BRIEF COMMUNICATION

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Visualization of Postmortem Chondrocyte Damage by Vital Staining and Confocal Laser Scanning 3D Microscopy

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ABSTRACT: The present study was designed to investigate whether the combination of vital dyes [calcein acetomethyl ester and ethidium homodimer (LIVE/DEAD® Viability/Cytoxicity Kit)] together with confocal laser scanning 3D microscopy was a suitable process to detect postmortem chondrocyte damage, and whether this process could be used to establish postmortem interval.

Human knee cartilage from 13 autopsies (postmortem interval from 1 day to 2.5 months) was incubated with the two dyes. The chondrocytes revealed intense staining according to their vitality. For those cases that were stored mainly at 4°C there was a vitality of approximately 88 to 96% within the first 4.5 days, which decreased to 58% after 6 days and to 9% after 1.5 months. After 2 days and 14 days at summer temperatures there were 70% and 8% vital chondrocytes respectively. Three of the 13 cases showed that altered body and storage conditions limited the efficacy of the method.

Initial data suggested a time and temperature dependent increase in cell breakdown. Under stable cooling conditions the use of vital dyes and confocal laser scanning 3D microscopy to measure chondrocyte loss may be a valuable tool for estimating the postmortem interval.

KEYWORDS: forensic science, postmortem chondrocyte damage, calcein, ethidium homodimer, confocal laser 3D microscopy

Introduction

Death can be regarded as a process with increasing cellular breakdown culminating in cytolysis. Determining the time of death using conventional staining and histochemical methods is considered unreliable due to a variety of factors, including the functional state of the cell, and disease and storage conditions (1). One report (2) has suggested that the loss of nuclear basophilia may be more reliable in estimating the time of death in stillborn fetuses. The immunohistochemical detection of hormones, for example thyroglobulin and insulin, may be a useful additional criterion (3,4). In addition, decreasing skin vitality detected by using vital dyes and fluorescence emission spectroscopy may be a promising approach for estimating the time of death (5).

The primary aim of the present study was to determine if the postmortem decrease of cell vitality could be visualized using fluoroprobes and confocal scanning 3D microscopy. Cartilage, a slow growing, so-called bradytrophic tissue, was selected as the tissue of choice. Because chondrocytes are uniquely fed by synovial fluid and are relatively resistant to oxygen starvation and acidosis, they are expected to be stable for postmortem intervals of more than a few days. Cartilage is an isolated avascular compartment away from other tissues, and therefore is protected from putrefaction. Chondrocytes are spread over cartilage in small groups and are easily identified using vital dyes.

The fluoroprobes calcein acetoxymethyl ester (Calcein/AM) and ethidium homodimer (EthD-1) are available as a double staining kit, and can be used to visualize cell vitality and cell breakdown (6). Calcein/AM is converted by intracellular esterases into the polyionic calcein. When activated at 488 nm, the dye shows a green fluorescence covering the entire cell representing intracellular esterase activity indicative of cell vitality (7,8). EthD-1 is a high affinity DNA dye that enters the cell through altered cell membranes. Staining with this substance is an indicator of cell damage and death (9). There is a 40-fold enhancement of the red fluorescence when it binds to nucleic acid.

Confocal laser scanning 3D microscopy is used as the detector. The result can be saved as a scanner file, projection image, and/or 3D animation for evidentiary purposes.

Materials and Methods

Cartilage samples from 13 autopsies were examined (Table 1); the postmortem interval ranged from 1 day to 2.5 months. In nine cases the bodies were stored at 4°C for the majority of time. Five bodies (Cases 1,5,6,7,9, and 12) were transported immediately to 4°C storage and four bodies [Cases 2,10,11 (from the spring and summer) and 13 (from the winter)] were never or only for a short time stored at 4°C.

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TABLE 1—*Cause of death and postmortem environmental factors in 13 cases.*

Case	Age/Sex (State of Decay)	Cause of Death	Place and Month of Death	Total pm Time Period from Death to Excision of Cartilage in Hours Including (Storage-Time at 4°C)
1	32/m	Multiple trauma	Highway January	29 (28)
2	81/f	Myocardial infarction	Home/mortuary June	48 (0)
3	49/m	Intoxication	Garage January	63 (50, 5)
4	38/f	Gunshot wound	Woods May	81 (72)
5	44/m	Craniocerebral trauma	Home January	102 (100)
6	60/m	Cachexia	Home January	105 (103)
7	55/m	Myocardial infarction	Home July	107 (105)
8	43/m	CO-poisoning	Fire scene June	134 (120)
9	63/m	Myocardial infarction	Home July	145 (144)
10	54/m (colored skin)	Subdural hemorrhage	Home July	168 (0)
11	50/m (putrefactions, maggots)	Metabolic crisis	Shed (wood) July	336 (72)
12	73/m (colored skin)	Pneumonia	Home January–March	1080 (1078)
13	40/m (mummification)	Massive cerebral hemorrhage	Home November–February	1776 (24)

In three cases, the conditions surrounding death and body storage were different. In one (Case 3) the deceased was found dead on the garage floor approximately 12.5 h after he was heard returning by car. The temperature during the night was close to freezing. After being found, the body was stored at 4°C for 2 days. In the second case (Case 4) the deceased was found about 9 h after last being seen alive and it was extremely warm. The body was stored at 4°C for 3 days after it was found. The final case (Case 8) was that of a fire victim whose body was found close to the origin of the fire about 14 h after the fire was reported. In this case the body was stored at 4°C for 5 days.

Only intact knee joints and macroscopically undamaged cartilage were used. After washing with phosphate buffered saline (PBS) horizontal slices ($0.5 \times 0.5 \times 0.5$ cm) were excised from the middle layers of cartilage on the lateral side of the femur condyle, a location expected to be one of minor pressure (10,11).

After washing, the preparations were incubated for approximately 40 min at room temperature with calcein/AM (1:5000) and EthD-1 (1:2000). Depth dependent serial sections of the unfixed cartilage were taken by a confocal laser 3D scanning microscope (Leica TCS SP, Argon-Krypton Laser). Both dyes were excited at 486 nm and the fluorescence images produced by calcein/AM recorded between 501 and 532 nm, and those for EthD-1 between 618 and 671 nm. Both projection images and 3D animation were produced and for each sample approximately 1000 cells were counted.

Results

After incubation the cytoplasm and nucleus of the chondrocytes showed intense staining according to the functional state of the cell (Fig. 1). Cells with green fluorescent cell bodies were noted as vital, while those without evidence of a cell body and red nuclear fluorescence were noted as dead. Those cells with green cell bodies and red nuclear fluorescence were noted as damaged.

As expected, the middle layers of cartilage decreased in vitality with increasing postmortem interval (Fig. 2). For those cases that were immediately stored at 4°C the chondrocytes had a vitality of 88 to 96% between 29 and 107 h. After 145 h, 58% of the chondrocytes were considered vital, and after 1.5 months approximately 9% were considered vital. Those cases that were not stored at 4°C had a vitality of 70% after two days (Case 2), and 20% after seven days (Case 10) at summer temperatures. One case (Case 11) had a vitality of 8% after eleven days at summer temperature and three days at 4°C.

For those three cases where the body and storage conditions were different, there was a vitality of almost 90% after 2.5 days including 12.5 h in temperatures close to freezing (Case 3), of 80% after 3.4 days including 9 h in warm conditions, and of 41% after 5.6 days including 14 h at a fire scene.

After an interval of months (Cases 12 and 13) there was a less structured fluorescence, with a high background, within the lacunae. However, structures resembling cells were identifiable.

Discussion

Postmortem chondrocyte damage could be visualized using a combination of staining with calcein/AM and ethidium homodimer and confocal laser scanning 3D microscopy (Figs. 1 and 2). Loss of chondrocyte vitality appears to be both temperature and time dependent. As with other temperature dependent methods (12) there are limitations to this method. These are apparent for Cases 3,4, and 8.

When the postmortem interval is several months, the interpretation of fluoroprobe staining is more difficult because of autofluo-

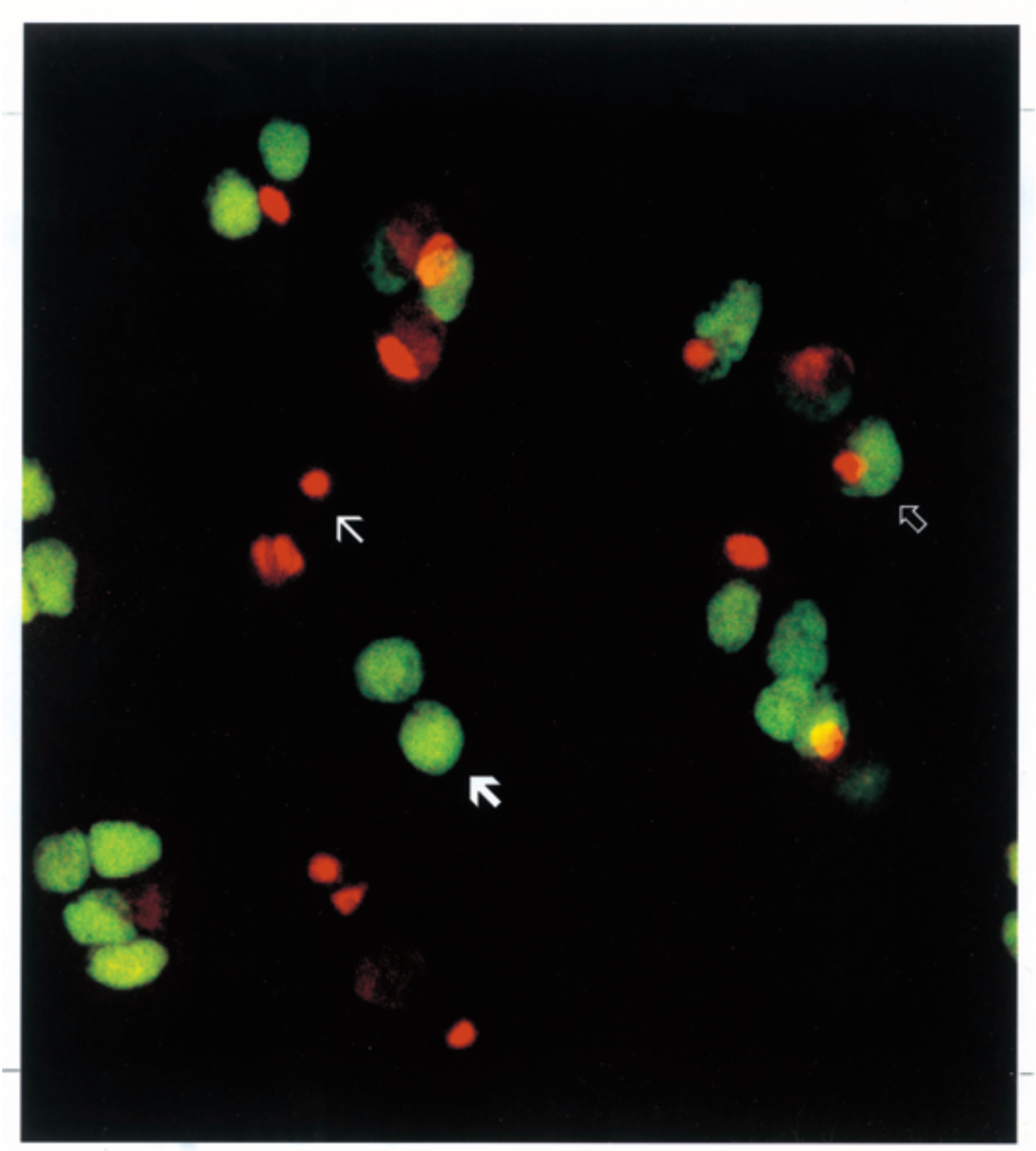

FIG. 1—*Confocal laser-scanning 3 D microscopy. Human cartilage (Case 8, pm interval about 5,5 days); three-dimensional reconstruction (projection image), step-size 0.4 μm. 20 × objective, zoom factor 4.*

➔ *green fluorescence of the chondrocyte cell body = vital cell.*
⇨ *chondrocyte cell body with green fluorescence, red fluorescent nucleus = damaged cell.*
→ *isolated red nucleus, loss of green fluorescence within the cell body = dead cell.*

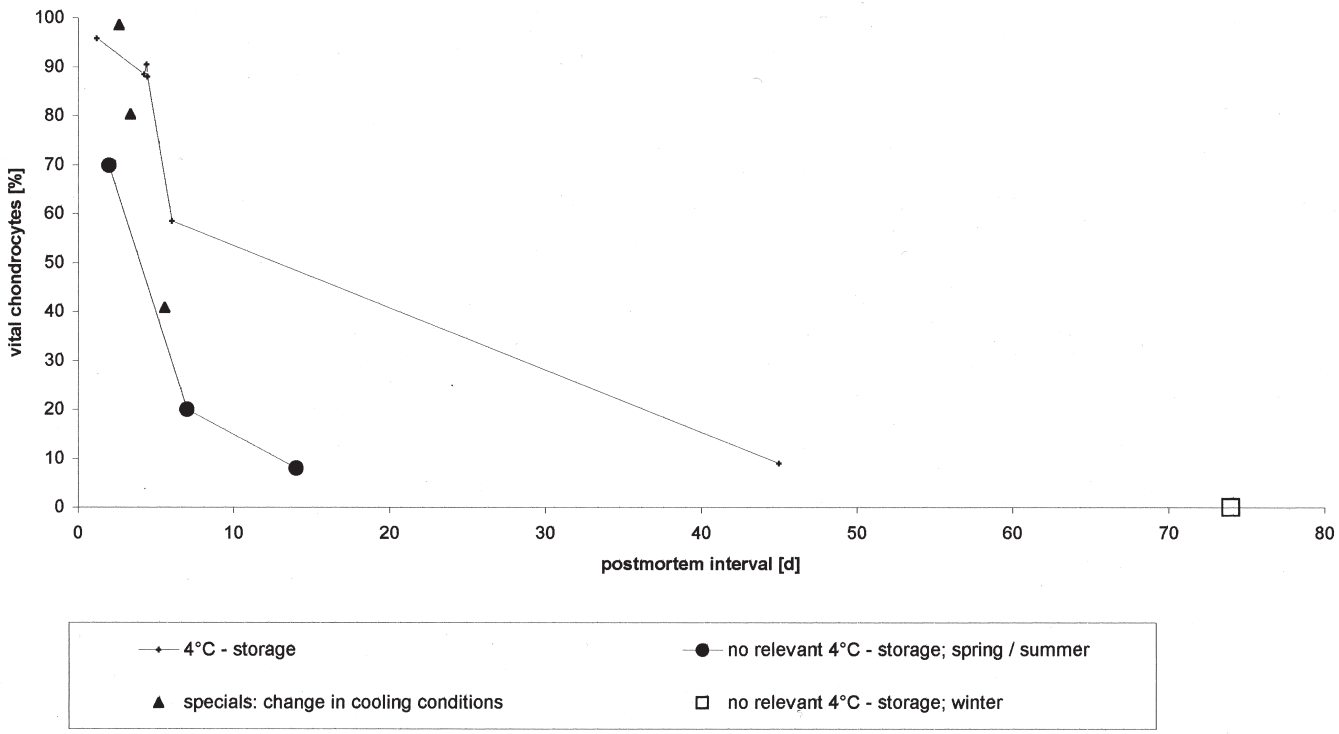

FIG. 2—*Cellular breakdown and increasing postmortem interval.*

rescence due to putrefactive changes in tissues. However, fluoroprobe staining appears to be promising for cases where the postmortem interval is days to weeks and when environmental and cooling conditions are stable. Presently, fluoroprobe staining cannot be used at the death scene to estimate the time of death, like the temperature-based nomogram (12,13).

The small number of cases that were examined limits the present study. Further studies are ongoing to examine the statistical relationship between the time of death and cell damage in human and porcine cartilage. These studies are also considering the importance of disruptive factors such as apoptosis, autofluorescence, trauma, and temperature at the scene and during storage.

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