Investigating the post mortem molecular biology of cartilage and its potential forensic applications

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ABSTRACT

This study investigated the post mortem molecular changes that articular cartilage undergoes following burial. Fresh pig trotters were interred in 30cm deep graves at two distinct locations exhibiting dissimilar soil environments for up to 42 days. Extracts of the metacarpophalangeal (MCP) and metatarsophalangeal (MTP) joint cartilage from trotters disinterred weekly over 6 weeks were analysed by Western blot against the monoclonal antibody 2-B-6 to assess aggrecan degradation. In both soil conditions, aggrecan degradation by-products of decreasing molecular size and complexity were observed up to 21 days post mortem. Degradation products were undetected after this time and coincided with MCP/MTP joint exposure to the soil environment. These results show that cartilage proteoglycans undergo an ordered molecular breakdown, the analysis of which may have forensic applications. This model may prove useful for use as a human model and for forensic investigations concerning crimes against animals and the mortality of endangered species.

KEYWORDS:

Aggrecan, Cartilage, Glycosaminoglycans, Porcine, Post Mortem Interval, Soil Environment

Articular cartilage is an avascular tissue rich in extracellular matrix (ECM) and with an exceptionally low cell density rendering it much more durable than most soft tissues (1). In addition, the surrounding soft tissues (membranes, muscles, ligaments) and bone provide articular cartilage with protection from the external environment, making it a suitable candidate for taphonomic research (1). The ECM of this tissue is highly hydrated and is formed of a complex network of collagens, proteoglycans and other non-collagenous proteins (2, 3) (Fig. 1) that are synthesized and maintained by a single cell type, the chondrocyte.

In mammals, two important and abundant structural components of hyaline articular cartilage are type II collagen and a large aggregating proteoglycan termed aggrecan. Type II collagen comprises about 60% of the dry weight of the tissue and forms a dense and ordered network of fibrils that provide the tissue with tensile properties. Aggrecan is the most abundant non-collagenous proteoglycan forming some 30% of the tissue by dry weight. The porcine core protein consists of 2,284 amino acid residues, of which a large extended section is richly decorated with negatively charged polysulphated glycosaminoglycans, mostly chondroitin sulphate (4, 5) which are responsible for the hydration of the tissue. Aggrecan therefore provides the cartilage with its ability to withstand compressive forces. Aggrecan is readily susceptible to degradation by proteolytic activity (7) which increases in degenerative conditions such as osteoarthritis (reviewed in (2)). It has therefore been extensively studied as a molecular marker for degenerative conditions and numerous molecular tools exist for detailed analysis of cartilage degradation (8, 9). In concert, these facts make cartilage a compelling candidate for use in forensic science.

Forensic taphonomy is concerned with the ways in which different environments alter the integrity of corporeal remains, and *vice versa*. These changes can be used as evidence in medicolegal contexts. This subject involves simulated reconstructions of environmental conditions (such as geographical location, temperature, humidity, soil chemistry, organism activity) for the purposes of investigating the decomposition process (10).

A longstanding aim of forensic taphonomists is to establish sound methodologies for estimating post mortem interval (PMI) (11). Information regarding the amount of time that has

elapsed between physiological death and medicolegal examination (12) is crucial to criminal investigations and legal proceedings as it establishes a time frame for when the decedent died. Furthermore, it also provides a point of reference for law enforcement officers to consider against both suspect and witness testimonies.

At present, forensic examiners employ a variety of physical and chemical techniques, few of which rely on the use of micro molecular techniques (13-20). Majority of the soft tissue methods used are restricted to bodies found above ground and are limited to providing acceptable PMIs that extend beyond the 48- and 100-hour margins. The principles that govern observations of insect activity above ground (21-23) are often used for longer time frames (days to months) and are not applicable to bodies buried under ground. New methodologies for long-term remains buried below ground involve use of a post mortem interval formula that takes burial accumulated degree days (BADD), extent and rate of soft and hard tissue decomposition, percentage of adipocere, temperature, and soil moisture into account (24), and *odor mortis* which involves the release of some 478 different volatile organic compounds (VOC) from decomposing bodies over time (25-27). Numerous intrinsic and extrinsic factors, such as illness/disease, climate, geographical location, and burial environment, greatly affect the rate at which soft tissues decompose (28-31). As a result, the above methodologies are often rendered ineffective for determining the PMIs that extend beyond 48 hours. Research conducted by Ferreira and Cunha (32) exploring the practicality of using decomposition rates to establish PMI, concluded that it is impossible to achieve accurate PMIs for buried human remains. Therefore, it is of crucial importance that alternative approaches capable of accurately determining longer PMI intervals are explored.

Until recently, the pathological properties of decaying hyaline cartilage remained a largely unexplored tool for forensic investigations. In 2002, Lasczkowski et al. (33) examined the post mortem (PM) viability of chondrocytes using fluoroprobes and discovered a correlation between chondrocyte loss and PMI, where the percentage of viable chondrocytes decreased with increasing PMI. In support of this finding, Rogers et al. (1) and ten Broek (34) have noted systematic changes in colour and robustness of PM cartilage with increasing time, where the tissue gradually becomes dark

pink in colour and thinner as PMI increases. These studies highlight the cellular and macroscopic changes that porcine cartilage undergoes PM.

The current study was a biochemical investigation of PM cartilage obtained from porcine (*Sus scrofa*) trotters interred in distinct soil environments for up to 6 weeks. Separation and visualization of PM cartilage protein relies upon the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot techniques. Our data demonstrate that the ordered degradation of aggrecan has the potential to be used as a forensic tool. Porcine material was chosen as a model for human decomposition because its biogeochemistry is similar to that of humans (22, 35, 36). The results presented demonstrate the changes that the extracellular matrix undergoes with increasing PMI.

Material and Methods

Study Sample and Burial Sites

Dismembered adult porcine (*Sus scrofa*) trotters were collected from a local abattoir within an hour of slaughter. The total number of trotters used in the experiments below is 19. Control samples consisted of trotters collected from the forelimbs of 5 different subjects to minimise mechanical variation and determine whether individual wear-and-tear among species population would present variable results. Post mortem samples came from a mixture of fore and hind limbs. Interments were conducted at two distinct locations: The University of Wolverhampton's Crop Technology Unit at Compton Park (Wolverhampton, West Midlands, UK; SO888988), and the Grange Farm Bungalow (Hilton, Shropshire, UK; SO781949). The site at Compton Park (soil environment 1 – SE1), is located at ground level and exhibits soil that is moist and nutrient rich. The Hilton (soil environment 2 – SE2) burial plot is situated at the top of a hill (approximately 66m above sea level) and contains nutrient deficient soil that does not retain moisture. Soil at SE1 and SE2 burial plots were both characterized as sandy loam (as assessed by thermogravimetric, X-ray fluorescence and X-ray diffraction analyses) (37). Several interments were conducted at different periods during the course of this three year study. However, the results presented serve as an exemplar for samples subjected to

soil temperatures ranging from 9.0-18.0˚C (at 30cm below ground) and ambient air temperatures from 8.0-28.0˚C. The average amount of weekly precipitation for this 6-weeks experiment was 4.2mm.

Isolation and Storage of PM Cartilage Proteoglycans from Degraded Trotters for Western Blot Analysis

Dismembered trotters were interred as pairs in six distinct graves, with dimensions of 30.5cm x 20cm x 30.5cm (length x width x depth) at the SE1 and SE2 sites during the spring of 2011 (March to May). Samples were left to decay belowground for designated periods of time. Weekly, a pair of trotters was disinterred from a single grave and general observations about the physical state of their skin surface, internal soft tissue and joint exposure were recorded. Skin surface was described as intact (uniform with no discontinuities along the surface) or broken (lacerations or tears exposing internal soft tissues/bones present) and thick (firm, providing a cushion around the bones) or thin (where bone could be felt directly under the skin once muscle and tendons liquefied and skin exhibited loss of elasticity) and fragile (easily broken or torn upon handling). Joints were described as partially exposed if skin exhibited superficial tears but remained surrounded by the presence of soft tissue and enclosed by the synovial membrane, whereas joints whose synovial membrane were disrupted and exhibited penetration of soil into the cavity were described as completely exposed (Table 1). MCP/MTP joints were dissected with a surgical scalpel to excise cartilage samples (1, 34). Control samples were obtained from fresh trotters collected on the day of slaughter (0 days PM). All cartilage samples were stored in airtight plastic vials (-20˚C) before lyophilisation (Edwards Modulyo EF4 Freeze Dryer).

Extraction and Preparation of Proteoglycan Samples for Western Blot Analysis

Post mortem cartilage samples underwent proteoglycan extraction using the protocol of Dudhia et al. (38) in conjunction with 25mM EDTA and 1% v/v Protease Inhibitor Cocktail I (Calbiochem). Proteins in the soluble fraction of the cartilage extracts were precipitated with 9.5 volumes of ethanol containing 50mM sodium acetate solution at -80˚C before centrifugation at 13,000

rpm for 10 minutes at room temperature (Progen Genfuge 24D Microcentrifuge). Supernatants were discarded and the precipitates washed twice in the ethanol solution before drying in a heating block at 37˚C and resuspension in 50mM Tris, 60mM sodium acetate (Tris acetate buffer), pH 8.0. Each protein sample (5µL) was digested with 0.05 units Chondroitinase ABC (*Proteus vulgaris*, Sigma-Aldrich, UK), 0.01 units of Keratanase *Pseudomonas sp*. (Sigma-Aldrich, UK) in the presence of 11.25µL Tris acetate buffer at room temperature for 3.5 hours. Total protein in solution was determined by Bradford assays (39) using the modified protocol established by Zor and Selinger (40).

The enzyme treated cartilage (20µg protein in 10µl of 1X loading buffer containing 5% [w/v] SDS and 4% [v/v] of 2-mercaptoethanol, the reducing agent) was subjected to SDS-polyacrylamide gel electrophoresis (41) using 9% (w/v) gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) (42). Membrane blots were blocked overnight with a 10% skimmed milk and 2% bovine serum albumen (BSA) solution prepared with distilled water. Membranes were then incubated with a 1:500 dilution of monoclonal antibody (MAb) 2-B-6 (mdbioproducts, UK) for one hour, followed by incubation with horseradish peroxidase-linked anti-mouse IgG antibody (1:1000 dilution, Cell Signaling Technology, UK) for one hour. The presence of bound antibody was visualised by autoradiography using a horseradish peroxidase (HRP) chemiluminescence kit (EZ-ECL; Geneflow, UK), Kodak Biomax MS Film, developer and fixer (Kodak GBX; Sigma-Aldrich, UK) (42).

Results

Analysis of Fresh Cartilage Samples.

A determination of individual variation among cartilage samples may be a factor in producing significantly variable results. Hence, within 3-4 hours of slaughter, a series of five forelimbs were randomly selected from five pigs and dissected for control cartilage samples. Protein was isolated and probed via Western blots with MAb 2-B-6 (section 2.2) (Fig. 2). This demonstrated that not only did 2-B-6 cross-react with porcine cartilage extracts, but also that there was a consistent and comparable

immune-reactive polypeptide distribution between samples. The band pattern for the samples further demonstrated uniformity with no significant variability observed within the samples. Samples 1-5 each yielded high molecular weight bands greater than 175kDa which is indicative of the intact core protein of aggrecan after deglycosylation. Additional smaller but fainter fragments (100 – 150kDa), were observed in later PM periods. A prominent band of approximately 27KDa was also observed, although the identity of this band is not clear.

Post Mortem Degradation

For a period of six weeks, a pair of trotters was disinterred from the SE1 and SE2 burial plots at corresponding PM intervals on a weekly basis. Observations of the physical condition of PM trotters prior to and during dissection were noted (Table 1). With increasing PMI, skin became thinner and the integrity of muscles and tendons gradually diminished. These changes were most notable at 30 Days PM, by which time the skin had become extremely delicate and susceptible to tearing upon manipulation, or was already broken around the joint areas at the time of disinterment. The muscles and tendons felt softer than PM trotters disinterred 7-21 days PM and showed some signs of liquefaction.

Cartilage samples were excised from the MCP/MTP joints for comparative analyses across PM weeks and burial location. The PM samples were processed in the same manner as the control samples to reveal aggrecan polypeptides. Western blots for PM samples disinterred from SE1 (Fig. 3a) and SE2 (Fig. 3b) also identified the presence of high molecular weight bands larger than 175kDa and the presence of low molecular weight bands at approximately 28kDa for up to 21 Days PM. For cartilage samples extracted 30-42 Days PM, there was an absence of immune-reactive bands. Absence of bands in samples disinterred between 30-42 Days PM coincided with observations of extreme soft tissue degradation that resulted in the exposure of joints to the surrounding soil environment. For PM samples collected from SE1 (Fig. 3a) and SE2 (Fig. 3b), high molecular weight (>200kDa) bands representative of intact aggrecan were observed but interestingly, in the SE2 samples there was an increasing heterogeneity of size with time, as observed by the presence of

fragments between 80 – 200kDa at 14 and 21 days. At 30 Days PM and beyond, both high and low molecular weight bands were absent.

Discussion

This investigation is the first to explore the biochemistry of PM cartilage and its potential forensic applications. The data reported illustrates the general trends observed for weekly samples collected March-May (2011) over the course of 42 days. They are representative of results obtained from numerous sample sets disinterred throughout the year and reveal a temporal PM degradation of aggrecan. With increasing time, the presence of aggrecan becomes undetectable for samples disinterred more than 3 weeks PM.

The observed changes among the protein bands with increasing PMI could be attributed to the experimental conditions because cartilage excised from fresh trotters showed a consistent and uniform banding pattern. Although semi-quantitative, the 200kDa band was more intense than in the experimental samples suggesting a lesser amount of intact aggrecan with growing PMI.

The appearance/visibility of protein bands for samples disinterred from SE1 and SE2 illustrates that MAb 2-B-6 reacts with chondroitinase digested aggrecan motif. This motif is common to higher mammals, such as porcine and humans (43). In addition, porcine and human (43) aggrecans also share highly similar amino acid sequences. These combined features make this experiment an acceptable model for studying human PM samples. Moreover, the similarities in amino acid sequence between porcine and human aggrecan present opportunities for the analytical tools used in this experiment to be further expanded with use of different antibodies recognising specific cleavage sites along the core protein. This study opens an avenue for further refinement of PMI obtained using cartilage samples and lends more evidence that porcine is an acceptable model for humans.

An absence of protein bands detected by MAb 2-B-6 in Western blots of cartilage extracts collected from trotters disinterred 30, 36 and 42 days PM suggests a complete disintegration of the core protein into fragments too small to be resolved in 9% gels. This observation corresponds with a

decline in the physical state of the trotters whereby joint exposure occurred when soft tissue surrounding the joint deteriorated, leaving the joint membrane or the synovium exposed to the external environment. Trotters exhumed at these intervals possessed significantly thinner skin, muscles and tendons on the verge of liquefying or completely liquefied, and bones that showed signs of disarticulation at the epiphyseal-diaphysis junction of the metacarpal/metatarsal and phalangeal joints. Joints disinterred from SE1 showed the most pronounced change which most likely was due to the moist soil at this burial site accelerating soft tissue decomposition. This was one week earlier than the Hilton site where the soil was classified as extremely dry and nutrient deficient (lacking sufficient humus). Deterioration of the soft tissues surrounding these joints may have facilitated the direct access of soil microbes to cartilage and microbial metabolic activity in accelerating the degradation of aggrecan (44).

Protein bands with molecular weights greater than 175kDa were observed among the Western blots for control and PM samples. These bands are characteristic of deglycosylated PGs whose protein core has a molecular weight of approximately 230kDa (45-47) and fall within the expected range for viewing structural and catabolic neoepitopes of aggrecan probed with MAb 2-B-6 (48). Kashiwagi et al. (49) examined the proteolytic effects of altered forms of the ADAMTS-4 enzyme on aggrecan degradation, using MAb 2-B-6, and illustrated the appearance of lower molecular weight fragments when subjected to prolonged incubation times with the enzyme (48). These low molecular weight fragments most likely represent small quantities of degrading aggrecan that are known to accumulate in cartilage with increasing age (2).

The catabolism of proteins relies on enzymes that have the ability to split apart the peptide bonds of a protein by incorporating water between the adjoining amino acids that form the protein. A moist environment would facilitate the fragmentation of aggrecan into lower molecular weight fragments by maintaining the water levels necessary for proteolysis, whereas trotters buried in soil environments that are dry, may result in the leeching-out of bodily fluids and nutrients (50). Moreover, changes in soil moisture content are associated with fluctuations in the microbial biomass, whereby increased water content results in proliferation of microbial activity (11, 50). Furthermore,

water allows soil microbes and/or their proteolytic secretions to be better transported deeper into the soft tissues of decomposing remains.

Despite the distinct locations the biochemical degradation pattern of aggrecan pursuant to death is remarkably similar. Initially the autolysis of cartilage might be expected to occur with great variability as a result of various environmental factors influencing the rate of soft tissue decomposition. However, the situational (ground-level versus hill-top) and environmental (moist and nutrient-rich versus dry and lacking nutrients) differences that existed between the SE1 and SE2 burial plots appeared to have little influence on the decay of the cartilage. The basis of cartilage degradation from a PM standpoint is that the sizeable proteoglycan aggregate dissociates and degrades in an orderly manner, whereby intact aggrecan gradually fragments into lower molecular weight by-products until the time when the joint is exposed to the external environment; the degradation bands observed in the intact joints is owed to the slow activity of proteolytic enzymes that accumulate in the ECM. This finding lends further support to previous studies conducted by Lasczkowski et al. (33) who observed a relationship between chondrocyte viability and PMI in controlled environments and Rogers et al.'s (1) report which underscored the systematic changes in colour and robustness that cartilage underdoes, as well as loss of nucleic material, with increasing time. Furthermore, the biochemistry of degrading cartilage exhibits little to no variation for samples collected from differing soil environments at the same time of year. This consistency among the data sets may be accredited to the protection that cartilage is afforded from the immediate environment as a result of its situation in relation to the surrounding soft tissues, and the low cell density of the tissue.

Unlike previous studies that have explored the cellular degradation of PM cartilage in manipulated or controlled settings (32, 51, 52), this study was conducted in both laboratory and field environments where dismembered trotters buried in soil plots were left exposed to the natural elements for various lengths of time, undisturbed by animal activity. Although the intention of this study was to explore the long-term biochemical properties of PM cartilage in forensic contexts, the results do not consider how variations in soil depth or different soil chemistry affect cartilage degradation. Likewise, human remains are found in a variety of different contexts where bodies may

be found aboveground where the ambient temperatures tend to be warmer or in large bodies of cool water, and are usually fully intact. Further research on the PM degradation of cartilage should consider how these factors affect the biochemistry of the avascular tissue. Moreover, use of MAb 2-B-6 serves to highlight only 87% of the PG core protein surface area and does not account for the entire structure of aggrecan that includes the HA backbone to which PG monomers are attached by way of link proteins (47). Other antibodies specific to these areas should also be explored in order to achieve a complete picture of the PM degradation that aggrecan undergoes for further realization of this tissue's relevance to forensic investigations.

Conclusions

The molecular breakdown of cartilage PGs has the potential to be used as a reliable indicator of PMI irrespective of differing soil environments for up to 3 weeks post mortem at soil temperatures ranging from 9.0-18.0˚C (buried at a depth of 30cm) and ambient temperatures between 8.0-28.0˚C. This finding extends the potential method for PMI determination well beyond 48-hours. Because the structural similarities of PGs derived from porcine and human subjects are very identical, the results obtained herein could serve as a model for the PM degradation of human cartilage and thereby validate the practicality of using PM cartilage in forensic investigations. Although porcine specimens are used as analogues for human decomposition in forensic research because it is readily accessible, examination of human cartilage would be ideal as it is necessary to validate the usefulness and applicability of these findings to cases involving human remains. Future work would involve application of the methodologies described in this article to human samples collected at roughly the same PM interval. Finally, the results obtained may also prove useful for forensic investigations concerning crimes against animals which involve the illegal trade and mortality of endangered species or detection of food crimes (53).

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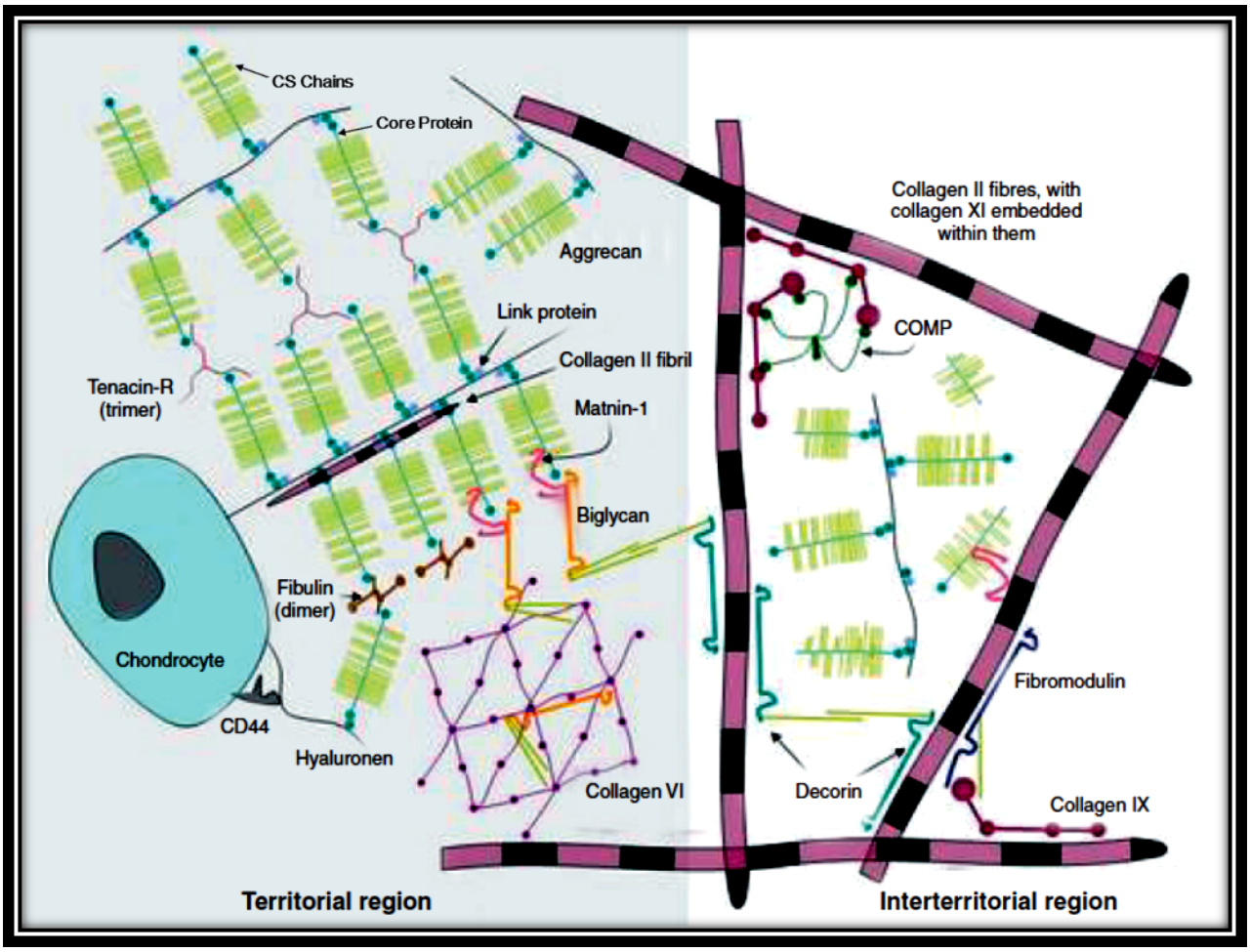

FIG. 1 Simplified schematic representation of cartilage components and their interactions.
Cartilage is comprised of an extracellular matrix which is synthesised and maintained by the chondrocytes, the cellular matrix component. Its major components include aggrecan, proteoglycan, and smaller proteoglycans (biglycan, decorin, fibromodulin); numerous collagens (predominantly type II); and a series of non-proteoglycans such as cartilage oligomeric matrix protein (COMP) and link protein. The territorial region of the extracellular matrix is characterised as proteoglycan-rich and is situated directly outside the chondrocytes, whereas the interterritorial region is located between the territorial matrices. Aggrecan, the structure providing cartilage with its elasticity and ability to resist compressive forces, contains a protein that is indirectly attached to a hyaluronic acid backbone by way of link protein. This core protein also consists of CS side chains. Modified and reprinted with permission from Dudhia [2]. Copyright 2005 Cellular and Molecular Life Sciences.

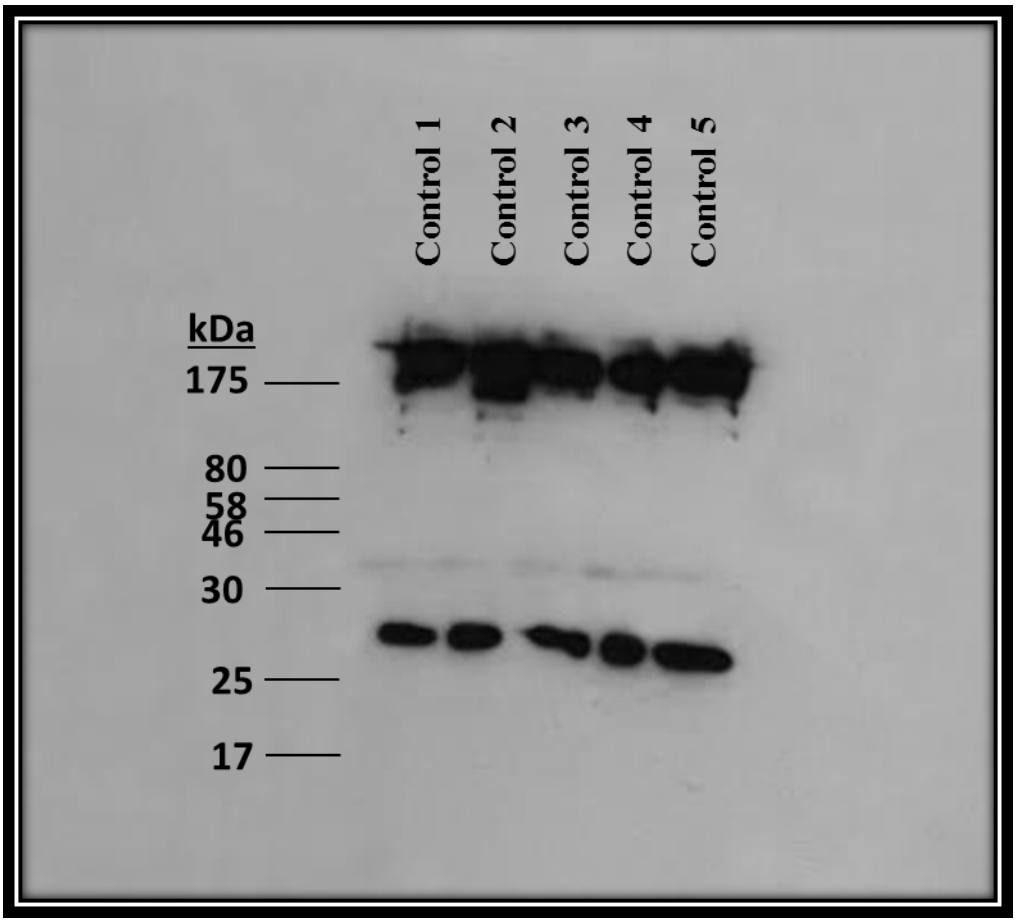

FIG. 2 Immunoblot of a 10% SDS-polyacrylamide gel illustrating control cartilage samples (0 Days PM). Columns from left to right represent control samples 1-5 collected from a forelimb of 5 different pigs within hours of slaughter. Cartilage extracts were treated with chondroitinase ABC to digest the chondroitin sulphate chains of aggrecan to enable entry of the aggrecan core protein into the gel medium and immunodetection with MAb 2-B-6 antibodies. Separation of polypeptides by SDS-PAGE was followed by transfer to a PVDF membrane (Western blot) and immunodecoration with MAb 2-B-6 which recognises the chondroitin-4-sulphate (C-4-S) and dermatan sulphate (DS) (chondroitin-0-sulphate) stubs that remain after digestion with Chondroitinase ABC, thereby enabling visualization of the protein core situated between the G2 and G3 domain of aggrecan. Control samples (0 Days PM) labeled 1-5 were loaded in adjacent wells (20μg/10μL) of a 9% gel and separated by SDS-PAGE and Western blot as previously described, followed by secondary anti-mouse antibody conjugated with horse-radish peroxidase and visualised by chemiluminescence.

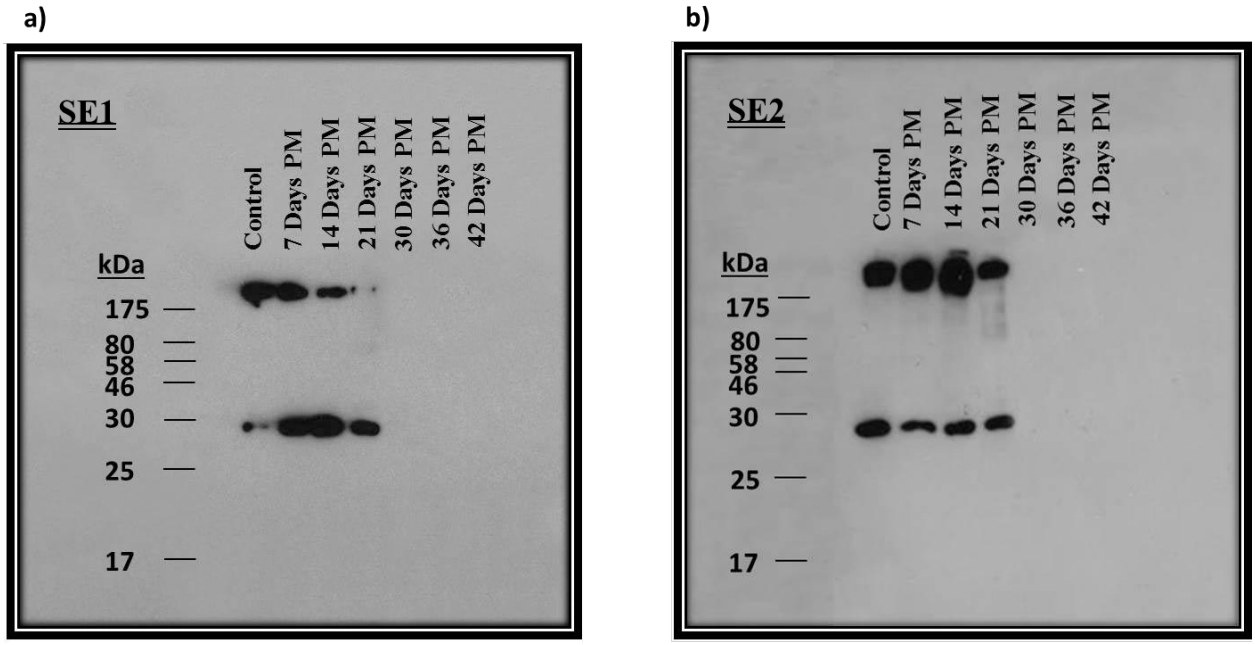

FIG. 3 Western blots of post mortem degraded proteoglycan extracted from cartilage samples at weekly intervals from **a**) SE1 and **b**) SE2 burial plots. Cartilage samples were collected from trotters disinterred at 7, 14, 21, 30, 36 and 42 days PM. Extracted protein samples were digested with chondroitinase ABC prior to electrophoresis in 10% gels and immunoblots probed with MAb 2B6. Proteoglycan extracts contain 20µg of protein.

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ɔservations for the physical properties of post mortem trotters disinterred prior to cartilage extraction.

Site	PM Sample	Skin Surface	Joint Exposure	Internal Soft Tissue
Compton	0 Days	Intact - Thick	No exposure	Solid Tissues
	7 Days	Intact - Thick	No exposure	Solid tissues
	14 Days	Intact - Thick	No exposure	Solid tissues
	21 Days	Intact - Beginning to thin	No exposure	Solid tissues - Softening; Darkened muscles
	30 Days	Broken - Notably thinner and fragile	Exposured - Slightly; synovial membrane ruptured	Liquefying- Some solid muscles but tissues liquefying
	36 Days	Broken - Significantly thinner, little remaining	Exposed - Partially; still surrounded by soft tissue	Liquefied - muscles completely putrefied, tendons much softer
	42 Days	Completely degraded - Internal soft tissues and bones exposed	Exposed - Completely	Liquefied - Completely; extremely thinned tendons remain
Hilton	0 Days	Intact - Thick	No exposure	Solid Tissues
	7 Days	Intact - Thick	No exposure	Solid tissues
	14 Days	Intact - Thick	No exposure	Solid tissues
	21 Days	Intact - Beginning to thin and slightly dry	No exposure	Solid tissues - Softening; Darkened muscles
	30 Days	Broken - Small breaks in skin around carpal joints; thinner and slightly drier	No exposure	Liquefying- Mostly solid muscles but tissues beginning to liquefy
	36 Days	Broken - Larger breaks around carpals; increasingly thinner and drier	Exposed - Partially	Liquefied - Some solid muscles among putrefying soft tisses, tendons much softer
	42 Days	Near Completely degraded - very thin; internal tissues and bones exposed	Exposed - Completely	Liquefied - Completely; tendons almost completely liquefied