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The mechanical properties of artificially aged bone: Probing the nature of the collagen–mineral bond

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ABSTRACT

The past two decades have seen enormous advances in our understanding of the diagenetic changes that bones undergo in the archaeological record and the potential for survival of biochemical, isotopic and genetic trace evidence in excavated human and animal bones. What remain relatively poorly understood are the very early changes that take place following skeletonisation because these changes are overprinted by the slower but more dramatic modifications arising from microbial degradation and other diagenetic effects. These very early changes are of interest because of their potential impact on the longer-term survival of biomolecular evidence such as DNA. The mechanical properties of bones, in particular tensile strength, are interesting because they are sensitive to changes in the collagen fraction of bone tissues and the integrity of the protein–mineral bond. In the present study, data is presented on standard samples (N = 220) of modern bovine metapodial bone artificially aged in water at 60 °C for up to ~200 days. Changes in tensile strength were evaluated using the indirect diametral compression test (Brazilian test). In the control samples tensile strength was 74.14 (SD 12.9) MPa parallel to the long axis of the bone and 57.52 (SD 6.7) MPa tangential to the mid-shaft. Tensile strength shows rapid reduction with artificial ageing, losing ~27% after 196 days compared to the controls. Interestingly, there was a rapid deterioration (~16%) in mechanical properties for the initial four days, but this recovers to ~96% at 8 days and then declines more slowly. The reasons behind this behaviour are unclear but, if real, may represent changes in the "straight-jacketing" effects of HAP crystallites within and around collagen fibrils. High resolution SEM images of the fracture surfaces show evidence for partial demineralisation of collagen bundles and re-precipitation of crystallites on individual collagen fibrils.

1. Introduction

The work described in this paper builds upon previous research into the tensile strengths of modern and archaeological bones (Turner-Walker and Parry, 1995). Amongst other findings published in this earlier paper was a plot of tensile strength of bone samples against bulk density that suggested there was a very rapid decline in tensile strength soon after death and this interpretation was in line with a theoretical model of post-mortem deterioration of bone collagen (Collins et al., 1995). However, more recent work on the stability of mineralised collagen has cast doubt on the original model for the degradation of collagen in ancient bone and this has stimulated a return to look at early diagenesis of mineralised collagen in greater detail. This early diagenesis is of interest because results from the experimental burial of bovine bones in bogs show that DNA (or at least amplifiable DNA) also shows a very rapid deterioration during the first year of burial, followed by a much slower deterioration over subsequent years. This data also suggested that long-term survival of DNA may be linked to the mineralisation state of collagen (Turner-Walker et al., 2008).

The early post-mortem deterioration of bone tissues inside a decaying corpse or carcass is extremely complex due to rapidly changing pH, oxygen content and microbial populations in the putrefying soft tissues that surround the skeleton. These parameters can show considerable variation, even within a single skeletal element, and archaeological bones frequently exhibit differential preservation, although it is not always easy to ascribe these differences to either taphonomic or diagenetic influences. To simplify the experimental procedure and reduce the number of possible variables specimens of bone were artificially aged in sealed vials of pure water. This approach has been used by previous researchers when investigating the dynamics of collagen- (Dobberstein et al., 2009) and DNA degradation (Waite et al., 1997). The bone specimens were pre-machined into small test discs before ageing. In this way all the discs would experience similar deterioration throughout their volume and problems associated with preparing degraded, and therefore potentially fragile, pieces of bone could be circumvented.

The object of the work presented herein was to investigate early changes in the integrity of the collagen–mineral bond using measures of the samples' tensile strength. Changes in the porosity of the samples were also evaluated using a simple water absorption method.

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2. Materials and methods

The source material used came from modern cow metapodials. These were collected as frozen lower legs from a local slaughterhouse in Taiwan, then packed with dry ice in insulated boxes for transfer to the laboratory where they were stored at −22 °C until the metapodial diaphyses were dissected out. The diaphyses were cleaned mechanically to remove marrow and washed under running tap water. The bones were then allowed to air-dry at standard temperature and humidity (20 °C and 55% RH).

Because fresh bone specimens used as experimental samples are often frozen prior to use it is worth examining the issue of the possible impact of freezing on bone tissues, and more especially on the physical properties of bone. Previous work on the effects of freezing on the mechanical properties of bones has shown slightly conflicting results. Some report a small decrease in tensile strength after freezing and thawing (Abramov, 1975; Cowin 2001). Kayurapan et al. (2009) working on whole human femurs reported up to 30% reduction in the strength of human femurs after freezing but identified no significant difference in the strengths of bones frozen rapidly and those frozen more slowly. However they did report that freeze-drying resulted in a 90% decrease in strength under 3-point bending tests. Pelker et al., (1983) working on whole bones from rats found that freezing of specimens at −20 °C, −70 °C, or −196 °C did not adversely affect the strength of long bones tested in torsion or of vertebral bodies tested in compression. In work on segments of human vertebral columns there was no significant difference between the fresh and frozen columns in terms of amplitude and rigidity in flexion/extension and right/left lateral flexion after three months of freezing at −18 °C (Gleizes et al., 1998). The authors concluded that simple freezing at −18 °C was a reliable mode of storage of vertebral segments before their employment for biomechanical tests. Some authors have reported an increase in bone strength following freezing and thawing. Three-point bending tests on sheep femurs showed that frozen femurs (−20 °C for 60 days) were significantly stronger than either fresh or autoclaved femora (Moreno and Forriol, 2002).

It should be noted that the majority of the mechanical testing work cited above was undertaken on the whole bone specimens which may be more susceptible than smaller samples to early failure arising from local damage induced by pre-treatment. In contrast, the work presented here is based on numerous small specimens machined from each bone and in which local damage may be expected to have less influence on overall results. Furthermore, all of the samples, including the controls, were subjected to the same freezing regime and the data is presented as changes from baseline (controls). Less confidence can be placed on the absolute values of tensile strength measures because the indirect method adopted appears to underestimate the measured values compared to other techniques that measure tensile strength directly.

Tensile strength was measured using the indirect, diametral compression test or *Brazilian test*. This is normally used when determining the tensile strength of brittle solids such as rock and concrete. Here it was adopted because small samples could be used and therefore many specimens could be obtained from one bone — thus improving statistical significance in the results obtained. Small cylindrical cores were drilled from the mid-shafts of the bones using a purpose-made coring drill and support. Each bone was marked with lines parallel to the long axis of the bone prior to the cylinders being drilled so that orientation of the specimen could be preserved throughout the drilling and grinding process, and during subsequent tensile testing experiments. Parallel faces were then ground onto the ends of the bone cylinders using a lapping machine and purpose-made stainless steel holders. The resulting bone discs had diameters of ~6.30–6.60 mm and thicknesses ranging from ~2.00 to 4.00 mm.

In total, more than 250 discs were prepared from the three metapodials and these were pooled and randomised, then divided into 10 groups for artificial ageing plus two groups for controls. By randomising the discs it was hoped that any differences in microarchitecture and porosity arising from each disc's exact location in the diaphysis would be spread equally amongst the 10 groups. That such differences between discs existed is probably reflected in the large standard deviations in the measured parameters. Each disc was labelled and measured parallel to the bone's long axis and normal to the long axis, and its thickness measured (±0.01 mm). They were also individually weighed (±0.0001 g) after equilibration to standard temperature and humidity (20 °C and 55% RH). The 10 groups were then sealed in screw-top glass vials with 20 ml ultrapure water and placed in an oven at 60 °C for time periods of 24 h to 196 days with intervals increasing geometrically (1, 2, 4, 8 days etc.). A temperature of 60 °C was chosen because this is below the gelatinisation temperature of mineralised collagen but high enough to prevent microbial degradation of the bone specimens. After artificial ageing the discs were removed, rinsed twice in clean water and blotted — then left to air dry. The supernatant liquids were preserved for future chemical analysis.

In the Brazilian test discs are compressed along the vertical axis and the specimen becomes elongated at right angles to this — eventually fracturing along the loading axis. Discs were tested by loading them between specially machined concave anvils with a radius of 10 mm according to the protocols described by Turner-Walker and Parry (1995). Discs were initially loaded to failure in both the long axis direction (parallel to the long axis of the bone) and normal to this orientation. Discs loaded normal to the long axis of the bone exhibited visco-elastic deformation and did not fail abruptly, making determination of tensile strength problematic. Discs tested along the long axis, however, provided good, reproducible results and all subsequent tests were done parallel to the long axis. Failure parallel to the long axis was taken to reflect a decrease in the cohesive strength of the collagen fibre bundles — and by implication as a measure of the strength or integrity of the collagen–mineral bond. All samples were tested to failure over the space of two days after re-acclimatising to 55% RH and 20 °C.

Previous work had demonstrated the significance of the porosity and bulk density (itself related to porosity) of bones samples in tensile strength (Turner-Walker and Parry, 1995). This work had demonstrated that total pore volume could be determined with reasonable accuracy by measuring the weight gain after absorption of water — the pore volume being equivalent to the mass of water taken up by oven-dried bones when soaked in water. To investigate changes in porosity 8 discs that had already been tested for tensile strength were selected from each group of time intervals and these were dried at 105 °C for 24 h and then immediately weighed. The discs were then vacuum-impregnated in pure water for 4 h then removed, blotted dry and re-weighed. After weighing they were returned to water for a further 20 h and the procedure repeated. For each disc, porosity as volume percent was calculated from the differences between saturated and oven-dried weights of the discs and from the measured disc volumes. During analyses of the results means and standard deviations of all discs from each time interval were used.

To investigate the nature of any changes in fracture mechanism following artificial ageing a small selection of samples were examined at various magnifications using scanning electron microscopy (SEM). Imaging was done on uncoated samples in a Zeiss Supra 55, low vacuum field emission SEM (LVFESEM) at an accelerating voltage of 2 kV. Examining uncoated samples permitted extremely high resolution images to be obtained. These were captured and stored as TIFF files.

3. Results

Despite considerable scatter in the data, tensile strength of the artificially aged discs exhibited the anticipated decline with increasing ageing time (Fig. 1). The initial decline in these measured parameters was surprisingly rapid, with an approximate 16% reduction in tensile strength after only four days. What was completely unexpected, however, was