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TECHNICAL NOTE

Pathology/Biology

Postmortem chondrocyte viability in porcine articular cartilage: Influence of time, temperature, and burial under winter conditions

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Abstract

The aim of the present study was to investigate the effects of time, temperature, and burial in a natural environment on the viability of chondrocytes in porcine femoral condyles using confocal laser scanning microscopy. Hind trotters from 10 pigs were buried or left unburied. Samples were collected daily and stained with a combination of vital dyes (calcein-AM and ethidium homodimer-1). The chondrocytes showed an intense staining corresponding to their vitality. In the first 3 days, viability decreased slowly and showed no statistical difference between buried and unburied samples. After the first 3 days, it decreased rapidly, with the viability of the buried samples being 66% on day 4, decreasing to 25% on day 8 and to 16% on day 10, while in the unburied samples it decreased to 43% on day 4, 13% on day 8 and 5% on day 10. Our results indicate a time, temperature, and burial dependent decrease in chondrocyte viability and suggest the use of chondrocyte viability as a marker for estimating PMI in both the natural environment and in animals, as well as its potential use in humans.

KEYWORDS
burial, calcein-AM, cartilage, confocal laser scanning microscope (CLSM), ethidium homodimer-1, postmortem interval (PMI)

Highlights

- The influence of time, temperature, and burial on the viability of chondrocyte is investigated.
- Cartilage in the natural environment showed lower viability compared to in vitro studies.
- Burial has a significant effect on the long term, but not on the short-term viability of chondrocytes.

1 | INTRODUCTION

Immediately after death, progressive physical and biochemical postmortem changes such as autolysis, cooling of the body, stiffening, and putrefaction occur in the body in a specific order [1]. Knowing the exact order in which they occur and the internal and external influences on these changes is critical to determining the time that has elapsed since an individual's death, i.e., the postmortem interval (PMI), an essential component of both human and veterinary forensic examination.

The PMI is traditionally divided into an early (up to 72 h after death) and a late (72 h until complete decomposition of the body) period [2].

The overall accuracy of PMI determination decreases with increasing time since death [3]. To complicate matters, the environment

in which the corpse is found influences the rate of decomposition through factors such as temperature, humidity, burial, presence of insects, scavenger activity, etc., which are intertwined and often influence each other [4]. Over the past decade, the accuracy of methods for estimating PMI, especially late PMI, has not improved significantly, measurement errors remain high, and only approximate PMI estimates can be made. Therefore, despite decades of research, estimation of PMI at the crime scene is often not an exact science [4–7].

Many methods for the determination of PMI have been developed under in vitro conditions without taking into account the numerous environmental factors mentioned above and in particular their interactions [4]. For this reason, many of them are suitable for research purposes, but have little practical value [5, 8, 9]. Madea et al. (2005) and other authors suggest the use and development of methods that allow quantitative assessment, whereby a mathematical description of changes should be possible and influencing factors should be considered quantitatively [5, 7, 10, 11].

To reduce the error caused by the aforementioned factors, tissues that are only minimally affected by the environment could be used [9], for example, the vitreous humor, the synovial fluid, or the odontoblasts in the dental pulp [12–14].

Another such tissue is cartilage, a highly specialized tissue consisting of chondrocytes, cells with low metabolic activity and low nutrient and oxygen requirements, surrounded by an abundant extracellular matrix (ECM) [15, 16]. The ECM is rich in nutrients and various solutes that diffuse to the chondrocytes even after an individual's death, allowing chondrocytes to survive for several days after death [17–19].

Studies on human samples suggest that the viability of human chondrocytes in an in vitro environment is time and temperature dependent, suggesting that cartilage could be used to determine PMI in the late postmortem period, particularly when temperatures are stable. In some studies, the viability of chondrocytes from human osteochondral allografts (cartilage tissue used to treat large cartilage defects) remained high at up to 70% even after 1 month of storage at 4°C [9, 17, 19–22].

The long lifespan of chondrocytes after death and the gradual decrease of their viability under the influence of time and temperature make cartilage a promising tissue for the determination of PMI for forensic purposes.

The aim of our study was to determine the effects of time, temperature, and burial in a natural environment on the viability of chondrocytes in porcine femoral condyles using confocal laser scanning microscopy (CLSM). To the best of the authors' knowledge, this is the first study of its kind.

2 | MATERIALS AND METHODS

This work was carried out in the Institute for Pathology, Wildlife, Fish and Bees at the Faculty of Veterinary Medicine, University of Ljubljana between January 20 and 29, 2023.

2.1 | Sample procurement

10 pigs of the breed Landrace weighting between 20 and 35 kg were used in this study (permit no. U34453-38/2021/5). All pigs were euthanized by intravenous administration of sodium pentobarbital (Richter Pharma AG, Wels, Austria) 40 mg/kg body weight, after having been deeply anesthetized with intravenous administration of ketamine (Vetoquinol UK Limited, Towcester, UK) 20 mg/kg body weight, because of agony due to untreatable diseases. Necropsies were performed to determine possible diseases that could affect cartilage health; hind trotters were removed and labeled. The first cartilage samples (osteochondral cylinders) were collected from the knees of both removed hind trotters (femoral condyles) of each animal within 5 h of death, as this proved to be an ideal site for cartilage collection due to its compartmentalization from other tissues, ease of access and larger sampling area [20, 23]. Sampling was performed in the autopsy room at 20°C. The articular surfaces of the femoral condyles were exposed by transverse arthrotomy. Cartilage samples were collected using the Osteochondral Autograft Transfer System- OAST (Arthrex, Naples, Florida, USA) and stored in phosphate-buffered saline-PBS (Thermo Fisher Scientific, Waltham, Massachusetts, USA) until further processing. After sampling, the knee joints were carefully closed and tightly wrapped with a Coban bandage (3 M, Saint Paul, Minnesota, USA) to maintain closure. In a shady forested region in central Slovenia, one hind trotter of each animal was buried 50 cm deep into the ground, while the other was left on the surface. The soil type was sandy loam with a pH of 4.1. The experiment took place in winter from 20 to 29 January 2023. Due to the size of the cartilage in the knee joint, only 7 samples could be collected. Therefore, based on a previous pilot study, samples were collected when the pigs were euthanized (day 1) than at 1-day intervals for 4 days, on the 8th and 10th day after the animals death, with samples collected on site between 5:00 and 5:30 am. After sampling, each knee joint was closed, wrapped with a new Coban bandage and reburied or placed on the surface. During the experiment, surface temperature and temperature at the depth of the trotters were measured daily at the time of sampling with a buried thermometer (TFA Dostmann GmbH & Co. KG, Reicholzheim, Germany); temperature and humidity data were also collected from a local government weather station at the distance of 3.4 km. The exact temperatures and humidity are listed in Table 1.

2.2 | Chondrocyte viability assay

Tissue samples were stained and analyzed using an established protocol used in similar studies [17–19, 24].

For CLSM, osteochondral cylinders were removed from the storage medium (PBS) and three 250 μm thick slices were cut vertically through the entire cartilage thickness, starting at the synovial surface, and ending just above the subchondral bone junction. To minimize the artifacts from sample manipulation, sections were made at the centre of each sample, using a Leica VT1000 S vibrating

TABLE 1 Temperatures at the burial site at the time of sampling and data for humidity, precipitation, snowfall, and maximum, minimum, and daily mean temperatures from a local government weather station.

Date	Temp. in depth (°C)	Temp. on surface (°C)	Temp. mean (°C)	Temp. max. (°C)	Temp. min. (°C)	Humidity (%)	Snow	Rain
January 20	2.5	−4	0.1	1.7	−0.5	86	Yes	No
January 21	3	−1.5	0.2	3.7	−5.1	76	No	No
January 22	2.8	−1.1	0.6	1.8	−0.6	83	No	No
January 23	4.1	−0.5	1.6	3.4	0.4	89	Yes	Yes
January 24	4.4	−0.5	2	3.5	0.9	89	No	Yes
January 27	5.2	−0.5	1.1	3.9	0.1	71	No	No
January 29	5.3	−0.8	0.9	2.5	−0.1	77	No	No

blade microtome (Leica Biosystems, Wetzlar, Germany) and immediately stained with Calcein-AM (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and ethidium homodimer-1- EthD-1 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Calcein-AM (Ca-AM) is a membrane-permeable, non-fluorescent live-cell labelling dye that diffuses passively into the cytoplasm. Within the cell, the non-fluorescent Calcein-AM is hydrolyzed by nonspecific intracellular esterases to a green fluorescent calcein dye that is membrane-impermeable and therefore retained in the cytoplasm of cells with intact membranes. Calcein has a peak at 517 nm when optimally excited by blue light at 494 nm, indicating that the cell has an intact membrane and esterase activity and is therefore considered viable. Ethidium homodimer-1 (EthD-1) is impermeable to intact cell membranes but can diffuse through the porous membranes of dying or dead cells. This dye has a high affinity for nucleic acids and emits a bright red light at 617 nm when excited at 528 nm [19]. Cartilage slices were incubated for 45 min at room temperature in a 1.5-mL Safe-Lock tube (Eppendorf, Hamburg, Germany) containing a solution of 8 µL Ca-AM (1 mmol) and 2 µL EthD-1 (2 mmol) diluted in 1 mL PBS. Samples were protected from daylight during incubation and microscopic analysis. Using the described staining protocol, we detected viable and non-viable (dead) chondrocytes in each sample. Confocal micrographs were acquired with a laser scanning confocal microscope (LSCM 700) using 20× objectives (Zeiss, Stuttgart, Germany) and a 488-nm and 543-nm argon-krypton laser line (Leica, Wetzlar, Germany). Scans were acquired at 512×512-pixel resolution with the pinhole set at one Airy unit. Images were acquired in the central portion of the tissue sample at least 200 µm away from the slice margins to avoid any artifacts. Each region of interest was captured by eight stacked images at a depth of 60 µm, resulting in an optical slice volume of 0.011 mm^3 (field size of 424.27×424.27 µm and a depth of 60.1 µm). Confocal micrographs with green and red spots (live and dead cells) were analyzed using ZEN 2010 (Zeiss, Stuttgart, Germany) and ImageJ software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). Cells with green fluorescent cell bodies were classified as viable, whereas cells without signs of a cell body and with red nuclear fluorescence were designated as dead. Cells with green cell bodies and red nuclear fluorescence were classified as damaged and therefore counted as dead.

2.3 | Statistical analysis

Statistical analyses and graphs were generated using the programming language R for statistical computing, version 4.2.2 (R Core Team, 2022). First, the proportions of viable cells per sample were calculated and used for the calculation of descriptive statistics and further analysis. Differences in the proportions of viable cells between sampling days were assessed using the Kruskal-Wallis rank sum test and statistical differences between days were determined using the Kruskal-Wallis multiple comparison test. Differences between buried and unburied samples on the same sampling day were tested with a Wilcoxon rank sum test. In addition, the effect of time

and temperature on chondrocyte viability was assessed using a multiple linear regression model. For all statistical tests, a *p*-value of less than 0.05 was considered statistically significant [25].

3 | RESULTS

None of the pigs showed signs of arthritis or arthrosis in their hind trotters and were therefore suitable for further study.

After incubation with Ca-AM and EthD-1, the cytoplasm and nucleus of the chondrocytes showed intense staining according to the functional state of the cell (Figures 1 and 2). In the later stages of decomposition (day 8 and 10), groups of green fragments without red signal were detected, which were probably membrane particles from decaying cells and were therefore counted as dead. On day 10, a less structured fluorescence was observed with more background within the lacunae; however, cell-like structures were still visible.

Analysis of osteochondral cylinders by CLSM confirmed a statistically different percentage of viable cells in the cartilage samples during testing over a 10-day period after euthanasia. The descriptive statistics (minimum, quantiles, median, mean, standard deviation (SD), and maximum) of the results per day for all samples are shown in Table 2. The differences in the proportions of viable chondrocytes between days were statistically significant ($p < 0.0001$, Kruskal-Wallis rank sum test). In particular, the differences were significant when pairs of the first three and last three sampling days were compared, whereas there were no differences between adjacent days.

Figure 3 shows the difference in the distribution of results between buried and unburied samples per sampling day. There were no statistically significant differences between buried and unburied samples at the immediate time of storage (day 1) and until day 3 with *p*-values ranging from 0.0524 to 0.4813. After day 3 for the unburied and day 4 for the buried samples, the chondrocyte viability started to decrease. When comparing the results of buried and unburied samples from day 4 to day 10, the buried samples had a statistically significantly higher percentage of viable chondrocytes than the unburied samples, with p-values ranging from 0.0005 on day 4 to 0.0310 on day 10. At day 10, the median viability was higher in the buried samples (14.25%, interquartile range (IQR): 7.70–21.42) than in the unburied samples (3.06%, IQR: 0.10–9.59).

The mean temperatures at the surface and at the sample depth and the mean and standard error of the proportions of viable chondrocytes per day for buried and unburied samples are shown in Figure 4. Regression analysis showed that the two independent variables, time, and temperature, included in the final model, had a significant effect on the proportion of viable chondrocytes. Both were highly significant with $p < 0.0001$. The results showed that the proportion of viable chondrocytes decreased on average by 8.51% each day. On the other hand, a 1°C increase in temperature resulted in a 2.12% increase on average in the proportion of viable chondrocytes. The model explained 79% of the variation in the dependent variable.

4 | DISCUSSION

Although chondrocytes and cartilage in general are highly resilient cells with a long life-span protected by a rich ECM, they have received little attention in forensic medicine over the last decade.

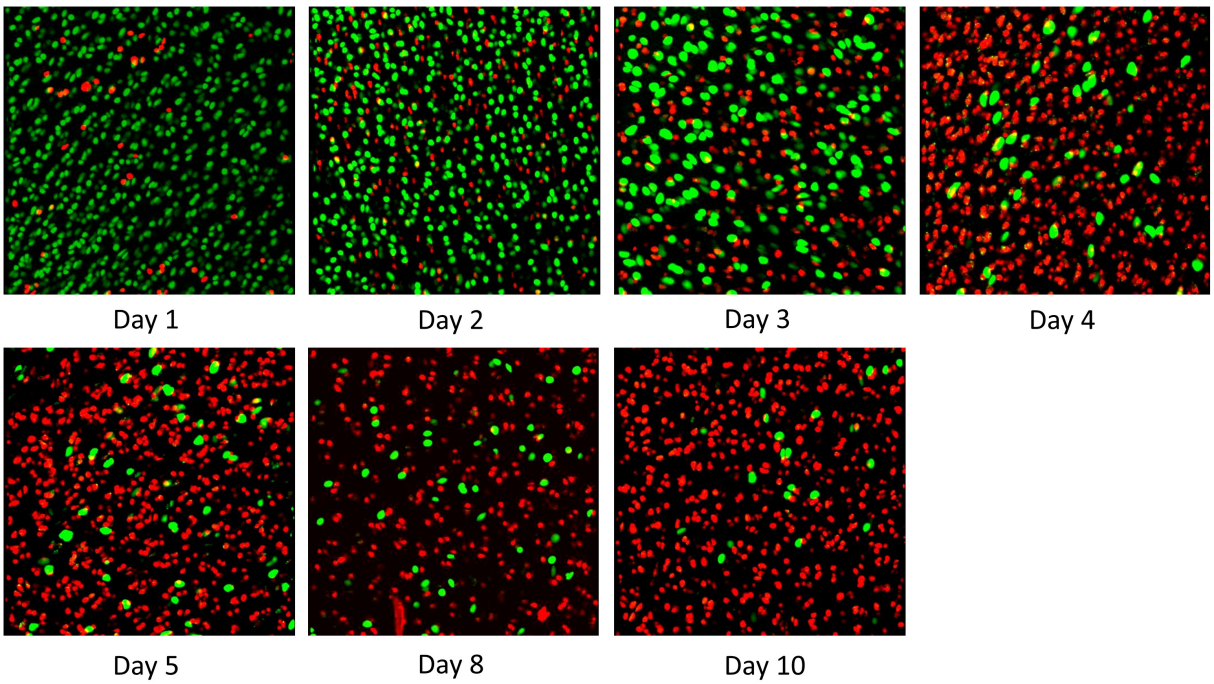

FIGURE 1 An example of confocal microscopic images of staining of chondrocytes in unburied samples for all sampling days. Samples are stained for live (green) and dead (red) cells.

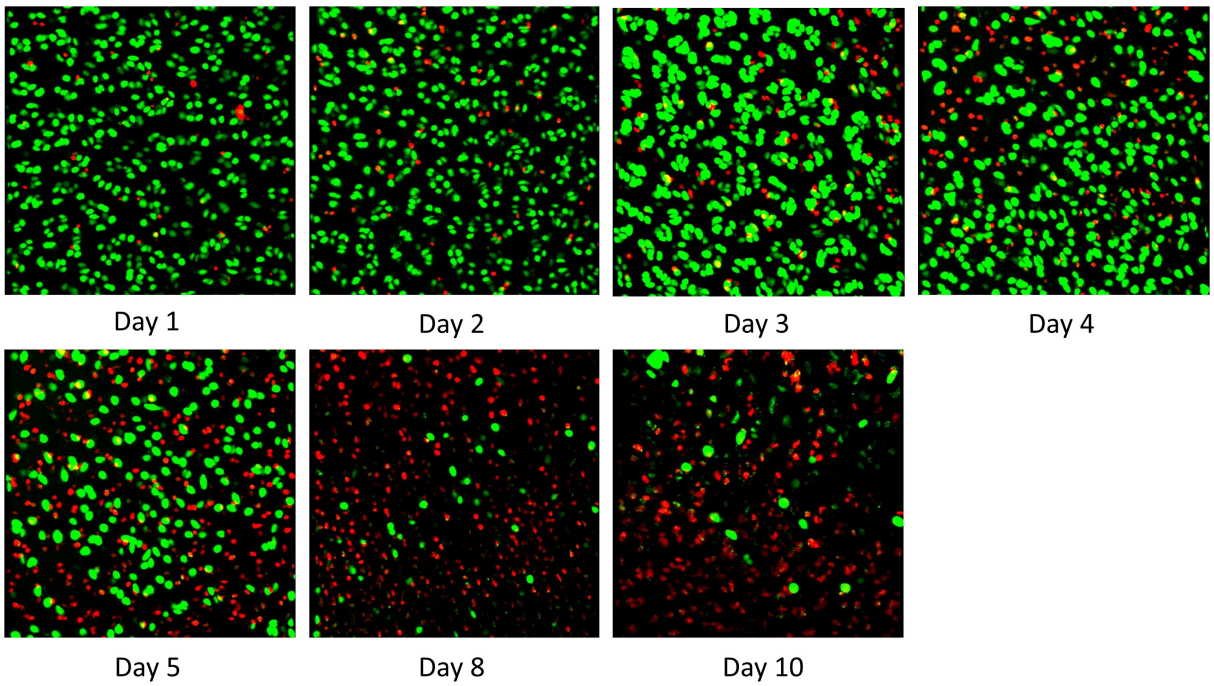

FIGURE 2 An example of confocal microscopic images of staining of chondrocytes in buried samples for all sampling days. Samples are stained for live (green) and dead (red) cells.

TABLE 2 Sum of viable burried and unburied chondrocytes (%) by sampling day using confocal laser scanning microscopy.

Date	Day	Minimum	1st Q	Median	Mean	SD	3rd Q	Maximum
January 20	1	48.21	62.57	71.85	72.15	12.55	82.09	94.29
January 21	2	63.73	73.07	76.15	76.86	7.55	78.25	96.19
January 22	3	56.35	62.65	68.07	68.03	6.91	73.24	78.46
January 23	4	13.07	49.7	58.14	54.41	18.18	66.68	80.91
January 24	5	8.82	26.09	37.09	38.62	18.33	53.62	71.74
January 27	8	1.12	10.94	17.40	19.22	12.35	25.83	44.01
January 29	10	0	2.56	7.93	10.59	10.89	15.14	39.53

Abbreviations: Q, Quantile; SD, standard deviation.

Existing studies have mainly been conducted in controlled in vitro environments, which limits the consideration of various factors, not to mention their mutual influence.

In the present study, we sought to address this knowledge gap by investigating the effects of burial on chondrocyte viability, as this is an often overlooked factor that is difficult to reproduce in in vitro studies. In addition, we wanted to determine the role that time and temperature play in a natural environment and to verify whether viability decreases in the natural environment in the same way as under in vitro conditions. Pigs were chosen as analogues to human tissue because their anatomical and physiological similarity to humans provides a comprehensive experimental basis for empirical studies of decomposition in forensic entomology, taphonomy, and ecology [26]. The peripheral articular cartilage of the knee was used because, unlike the cartilage tissues of the head, neck or torso, it is not in close contact with the commensal bacteria reservoir [19, 20].

Initially, the count was carried out using operator-independent software (ImageJ). When comparing the results with the manual count, we found discrepancies caused by the threshold calculation, which led to a systematic error. In addition, the software counted the aforementioned cell membrane fragments as viable, which led to false positive results. To solve these problems, the counting was performed manually.

Given the limited knowledge of the lifespan of cartilage cells in the natural environment, we compared our results with in vitro studies on human cadavers and with studies on human allografts performed for tissue banking requirements and for clinical purposes. In these studies, the samples were stored under optimal conditions, which allowed for a longer survival time [19, 21, 22, 27]. In the in vitro human cadaver studies, although the cartilage was stored under conditions similar to those of a deceased person, the environment did not resemble the conditions typically found in forensic scenarios, with the exception of varying temperatures [17, 18, 24].

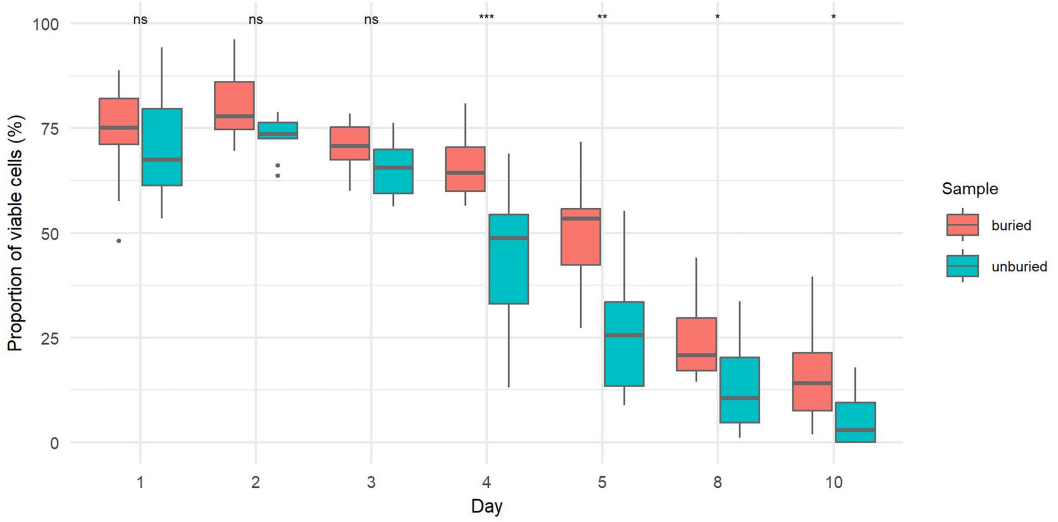

FIGURE 3 Comparison of the proportion of viable chondrocytes between buried and unburied samples by sampling day using confocal laser scanning microscopy. Above each pair of boxplots are the results of the Wilcoxon rank sum test; ns, not significant; $^{***}p < 0.0001$; $^{**}p < 0.001$; $^{*}p < 0.05$. The box represents the lower and upper quantiles, the line in the box denotes the median, the whiskers denote the minimum and maximum values without outliers, which are marked with dots.

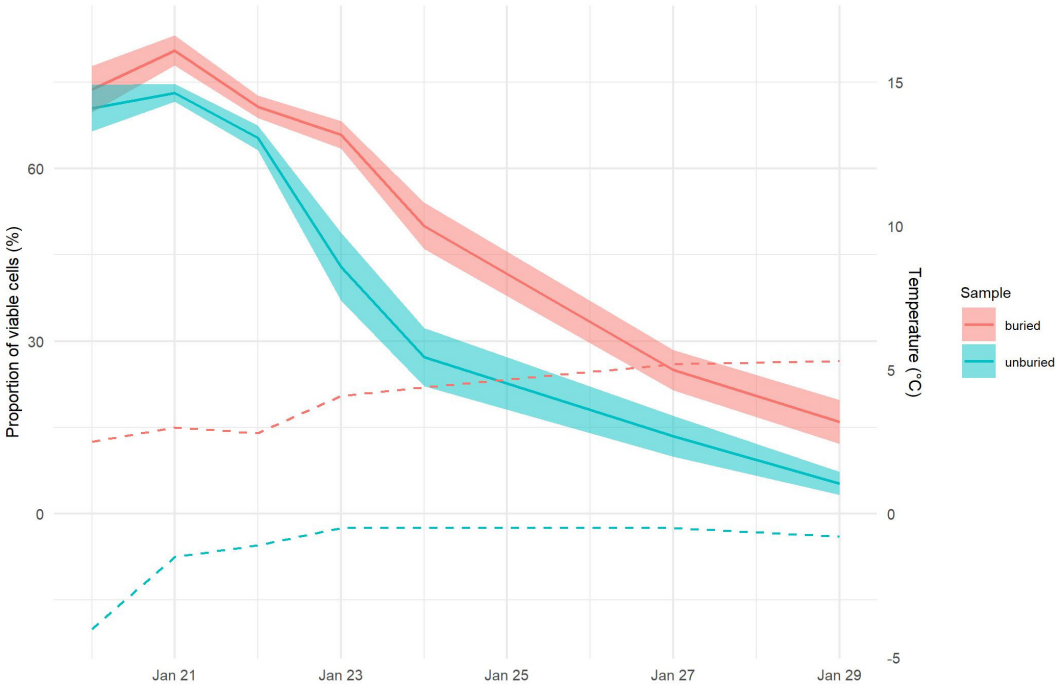

FIGURE 4 The curves show the mean and standard error of the proportion of viable chondrocytes in buried and unburied samples using confocal laser scanning microscopy. The dashed lines show the temperature on surface (blue) and in depth (red).

In the present study, the overall viability of both buried and unburied samples decreased over a 10-day period. During the first three days, viability decreased slowly, this phase could be described as a plateau. After the third day for the unburied samples and the fourth day for the buried samples, viability began to decrease rapidly. We hypothesize that the initial slow decline is due to the low nutrient and oxygen requirements of the chondrocytes and the abundant ECM, which contains various solutes that provide nutrients and oxygen to the chondrocytes and meet their postmortem needs [9]. Thus, rapid depletion of nutrients is probably the main cause of the overall rapid decline in the proportion of viable chondrocytes. The plateau phase has been demonstrated in in vitro studies on human cadavers, where it lasted between 7.5 and 15 days, and in studies on human allografts, where it lasted even longer, with chondrocyte viability remaining at 70% after 1 month in some studies. As mentioned by the authors, this was likely the result of the optimal storage medium

and stable storage conditions used in their studies [19, 21, 22]. In the study by Lasczkowski et al. (2002), human cadaver samples stored at 4°C showed a plateau phase of up to 5 days, with viability decreasing to 58% after 6 days and to 9% after 1.5 months. The cadavers were stored under controlled conditions to minimize environmental influences and ensure a constant temperature throughout the study [17]. The samples in our study were collected daily from knees that were exposed to fluctuating temperatures, rain, snow and other environmental factors. The plateau phase was therefore shorter and the initial drop in chondrocyte viability was more drastic compared to the previously mentioned in vitro studies.

Burial is one of the simplest methods of minimizing the effects of environmental factors on a corpse. It has been found that corpses buried in shallow graves (<30 cm) decompose faster than those buried at greater depths. This discrepancy results from the thinner layer of soil, which offers less protection from environmental factors and temperature fluctuations [8, 28, 29]. To investigate the effects of burial on chondrocyte viability, 50 cm deep graves were used in this study. When comparing the results of unburied and buried samples, no statistically significant difference was found in the first three days, suggesting that the tissue surrounding the cartilage and the ECM provide adequate protection regardless of burial. From the third to the tenth day, the difference was statistically significant, with a faster decrease in viability in the unburied samples and a statistically significant higher percentage of viable cells in the buried samples. Some studies on cartilage allografts have found that the optimal storage temperature is 4°C [17, 19, 22, 30–32], while Alibegovič et al. (2014) believe that the optimal temperature for in vitro storage is between 9 and 23°C (24). In the present study, the temperatures in the burial depth were significantly higher (between 2.5 and 5.3°C) than the surface temperatures (between −4 and −0.5°C). Based on our results and the studies mentioned above, we hypothesize that the higher viability in the buried samples is most likely due to the insulating properties of the soil, which acts as a barrier between the samples and the environment, maintaining a constant and higher temperature than in the unburied samples. In addition, the soil reduces the effects of other environmental factors, such as rain, which has been shown to accelerate decomposition by mechanically breaking up the flesh and keeping the soil around the cadaver moist, preventing it from drying out, promoting maggot and bacterial activity in the flesh, and further accelerating decomposition [8, 28, 33].

The results of our study confirmed a time- and temperature-dependent gradual decrease in chondrocyte viability in a natural environment, similar to the decrease observed in human chondrocyte samples under in vitro conditions. The discrepancy was mainly due to the duration of the plateau phase, which was particularly evident in the unburied samples. These results emphasize the potential utility of chondrocyte viability in cartilage as a marker for estimating PMI in both the natural environment and also in animals [17, 24]. To our knowledge, this is the first study to determine the viability of chondrocytes in porcine cartilage in a natural environment using CLSM. Rogers et al. (2011) conducted a study on buried pig trotters, in which macroscopic and histological changes in cartilage were analyzed [34]. Although those methods are less invasive, we believe that our method is less subjective and fulfills the requirements set by Madea et al. (2005) by providing a quantitative assessment with a mathematical description of the changes and a quantitative consideration of influencing factors such as temperature, humidity, precipitation, snowfall, and burial [5, 7]. Since porcine cartilage tissue is an analog to human tissue, we believe that the results of this study are applicable to both veterinary and human forensics.

With the results of our study, we were able to create a simple graph (Figure 4) that could help the user to determine PMI based on the percentage of viable chondrocytes up to day 10 after death and distinguish between the early and the first days of the late postmortem periods. Due to the small statistical difference in the percentage of viable chondrocytes between the first three days in the unburied samples and the first four days in the buried samples, the graph cannot distinguish between the first three to four days but becomes more reliable in the subsequent period.

It should also be noted that, as with all experiments in the natural environment, the results of this study are applicable to environments and temperature ranges similar to those in the present study and should therefore be used with caution when evaluating PMI. A more accurate assessment could be made if the graph is supplemented by the results of other methods such as forensic entomology and temperature nomogram. Therefore, to increase the accuracy of the graph, further studies should be conducted in the same environment and under the same conditions as in the current study, but at different times of the year. Similar studies should also be conducted in different environments and repeated over the course of several years. With the results of further studies, a scale could be created that could function like Henssge's temperature nomogram [35] by assessing PMI based on chondrocyte viability with corrections for factors such as temperature, burial, and precipitation.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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