Do chondrocytes within calcified cartilage have a higher preservation potential than osteocytes? A preliminary taphonomy experiment (Postprint)

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Abstract

Chondrocytes with remnants of nuclei and biomolecules were recently reported in two Cretaceous dinosaurs from North America and China. For multiple reasons, it was hypothesized that calcified cartilage (CC) had a better potential than bone to preserve ancient cells. Here we provide the first experimental test to this hypothesis by focusing on the most important variable responsible for cellular preservation: the postmortem blockage of autolysis. We compare the timing of autolysis between chondrocytes and osteocytes in an avian model (Anas platyrhynchos domesticus) buried for up to 60 days under natural conditions that did not inhibit autolytic enzymes. Within 15 days post-burial, almost all osteocytes were already cytolyzed but chondrocytes in CC were virtually unaffected. All osteocytes were cytolyzed after 30 days, but some chondrocytes were still present 60 days post-burial. Therefore, even in harsh conditions some CC chondrocytes still survive for months postmortem on a time scale compatible with permineralization. This is consistent with other data from the forensic literature showing the extreme resistance of hyaline cartilage (HC) chondrocytes after death and does support the hypothesis that CC has a better potential than bone for cellular preservation, especially in fossils that were not permineralized rapidly. However, because the samples used were previously frozen, it is possible that the pattern of autolysis observed here is also a product of cell death due to ice crystal formation and not strictly autolysis, meaning a follow-up experiment on fresh (non-frozen samples) is necessary to be extremely accurate in our conclusions. Nevertheless, this study does show that CC chondrocytes are very resistant to freezing, suggesting that chondrocytes are likely better preserved than osteocytes in permafrost fossils and mummies that underwent a freezingthawing cycle. It also suggests that cartilage (both hyaline and calcified) may be a better substrate for ancient DNA than bone. Moreover, even though we warrant follow-up taphonomy experiments with non-frozen samples paired with

DNA sequencing, we already urge ancient DNA experts to test CC as a new substrate for ancient DNA analyses in fossils preserved in hot and temperate environments as well.

Full Text

Do Chondrocytes Within Calcified Cartilage Have a Higher Preservation Potential Than Osteocytes? A Preliminary Taphonomy Experiment

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Abstract

Chondrocytes with remnants of nuclei and biomolecules were recently reported in two Cretaceous dinosaurs from North America and China. For multiple reasons, it was hypothesized that calcified cartilage (CC) had better potential than bone to preserve ancient cells. Here we provide the first experimental test of this hypothesis by focusing on the most important variable responsible for cellular preservation: postmortem blockage of autolysis.

We compared the timing of autolysis between chondrocytes and osteocytes in an avian model (*Anas platyrhynchos domesticus*) buried for up to 60 days under natural conditions that did not inhibit autolytic enzymes. Within 15 days post-burial, almost all osteocytes were already cytolyzed, while chondrocytes in CC were virtually unaffected. All osteocytes were cytolyzed after 30 days, but some chondrocytes persisted 60 days post-burial. Therefore, even under harsh conditions, some CC chondrocytes survive for months postmortem on a timescale compatible with permineralization. This is consistent with forensic literature showing the extreme resistance of hyaline cartilage (HC) chondrocytes after death and supports the hypothesis that CC has better potential than bone for cellular preservation, especially in fossils that were not rapidly permineralized.

However, because the samples were previously frozen, the observed autolysis pattern may also reflect cell death from ice crystal formation rather than strict autolysis. Therefore, a follow-up experiment on fresh (non-frozen) samples is necessary for greater accuracy. Nevertheless, this study demonstrates that CC chondrocytes are highly resistant to freezing, suggesting they are likely better preserved than osteocytes in permafrost fossils and mummies that underwent freezing-thawing cycles. It also suggests that cartilage (both hyaline and calcified) may be a better substrate for ancient DNA than bone. Although we

warrant follow-up taphonomy experiments with non-frozen samples paired with DNA sequencing, we already urge ancient DNA experts to test CC as a new substrate for ancient DNA analyses in fossils preserved in hot and temperate environments.

Keywords: experimental taphonomy, chondrocytes, osteocytes, postmortem autolysis, freeze-related cell-death, cellular and biomolecule fossilization, aDNA substrate

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Introduction

Seventy-five million-year-old chondrocytes preserved with their fossilized nuclei and chromosomes were recently reported in the calcified cartilage (CC) of the North American dinosaur *Hypacrosaurus* (Bailleul et al., 2020). Additionally, two fluorescent DNA stains were applied to a few *Hypacrosaurus* chondrocytes isolated from their extracellular matrix (ECM), and a few cells showed fluorescent intracellular DNA staining (Bailleul et al., 2020). More recently, a cell nucleus with chromatin-like threads that stained just like an extant avian nucleus with hematoxylin and eosin was reported in the CC of a 125 million-year-old oviraptorosaurid dinosaur (*Caudipteryx*) from Northeast China (Zheng et al., 2021).

These results suggested that some cell nuclei from Mesozoic cartilage cells retain remnants of original nuclear biomolecules (Bailleul et al., 2020; Zheng et al., 2021). Although such studies and results are highly controversial (Schweitzer et al., 2013; Bailleul et al., 2020; Zheng et al., 2021) because DNA sequencing has never yielded an authenticated sequence from any fossil this old (van der Valk et al., 2021), the data still suggest remnants of original DNA are preserved, even if chemically modified (Bailleul and Li, 2021).

Data from *Hypacrosaurus* led to the hypothesis that CC may be a better candidate than bone for cellular and biomolecule preservation. This hypothesis was based on noticeable tissue characteristics present only in cartilage that seemed favorable for molecular stabilization. Unlike bone, the ECM of cartilage (both uncalcified and calcified) has no vascularization, which provides less surface area for groundwater and microbes to deteriorate the tissue. CC also has a higher mineral:organic ratio than bone (Goret-Nicaise and Dhem, 1985), presumably giving further protection to chondrocytes from external attacks. Lastly, CC is a hypoxic environment (with sometimes only 1% oxygen (Pfander and Gelse, 2007)), and the authors (Bailleul et al., 2020) proposed this hypoxia may protect chondrocytes from oxidative damage.

It was later noted that an additional key characteristic further supported this hypothesis: chondrocytes naturally have an extensive postmortem delay of autolysis (Bailleul, 2021), at least for chondrocytes in uncalcified hyaline cartilage (HC) (Csönge et al., 2002; Lasczkowski et al., 2002; Williams et al., 2003; Drobnic et al., 2005; Pallante et al., 2009; Rogers et al., 2011; Alibegović, 2014; Paulis et al., 2016). Autolysis is the autodigestion of cells by their own lytic enzymes after somatic death, culminating in cytolysis (complete breakdown of all cell components). It usually begins extremely rapidly within a few hours after death in most cell types and depends on the metabolic rate of the cell (Clark et al., 1997; Powell, 2015). Cells with high metabolic rates autolyze fastest (Clark et al., 1997; Powell, 2015). During "exceptional" cellular fossilization, autolysis is blocked by external factors (e.g., rapid burial in an anoxic environment combined with reducing conditions that denature hydrolytic enzymes, or cold temperatures in permafrosts) (Raff et al., 2006, 2008). Without blocking autolysis, or at least delaying it long enough to allow permineralization and/or mineral replication (Briggs et al., 1993), cells and their biomolecules are not expected to enter the fossil record (Raff et al., 2006, 2008; Bailleul and Li, 2021).

Research on articular HC allografts from human cadavers transplanted into live patients with osteoarthritic joints has shown that many chondrocytes retain viability for long periods after somatic death (Csönge et al., 2002; Lasczkowski et al., 2002; Williams et al., 2003; Drobnic et al., 2005; Pallante et al., 2009). Chondrocytes function with anaerobic metabolism, are adapted to hypoxic environments, and can survive in modest conditions low in both nutrients and oxygen (even in full anoxia (Pfander and Gelse, 2007)). After somatic death, nutrients and oxygen stored in the avascular ECM of cartilage still diffuse to HC chondrocytes, allowing survival for several days to weeks or even months after the individual's death (Csönge et al., 2002; Lasczkowski et al., 2002; Williams et al., 2003; Drobnic et al., 2005; Pallante et al., 2009; Alibegović, 2014).

Such exceptional postmortem survival timing is apparently not seen to this extreme in any other human tissue (Pfander and Gelse, 2007). Unfortunately, although abundant data exist on postmortem autolysis timing in mammalian HC (Csönge et al., 2002; Lasczkowski et al., 2002; Williams et al., 2003; Drobnic et al., 2005; Pallante et al., 2009; Rogers et al., 2011; Alibegović, 2014; Paulis et al., 2016), virtually nothing is known about CC for any vertebrate taxon. Although never clearly stated or demonstrated, we can assume osteocytes die and autolyze much more rapidly than both HC and CC chondrocytes after death, since the former function with aerobic metabolism under basal conditions (Shapiro et al., 1982; Frikha-Benayed et al., 2016), directly receive nutrients and oxygen from blood vessels, and are not adapted to thrive under hypoxia. This was already assumed and proposed in a few studies (Bailleul, 2021; Bailleul and Zhou, 2021; Zheng et al., 2021), but (1) it was never clearly tested in a comparative setting with unequivocal histological images, and (2) contradicting data exist concerning bone cells, with a forensic study stating that cells of "skeletal tissues" (presumably bone cells) are among the last to autolyze (Clark et al., 1997).

Here, we perform a simple actualistic taphonomy experiment comparing autolysis timing between CC and bone in an avian model that can serve as a baseline for inferences concerning cellular and biomolecule fossilization in these two tissues. This will help test the original hypothesis that CC is a better candidate than bone for ancient cell and biomolecule preservation. The experiment was conducted under harsh conditions not comparable to "exceptional" fossilization (i.e., buried during summer, under oxic conditions, without antibiotics) to observe the "natural" autolytic behavior of chondrocytes and bone cells after death. For additional comparison, we also analyzed autolysis timing in HC, for which considerable data have been generated recently from clinical medicine and forensics. We used the webbed feet of domestic ducks (*Anas platyrhynchos domesticus*) that were buried for up to 60 days and histologically analyzed and compared the cells of the three tissues at the joint between the distal end of the third tarsometatarsus (TMT III) and the third proximal phalanx.

Materials and Methods

2.1 Materials

Three domestic ducks of unknown sex and 50 days old (~7 weeks) were obtained from a commercial bird farm in Linyi (Shandong Province). Specimens were close to skeletal maturity (bone growth being complete at 8 weeks in this species (Murawska, 2012)). Specimens were sacrificed according to local permits, then immediately transferred to a -18 ºC freezer within 1 hour of death. After a few days, they were shipped frozen to Beijing (~16 hour drive) and placed in another freezer at the Institute of Vertebrate Paleontology and Paleoanthropology, Chinese Academy of Sciences (IVPP). The ducks remained frozen upon arrival. In total, the ducks stayed frozen at -18 ºC for 26 days until the experiment began (at t = 0 days).

2.2 Experimental Setting

After 26 days in the freezer, three ducks were removed and their frozen feet and heads were sawed (note that the heads in Fig. S1 were buried for another study). One foot of the first duck (Duck 1) served as the control and was processed directly for histology after thawing (Supplementary methods). The sawed parts of the other two ducks (Duck 2 and Duck 3) were transported and buried approximately 20 cm deep in the soil (a sandy loam of pH ~7.8) of a nearby compound at the Beijing Zoo (Fig. S1).

Elements were placed approximately 10–15 cm apart in the soil (Fig. S1). The experiment took place between July 1st, 2020 and September 29th, 2020. Average precipitation for these three months was 128.2 mm and average temperature was 23.2 ºC in Beijing (source: http://www.nmc.cn/publish/forecast/ABJ/beijing.html).

2.3 Paraffin Histology

For the control foot of Duck 1 and the excavated feet of Ducks 2 and 3, tissue samples were cut from the articular surface of TMT III at the tarsometatarsophalangeal joint with a sharp razor blade. They were then placed in 10% Neutral Buffered Formalin and processed into paraffin slides stained with H&E and modified Masson's Trichrome (Supplementary methods).

2.4 Scanning Electron Microscopy

Some finished paraffin slides were deparaffinized and left to dry without a coverslip. These deparaffinized sections were then gold-sprayed and rapidly observed and photographed under the SEM at the Chinese Academy of Geological Sciences with an FEI Quanta 450 (FEG) at 20 kV. Images are shown in BSE mode (backscattered electrons).

Results

3.1 Gross Morphology

One frozen-thawed duck foot was not buried and served as the control (at t = 0 days) (Fig. 1A [Figure 1: see original paper]). Three other (previously frozen) feet were buried simultaneously but excavated at three different times: after 15 days, 30 days, and 60 days in the soil (Figs. 1B–D; S1). In the control foot, the skin was bright yellow and the articular HC on TMT III was transparent and glossy (Fig. 1A). After 15 days in the soil, the skin around the analyzed joint lost its bright yellow color and the HC turned white and cloudy (Fig. 1B). After 30 days, soft tissues and skin dried out and the HC was lost, leaving only exposed CC and subchondral bone (Fig. 1C). After 60 days in the soil, all surrounding soft tissues disappeared and the foot bones were disarticulated (Fig. 1D).

3.2 Histology and SEM Changes from t = 0 days to t = 15 days

At t = 0 days, chondrocyte lacunae of HC were filled with large chondrocytes that stained dark purple with hematoxylin and eosin (H&E) (Fig. 2C [Figure 2: see original paper]). At t = 15 days, chondrocytes had considerably shrunk and become light pink (indicating reduced stain uptake compared to controls; Fig. 2D). This loss of nuclear basophilia is known to occur in decomposing remains stained with H&E (Genest et al., 1992; Rogers et al., 2011; Dettmeyer, 2018; Alabbasi et al., 2022) and results from autolysis (Dettmeyer, 2018; Alabbasi et al., 2022). Chromatin dissolution had likely begun, and HC chondrocytes were no longer viable at t = 15 days.

SEM observations show these cells at t = 0 days were round and mostly smooth (Fig. 3A [Figure 3: see original paper]), but the shrunken, presumably nonviable cells at t = 15 days adopted a stellate shape with many "protrusions"

(Fig. 3B). These may represent extracellular secretions or breakdown products from autolysis itself.

In CC, no clear differences in staining intensity, cell shape, or cell size were observed between t = 0 days and t = 15 days (Fig. 2E, F). In both cases, cells were round and dark purple. No obvious surface morphology differences were seen under SEM either (Fig. 3C, D), suggesting CC cells were not affected by somatic death and were likely still viable at t = 15 days, unlike HC chondrocytes (Figs. 2C, D, 3A, B).

Subchondral bone has round osteocyte lacunae in many places resembling chondrocyte lacunae. This is characteristic of chondroid bone, a tissue common in rapidly growing juvenile ducks (Prondvai et al., 2020). At t = 0 days, bone cells from subchondral bone showed healthy, elongated morphology (Fig. 2G). At t = 15 days, many osteocyte lacunae were completely empty, indicating many cells had fully cytolyzed within 15 days post-burial (Figs. 2I, 3F). Remaining lacunae contained small round bodies representing autolyzed remnants not yet fully cytolyzed (Fig. 2H).

3.3 Histological Changes from t = 30 days to t = 60 days

No clear differences were observed between decayed tissues at t = 30 days and t = 60 days. All HC had disappeared through putrefaction, leaving only CC and subchondral bone (Fig. 4A [Figure 4: see original paper], B). Most CC lacunae were either empty or filled with contaminants like spores (seen in both paraffin slides and SEM observations, Fig. S2), but a few lacunae still contained round chondrocytes staining light to dark purple (Figs. 4C, D; S3). The dark purple stain indicates nuclear basophilia was retained after 30 and even 60 days post-burial, suggesting a few final "surviving" CC cells may have remained viable (or at least were "damaged" cells sensu Lasczkowski et al. (2002) but not fully dead).

In subchondral bone, no osteocyte remnants were visible in any lacunae at either t = 30 days or t = 60 days (Fig. 4E, F).

Discussion

4.1 First Observed Pattern: Bone Cells Autolyzed Faster Than Cartilage Cells

At t = 15 days, a clear autolysis pattern emerged among the three cell types: almost all bone cells were fully cytolyzed, HC cells were present but showed loss of nuclear basophilia (a sign of karyolysis (George et al., 2016)), and CC cells were virtually identical in morphology, size, and staining intensity to controls (Fig. 2). This demonstrates a clear gradient in autolysis timing, with bone cells fastest to initiate and complete autolysis, followed by HC cells, and finally CC cells. We confidently estimate that within 1 week in soil, at least 50% of osteocytes were already completely cytolyzed in our samples. Inhibition of

autolysis for months post-burial (as seen in some CC chondrocytes) is highly compatible with permineralization timing, which can occur within just a few weeks in experimental studies (e.g., ~2–3 weeks (Martin et al., 2003, 2005; Chen et al., 2009)).

The differential autolysis timing among these three tissues can be explained by differences in cell biology and metabolism. As predicted, autolysis was more rapid in osteocytes than in HC and CC chondrocytes, likely because bone cells are not naturally adapted to hypoxia. However, we did not predict that CC cells would be even more resistant to postmortem changes than HC cells. Although quantitative comparative data are lacking, HC cells are generally reported as having the most exceptional postmortem survival timing in the body (Pfander and Gelse, 2007), and chondrocytes in CC are usually thought to die rapidly once embedded in a calcified matrix. This latter assumption is erroneous; nutrients and solutes can still diffuse through the calcified cartilaginous matrix (Arkill and Winlove, 2008), and our study shows CC chondrocytes have a lifespan of at least several months. Our results re-attribute the postmortem survival "record" from HC chondrocytes to chondrocytes embedded in calcified matrix.

It has been demonstrated that HC layers closer to the articular surface are more affected by postmortem environmental factors than deeper layers (i.e., closest to subchondral bone) (Drobnic et al., 2005). Moreover, recent studies show chondrocytes in deeper layers are more adapted and resistant to hypoxia (with a hypoxia-adapted phenotype) than surface chondrocytes (Brucker et al., 2005; Pfander and Gelse, 2007). Based on this, we hypothesize that CC chondrocytes have a unique hypoxia-adapted phenotype enabling postmortem survival that surpasses all HC cells (and, of course, bone cells).

Cells (including their biomolecules) with extended postmortem survival periods—where autolysis is blocked or not activated for long durations—technically have greater chances to enter the fossil record than cells that autolyze rapidly after death. The autolysis pattern observed here therefore supports our original hypothesis that CC is a better candidate than bone for ancient cell and biomolecule preservation.

However, this is a preliminary conclusion because our tissues were frozen before the experiment (at -18 ^{o}C for 26 days), meaning our results may show an unrepresentative decay pattern due to freeze-related cell death. Freezing causes mechanical cell damage from intra- and extracellular ice crystal formation (Jang et al., 2017), and although highly unlikely, we cannot technically rule out that osteocytes may be more cold-sensitive than chondrocytes. Cell damage was indeed observed in some control tissue cells (Fig. S4), indicating that more appropriate material for histotaphonomy experiments is fresh, non-frozen tissue where original cell viability is unaffected by freeze-thaw cycles. Samples should also undergo DNA sequencing analyses to test for correlation between cell integrity/presence and DNA/biomolecule preservation, as it has been hypothesized that DNA molecules can "leak" from cells and be adsorbed and retained within hydroxyapatite crystals after death (Götherström et al.,

2002).

4.2 Second Observed Pattern: CC Chondrocytes Are Quite Resistant to Freezing Temperatures

Although our experimental material does not fully support the primary hypothesis tested, our results nevertheless demonstrate that CC chondrocytes are quite resistant to very low temperatures. This taphonomical experiment may therefore serve as a preliminary model for permafrost mummies that undergo freezing-thawing cycles (with temperatures usually varying between 0 ºC and -15 ºC; e.g., Sazonova et al. (2004)). Our results suggest that chondrocytes of both HC and CC in permafrost-preserved fossils and mummies may be better preserved than osteocytes. Since DNA is found within cells, cartilage is likely a better substrate for ancient DNA (aDNA) than bone in permafrost mummies.

Perspectives

Taphonomy experiments aiming to better understand and interpret fossilization processes rarely involve histology, even though this method provides deeper understanding of tissues and cells than gross examinations. The histotaphonomical results of this experiment bridge multiple fields including taphonomy, paleontology, and potentially aDNA research.

In the field of aDNA, where the current limit of sequence authenticity is only around 1.2–1.65 million years old (van der Valk et al., 2021), great efforts are constantly made to identify the best high-purity "substrates" for aDNA recovery and the skeletal elements richest in DNA for a given taxon (e.g., Götherström et al., 2002; Hansen et al., 2017; Alberti et al., 2018). The best substrates are tissues and body parts with the highest percentage of endogenous aDNA and lowest percentage of contaminating DNA (Hansen et al., 2017; Sirak et al., 2020). Recent studies show the most suitable substrates for DNA analysis in ancient humans preserved in hot and temperate environments are cementum of tooth roots (Hansen et al., 2017), the petrous part of the temporal bone (Pinhasi et al., 2015), and auditory ossicles (Sirak et al., 2020). How and where DNA is found within these high-purity tissues is not clearly understood, but hypothetically the petrous bone is richer in DNA than other body parts because (1) it is the densest bone in the human skeleton and somewhat protected from taphonomical processes (Gamba et al., 2014; Pinhasi et al., 2015), and (2) it has many micropetrotic mineralized osteocytes that supposedly sequester DNA molecules (like "microniches" of DNA (Bell et al., 2008; Pinhasi et al., 2019)). This type of cell mineralization is also possible within CC (see references in Kierdorf et al. (2022)); recently, some calcified "micropetrotic" chondrocytes were reported in the CC of the fossil bird *Yanornis* (Bailleul and Zhou, 2021).

To our knowledge, CC has not been clearly tested as a potential high-purity substrate for aDNA analysis in either permafrost fossils or those preserved in hot and temperate environments, despite having all the tissue architecture and

proper cell metabolism to serve as such a substrate. Based on our reported cold-resistance of CC cells combined with the fact that CC is avascular (so it can be considered "dense") with high cellular density, there is no reason not to sample this tissue in permafrost fossils. Additionally, pending other taphonomical experiments using fresh (non-frozen) material, our preliminary data still suggest CC has potential as a new high-purity aDNA substrate (possibly providing better results than dense micropetrotic bone or cementum) for fossils preserved in hot and temperate environments.

We propose that fragments of CC covering joints of adult humans, or basicranial synchondroses of infants, should be sampled when possible and their DNA yield compared to other tissues. This would be especially important in fossils where bone fragments or cementum did not yield good results (only very incomplete DNA sequences). We also anticipate CC could have significantly higher DNA purity than bone in large species (such as large Pleistocene mammals) that naturally have very thick CC layers at their joints. Additionally, fossil chondrichthyans (which are entirely made of cartilage) may be excellent study material for further investigating these questions about DNA preservation.

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Supplementary files can be found on the website of *Vertebrata PalAsiatica* (http://www.vertpala.ac.cn/EN/2096-9899/home.shtml) in Online First.

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Note: Figure translations are in progress. See original paper for figures.