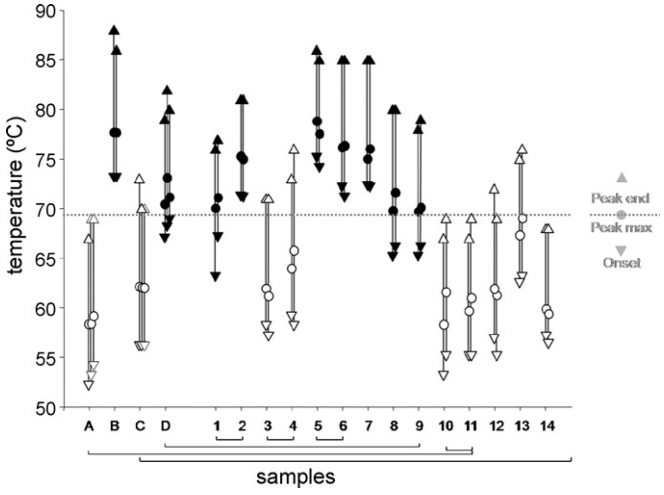

Fig. 4. A plot showing the DSC values obtained from all of the samples in the embalming case study. The onset (▼) peak maximum (●) and peak end (▲) values from each DSC scan are shown. Black indicates a DNA failure and white a success. The connector lines at the bottom of the graph indicate samples which came from the same bone (A and 11; C and 14; D and 9) and samples which came from the same individual but from different skeletal elements (1 and 2; 3 and 4; 5 and 6; 10 and 11) The dotted line represents the division in peak maximum values between the Camp Kokura samples which failed to yield amplifiable DNA and the rest of the samples, the peak maximum values are consistently elevated in these Camp Kokura samples.

and peak end (the point at which the DSC trace rejoins the baseline at the end of the endothermic transition) values from the DSC traces of each sample have been plotted together. The samples displayed a range of denaturation temperatures from 58 to 79 °C. A dividing line has been drawn between the peak maximum values of the samples for which DNA extraction was successful (plotted in white) and those Punchbowl samples which failed to yield amplifiable DNA (plotted in black); there is a clear relationship between the temperature of the endotherm and the DNA results. Samples with values similar to normal unaltered collagen allowed successful extraction of the DNA but those samples where the collagen had been stabilized through cross-linking failed to yield sufficient DNA for amplification.

Further details for each of the samples used in the case study are shown in Table 1. Bones which were reported to have been covered in the white powder are indicated, as are those that yielded amplifiable DNA. The DSC results showing the average peak maximum values for all the samples are also displayed in Table 1.

5. Discussion

Both TEM and DSC analysis revealed that samples A and C behaved as normal untreated collagen and DNA could be

Table 1
Details of the skeletal samples which were analyzed in the study and the DNA and DSC results

Label	Details	White powder?	DNA result	DSC result (peak max)
A	Control South East Asia	No	Success	$58.6 \pm 0.5^{\dagger}$
B	Korean War Punchbowl 1	Yes	Fail	$77.6 \pm 0^{\dagger}$
C	Control Korea	No	Success	$62.0 \pm 0.1^{\dagger}$
D	Korean War Punchbowl 2	Yes	Fail	$71.5 \pm 1.4^{\dagger}$
1	Korean War Punchbowl 3	Yes	Fail	69.99, 71.05
2	Korean War Punchbowl 3	Yes	Fail	75.24, 74.94
3	Control WWII	No	Success	61.89, 61.13
4	Control WWII	No	Success	63.93, 65.73
5	Korean War Punchbowl 4	Yes	Fail	78.79, 77.48
6	Korean War Punchbowl 4	Yes	Fail	76.13, 76.3
7	Korean War Punchbowl 5	Yes	Fail	74.97, 75.99
8	Korean War Punchbowl 6	Yes	Fail	69.74, 71.57
9	Korean War Punchbowl 2	Yes	Fail	69.65, 70.06
10	Control South East Asia	No	Success	58.25, 61.53
11	Control South East Asia	No	Success	59.63, 60.95
12	Control WWII	No	Success	61.86, 61.24
13	South East Asia Sample, possibly smoke cured	No	Success but difficult	67.28, 68.99
14	Control Korea	No	Success	61.57, 59.83

† $n = 3$ (±S.D.).

extracted from both of these bones. However, the differences in thermal stability and resistance to swelling between the two controls A and C cannot be readily explained, although it may be related to variable low level cross-linking that can occur as a component of environmentally induced diagenesis, in the absence of chemical treatment [12].

Samples B and D behaved differently from untreated bone collagen, both came from Camp Kokura and amplifiable DNA could *not* be successfully amplified from either (Table 1). The positions of the DSC peaks for these samples are consistent with a treatment which had stabilized the collagen—e.g., cross-linking. The peaks of these two samples were less sharp, and the denaturation temperatures were lower than the glutaraldehyde tanned control; this is to be expected. The stabilizing agent would have been applied to the skeletal remains and consequently tanning of the mineralized collagen would be anticipated to be less effective than tanning of non-mineralized collagen. Indeed it is remarkable that the bone collagen was so well tanned given the difficulty of penetrating the collagen/mineral composite.

DSC proved to be a useful method of identifying bones which had been treated with a cross-linking agent (Fig. 4 and Table 1). The denaturation temperature for untreated, fully hydrated, demineralized bone collagen at neutral pH is approximately 58–63 °C. All of the samples with similar peak maximum values to this came from individuals where no post-mortem treatment was suspected and DNA extraction had been successful (see samples A and C, 3, 4, 10–12 and 14). Conversely, all of the samples which failed to yield amplifiable DNA, had peak maximum values at or over 70 °C (see Table 1 samples 1, 2 and 5–9). One sample 13, which had not been processed through Camp Kokura had peak max values above that found in normal untreated bone collagen and just below the 'dividing line' between DNA success and failure; DNA extraction and amplification of this sample was very difficult but ultimately successful (using a new-demineralization protocol) [13]. This particular sample comes from the Vietnam War and is suspected to have been subjected to traditional folk preservation techniques involving smoking.

Willerslev et al. demonstrate an age-dependent increase in the number of inter-stand cross-links in DNA from permafrost [12]. Potential cross-linking agents exist both in soils and sediments and may be generated *in situ* from organic matter within the bone [14,15], creating Maillard-type reactions. DSC may therefore have wider application for the study of DNA recovery from cross-linked material than the unusual case presented here.

6. Summary

The Joint POW/MIA Accounting Command-Central Identification Laboratory and the Armed Forces DNA Identification Laboratory have been trying to identify the remains of American servicemen buried as ''unknown'' from the Korean War. The remains were tested for DNA, however, after rigorous extraction procedures, DNA could not be successfully amplified in the majority of cases. The remains had been processed through the Camp Kokura mortuary facility, and were treated with one or more mortuary compounds, as evidenced by the presence of white powder at the time of exhumation. This investigation targeted the collagen component of these bones to examine potential evidence of chemical alteration in the form of cross-linking or use of strong alkali (lime).

TEM analysis of collagen fibrils showed that the Punchbowl remains were more resistant to swelling and heat treatment than those from untreated material. This, combined with the denaturation temperatures obtained from the DSC analysis, strongly suggested the action of an aldehyde-based cross-linking agent.

A comparison of the denaturation temperatures with the results from the DNA amplification showed a clear correlation between successes and failures. Thus applying DSC to bone collagen could be used as a rapid screening technique to assess the likelihood of successful DNA extraction and amplification within this unusual burial context.

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