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The temporal degradation of bone collagen: A histochemical approach☆,☆☆

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ABSTRACT

As forensic anthropologists are currently unable to estimate reliably and quantitatively the postmortem interval (PMI) of skeletonized remains, the current study was conducted to determine if degradation of bone collagen over time could be quantified using sirius red/fast green staining, and whether the degradation would occur at a predictive rate such that it may be used to estimate the PMI of skeletonized individuals. Resin embedded 200–300 μm cross-sections of pig (*Sus scrofa*) long bones with known provenience and PMIs ranging from fresh to 12 months were stained using a histochemical reaction which differentially stains collagenous (Co) and non-collagenous (NCo) proteins. Spectrophotometry was used to determine the concentration of Co and NCo proteins in each bone section, after which the ratio of these proteins was calculated. The results of this study revealed a significant decline in the ratios of Co/NCo protein concentrations over the time period studied ($p < 0.001$). Furthermore, a significant negative correlation between the ratios of Co/NCo protein concentrations and time ($r = -0.563$, $p < 0.0001$) was observed. Despite a significant correlation, the moderate r-value obtained suggests that, at present, this method is useful primarily for detecting and quantifying the degradation of Co and NCo proteins in bones. Future studies that include shorter time intervals and environmental factors, such as soil pH, temperature, and hydrology may prove to be critical for using this method for PMI estimation.

1. Introduction

A central role of the forensic anthropologist is to aid in the identification of deceased individuals by constructing their biological profile – composed of estimates of their sex, age, ancestry, and stature – via the metric and morphologic analysis of skeletal remains. Yet, despite their understanding of human skeletal anatomy, a substantial challenge faced by forensic anthropologists is determining the postmortem interval (PMI) of skeletonized remains. As a result, even the most carefully and thoroughly compiled biological profile could potentially be of limited utility to law enforcement personnel if no reliable time frame exists to refer to when searching among missing persons records [1].

While a variety of techniques are available to estimate the PMI of skeletonized remains, the most commonly used methods vary widely in their applicability, reliability, and even field of study [2–18]. Many are of limited forensic utility, since they either use qualitative and subjective phase-based methods to generate their data (for example, Behrensmeyer's [2] widely cited method which estimates PMI by subjectively assessing the degree or stage of bone weathering), or they are not sensitive enough to distinguish between historical or forensically significant time intervals [12,19–24]. Those methods which do generate an estimate tend to yield rather broad PMIs ranging from months to years [2–8,12,13,17,20–25]. Finally, many of these methods do not directly test the skeletal remains but instead generate PMI estimates from items such as insects, soil, botanical, or faunal evidence which have been found in association with them [1,3–5,7–9,15–17,19]. As a result, these methods inherently assume that the remains have lain in their current location for the entire PMI and do not take into account the possibility that they may have been moved to the location where they were later discovered.

Several studies have been conducted which directly examine skeletal remains, including those which assess residual levels of radioisotopes [11–14], nitrogen, amino acids, and fluorine [20–23],

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lipids, proteins and minerals [25], hemoglobin [26–28], or citrate content in the skeletal material [29]. However, none of these methods are commonly used to estimate the PMI, because they were either never designed to do so [12,20–22,30] or because they have not been tested sufficiently and therefore remain unreliable for use by forensic anthropologists [10,25,27–30].

In light of these methods' limitations, an accurate and reliable method which estimates the PMI of skeletonized remains would be of great practical value. Additionally, to satisfy the criteria set for by the *Daubert* standard [31], any new method should be one which is objective, quantitative, yields narrow – i.e., practical – time ranges, and directly tests the skeletal elements.

Collagen is a ubiquitous protein in the human body, and exists in some 20 or more types, with Type 1 constituting approximately 90% of total body collagens. Like most proteins, it degrades over time [32–37]. Gutierrez [38] and Bell et al. [39] argue that the period between death and burial is likely the most critical phase in the diagenetic history of a given bone, as the species, age at death, cultural/funerary practices, cause of death and form of interment will all converge to play a part in shaping the process of diagenesis. Once placed in their depositional context, whether buried or fully exposed, bones become subject to the interplay of various extrinsic environmental factors, such as temperature, soil pH, and hydrology [32–35,39–43].

Traditionally, several researchers of bone diagenesis have concluded that no single factor can determine the level of preservation of a bone, and that the fate of a given bone is site-dependent [33–36]. More recently, however, Nielsen-Marsh et al. [43] and Smith et al. [44] examined the possibility that bones follow specific diagenetic trajectories defined by their general depositional environments. In their study, Smith et al. [44] were able to group 195 bones (both human and animal) into one of four diagenetic trajectories: well-preserved (WP), accelerated collagen hydrolysis (ACH), microbially attacked (MA), and catastrophic mineral dissolution (CMD). Like Bell et al. [39], Smith et al. [44] concluded that the factors which decide a given bone's diagenetic trajectory must also occur early in the diagenetic/post-depositional process.

Using the same sample as that of Smith et al. [44], Nielsen-Marsh et al. [43] examined which environmental and taphonomic factors help determine the trajectories identified by Smith et al. [44] and revealed the importance of soil types on the overall diagenetic process. Specifically, in a corrosive soil environment (i.e., one with low pH, high phosphate ion concentration $[PO_3]_4$, high exchangeable acidity, and low organic content) bones, regardless of their initial depositional context and state of preservation, tended to suffer CMD [43]. In benign soils (i.e., those with more neutral pH, high calcium ion concentrations $[Ca^{2+}]$, low exchangeable acidity, and higher organic contents), bones followed the WP or MA trajectories [43]. However, factor(s) which placed elements on the ACH trajectory could not be determined [43]. Together, these studies create a model which, rather than discussing site-specific examples of diagenesis, focuses first on the depositional environment and soil conditions followed by the role of various taphonomic factors in the process. Since they do not attempt to provide predictive rates of degradation, they could, if used for this purpose, only provide guidance to forensic anthropologists when estimating the PMI of skeletonized remains.

Given that bone's diagenetic trajectory appears to be decided early in the post-depositional period, it is plausible that the same factors described by Nielsen-Marsh et al. [43] and Smith et al. [44] have the same effect on forensically significant bones as those from archaeological contexts. The experiments reported here, seek to elucidate whether using sirius red/fast green histological staining followed by spectrophotometric analysis for quantification of protein levels could reliably detect and quantify the degradation of proteins – both collagenous (Co) and non-collagenous (NCo) – in bones whose depositional contexts have remained constant over a 12 month period of time. The experiments also investigated whether the degradation quantified by this method occurs at a constant-enough rate (i.e., linear) over the time period sampled such that, if successful, it could eventually be used to predict the PMI of a bone with an unknown date of deposition [45]. This approach uses a combination of histological stains, sirius red/fast green, applied to thin expoxy resin sections of mineralized bone. Because sirius red/fast green is designed to selectively stain collagenous proteins (all types) red, and non-collagenous proteins green in all tissue types, it was possible to determine the ratio of these proteins in a given bone section at a given point in time. While sirius red has been used since 1964 [46], the sirius red/fast green combination has been primarily used for pathology differentiation in biomedical and biochemistry studies since 1985 [47]. To date, no studies have been published which have applied sirius red/fast green to mineralized bone sections; though it has been successfully used in studies testing decalcified hyaline cartilage [48].

2. Methods

2.1. Sample preparation

As human skeletal remains were unavailable for testing, five purpose-bred pigs (*Sus scrofa*) were selected for this study. Animal carcasses were obtained from the Cummins School of Veterinary Medicine in Grafton, MA. All experimental animals were humanely euthanized by captive bolt at approximately 2 months of age (40 pb b.w.) at the Tufts University facility in full compliance with AVMA 2011 animal euthanasia guidelines and the Tufts University School of Veterinary Medicine Institutional Animal Care and Use Committee (IACUC). The animals were then delivered, on the same day, to the Boston University Outdoor Research Facility (BU-ORF) in Holliston, MA. To control for potential confounding variables, all animals shared similar parameters at death, including age, body mass, and manner of death. To prevent bone scatter and/or scavenging during the decomposition process, the animals were housed in medium-sized portable kennels and left to decompose on the surface of a field in the BU-ORF.

To avoid sampling from artificially disturbed remains, samples were collected only once from each pig. Since five pigs were sampled, and given the timing of their placement at the BU-ORF, samples were recovered at 2, 4, 6, 10 and 12 month intervals. Due to a gap in subject placement at the facility, a sample at the eighth-month interval could not be obtained. Two fresh juvenile pig bones were also obtained as a control for the theoretical upper limit of protein concentrations in a given bone. All samples were embedded and prepared for data collection at Boston University School of Medicine.

Two limb bones were taken from each sampled pig. Whenever possible, one long bone was taken from each of the fore and hind limbs. An exception was made in the case of the samples taken for the 2 month time-period, where only hind limb long bones (a femur and a tibia) could be collected. Long bones were selected for sampling because they are recovered most often owing to their relative size and robusticity, and have been documented as being more resistant to diagenetic effects [40,41]. As a result, they are more likely than other bone types to persist in destructive depositional environments.

Two-centimeter long samples were taken from each diaphysis using a band saw. Samples were then embedded in Buehler EpoThin™ Epoxy Resin (Lot no. 20-8140-128, Buehler, Illinois, USA) according to the manufacturer's protocol. The procedures of de Boer et al. [49] were followed for all other steps of the