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TECHNICAL NOTE

PATHOLOGY/BIOLOGY

Armin Alibegović,[1] M.D., Ph.D.; Jože Balažic,[1] M.D., Ph.D.; Danijel Petrovič,[2] M.D., Ph.D.; Nevenka Kregar Velikonja,[3] Ph.D.; Rok Blagus,[4] Ph.D; Dušan Šuput,[5] M.D., Ph.D.; and Matej Drobnič,[6] M.D., Ph.D.

The Optimal Combination of Cartilage Source and Apparatus for Long-Term *In Vitro* Chondrocyte Viability Analysis

ABSTRACT: Most studies of long-term chondrocytes survival were for tissue banks. They showed a gradual reduction in the viable chondrocytes percentage as a function of time and ambient temperature, but the samples were harvested under optimal conditions. The aim of our study was to determine the most reliable combination of cartilage source and assay for the *in vitro* postmortem chondrocyte viability analysis in the conditions that imitate a dead body. Osteochondral cylinders were procured from femoral condyles and talar trochleas of three male donors and stored in the cell culture media at 4 ± 2°C and 23 ± 2°C. The samples were analyzed by a cell viability analyzer and a confocal laser scanning microscope (CLSM) initially 24–36 h after death and then in 4-week intervals. The results reconfirmed the significant influence of time ($p = 0.0002$), but not of the temperature ($p = 0.237$). The largest reproducibility was presented for the knee joint and the CLSM.

KEYWORDS: forensic science, forensic pathology, time since death, articular cartilage, chondrocyte viability, temperature, confocal laser scanning microscope, cell viability analyzer

The chondrocytes in human joints retain their viability for a prolonged period after the death of an individual depending on the ambient conditions (1,2). Lasczkowski et al. (3) revealed that nearly 60% of chondrocytes in the human knee survived 6 days under appropriate conditions, and nearly 10% were still viable one and a half months after death. Studies on human osteochondral allografts revealed that around 70% of chondrocytes survived for 1 month and around 35% chondrocytes survived for 2 months if stored at 4°C in tissue banks under optimal conditions (4–6). These characteristics, the constant decrease in chondrocyte viability under the influence of time and ambient conditions, make cartilage an attractive tissue for the determination of time since death for forensic needs; however, systematic studies on this topic are not available. Because human corpses are exposed to a wide variety of ambient conditions, the *ex vivo* studies under a controlled laboratory environment need to be undertaken initially. The protocols for the analysis of chondrocytes' viability in *in vitro* conditions, which could help the study of postmortem interval (PMI), must be developed to the standardized and optimized conditions that try to imitate the conditions in a dead body as close as possible.

In our previous *in vitro* pilot study on human chondrocyte viability for its possible use in forensics, we tested three sources of cartilage (knee, ankle, and ear) and three chondrocyte viability analysis assays (manual counting under a microscope, cell viability analyzer (CVA), and a flow cytometer). Most of the auricular cartilage samples were infected, and therefore, their usage in forensics was abandoned because of the close contact to the commensal bacteria reservoir that spreads rapidly over the tissues. We assumed that a similar problem might also occur with other cartilaginous tissues of the head, neck, and torso. The CVA, among the above-mentioned assays, provided the most reliable measurements and the best cost–time performance, and it had the highest potential for further studies. All the viability analyses in the above-mentioned study, were conducted on cells isolated from the cartilage. Such processing for the physical separation of the cells from the tissue requires mechanical and enzymatic processing of the tissue, which may additionally damage some cells and consequently change their viability score.

The aim of further research was to compare the results from a CVA to another method that enables viability analysis on the cells *in situ*. The viability cartilage staining with fluorescent Live/Dead probes followed by the sample scanning under a confocal laser microscope has been used lately in this regard (3–5,7). The aim of the presented *in vitro* study was to follow the chondrocyte viability of human cadaveric knee and ankle samples under two temperature conditions over a period of 1 month after the donor's death comparing the CVA and the confocal laser scanning microscope (CLSM).

[1]Institute of Forensic Medicine, Medical Faculty, University of Ljubljana Korytkova 2, 1000 Ljubljana, Slovenia.
[2]Institute of Histology and Embryology, Medical Faculty, University of Ljubljana, Korytkova 2, 1000 Ljubljana, Slovenia.
[3]Educell d.o.o., Letališka 33, 1000 Ljubljana, Slovenia.
[4]Institute of Biostatistics and Medical Informatics, Medical Faculty, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia.
[5]Institute of Pathophysiology, Medical Faculty, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia.
[6]Department of Orthopedic Surgery, University Medical Centre Ljubljana, Zaloška 9, 1000 Ljubljana, Slovenia.

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Materials and Methods

Sample Procurement and Storage

The protocol followed the requirements of the National Medical Ethics Committee of the Republic of Slovenia (No. 25/09/08). Human cartilage specimens were obtained during the autopsies from three male donors (21, 45, and 50 years old). They had all died a sudden traumatic death (accidental drowning, head injuries in traffic accident, and suicide by hanging) and they had no medical history of any knee or ankle joint pathology, neither was there any record of a systemic illness that could result in cartilage deterioration. All three corpses were kept at 4 ± 2°C from the second postmortem hour until the autopsy. The cartilage samples were procured bilaterally from the knee and ankle joints within 24 h after death in aseptic conditions at room temperature (RT) (23 ± 2°C). The articular surfaces, which were exposed by a wide transversal arthrotomy, were macroscopically intact—ICRS grade 0 (8). Osteochondral cylinders were removed with the mosaico-plasty coring instruments without drilling (Helipro, Lesce, Slovenia) from both femoral condyles (Ø 6 mm, depth 20 mm) and from the talar trochlea (Ø 5 mm, depth 15 mm). Every osteochondral cylinder was immediately stored in a tightly closed 2 mL tube (Safe-Lock tubes; Eppendorf, Hamburg, Germany) filled to the top with Dulbecco's Modified Eagle Medium with L-Glutamine and 15 mM HEPES (DMEM/F12) supplemented with 50 μg/mL of gentamicin and 2 μg/mL of amphotericin B (all produced by Gibco, Paisley, UK). The cell preservation media was not changed throughout the experiment. The procured samples were, separately from the knees and ankles, randomly divided into storage at 4 ± 2°C or at RT (23 ± 2°C). The initial viability analyses were made 24–36 h after the procurement. The subsequent analyses were followed in weekly intervals for the next 28 days. Two chondrocyte viability assays were used: the CVA and the CLSM.

Cell Viability Analyzer

Specimens for the CVA (Vi-Cell XR; Beckman Coulter, Fullerton, CA) were processed under aseptic conditions. The chondral part was first split off the osteochondral cylinder using a scalpel blade. Every sample was washed with DMEM/F12 solution, diced to 1–2 mm^3 pieces, and enzymatically digested in 10 mL of collagenase II solution (1 mg of collagenase II in 1 mL of DMEM/F12, supplemented with 50 μg/mL of gentamicin; all produced by Gibco) by stirring for 18–21 h at 37°C. The degraded cartilage samples were afterward washed through a cell strainer with 40 μm pores and resuspended in a DMEM/F12 solution for the viability analysis. The chondrocyte suspension for the CVA was treated with trypan blue vital dye included in the kit for automatic dyeing (Vi-CELL Reagent Quad Pak; Beckman Coulter). Viable cells resist dye passage through the membrane; therefore, only dead cells are marked intensively blue. The CVA automates the trypan blue vital dye exclusion method for the determination of cellular viability. The size limits for cell capturing were set between 8 and 20 μm (9). The cell suspension obtained after tissue digestion of each examined tissue sample was analyzed with six parallel aliquots of 1 mL. Dead and living cells were counted and written automatically. The calculated costs and time consumption for the viability analysis with the CVA are given in Table 1.

Confocal Laser Scanning Microscopy

The osteochondral cylinders for the CLSM were removed from the storage media (DMEM/F12 with additives) on the day of the analysis. Six slices, each 300 μm thick, were cut out in the vertical direction perpendicularly through the whole cartilage thickness, starting at the synovial surface and ending just above the subchondral bone junction (Fig. 1). The slices were sectioned in the central part of each sample, to minimize the artifact from sample manipulation, with the vibratory microtome (Oxford Vibratome model G; Oxford Laboratories, San Mateo, CA). They were immediately stained with Live/Dead Viability/Cytotoxicity kit (Molecular Probes Inc., Eugene, OR). The kit includes two fluorogenic reagents: calcein-AM (Ca-AM) and ethidium homodimer-1 (EthD-1). Ca-AM is a membrane permeable nonfluorescent esterase substrate that passively

TABLE 1—*The average cost in € and $, and time consumption of the two viability assays used in the study: cell viability analyzer (CVA) and confocal laser scanning microscope (CLSM).*

	CVA	CLSM
Isolation		
Material costs		
(€)	9.6	0.7
($)	13.5	1
Time for a single sample (min)	50	16
Time for 15 samples (h)	10	4
Analysis		
Material costs		
(€)	5.6	0.1
($)	7.9	0.14
Apparatus rental price		
(€/h)	12	77.4
($/h)	16.9	109.1
Time for a single sample (min)	10	28
Time for 15 samples (h)	1	7

€, euro, the official currency of the European Union; $, United States dollar, the official currency of the United States of America.

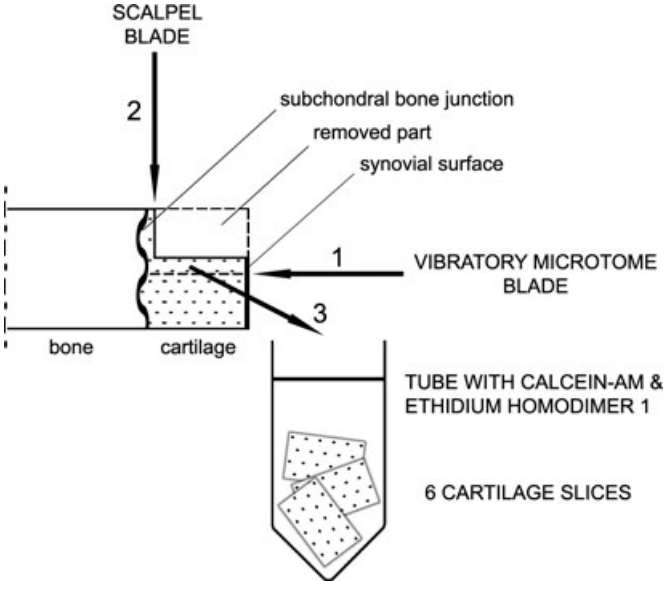

FIG. 1—*Schematic presentation of the samples removal from the cartilage. The bone portion of the osteochondral cylinder was clamped firmly perpendicularly in the direction of the vibratory microtome blade (1). The upper third of the cartilage sample was removed at the beginning so the slices for analysis were sectioned in the central part of the sample. Six slices were cut out in the vertical direction through the whole cartilage thickness, starting at the synovial surface. After cutting with the vibratory microtome blade (1) each slice was separated just above the subchondral bone junction with the scalpel blade (2) and placed in the tube with the prepared fluorogenic reagents of the Live/Dead Viability/Cytotoxicity kit.*

diffuses into cytoplasm. After intracellular enzymatic hydrolysis of the acetoxymethyl ester portion, the remaining calcein is impermeable and, therefore, trapped by intact cell membranes. Calcein emits a green fluorescence at 517 nm (from 505 to 535 nm) when excited by blue light at 494 nm indicating that the cell has an intact membrane and esterase activity and is, therefore, considered viable. EthD-1 is impermeable to intact cell membranes but is able to diffuse through the porous membranes of dying or dead cells. This dye has a high affinity to nucleic acids and emits a bright red light at 617 nm (from 605 to 635 nm) when excited at 528 nm (4). The cartilage slices were incubated for 90 min at 37°C with a solution of 1 µmol EthD-1 and 250 µmol Ca-AM diluted in saline (sodium chloride 0.9%; B. Braun, Melsungen, Germany). Both diluted dyes, 500 µL of each, and six cartilage slices were put together in a 1.5 mL tube (Safe-Lock tubes). The contents of the tubes were protected from daylight during incubation and microscopic analysis. With the described staining protocol, we were able to detect chondrocyte viability in every analyzed specimen. Three of six stained slices were randomly selected for the analysis with an apochromatic objective lens (HCX PL APO 40 × 1.25 OIL) on a DM IRBE inverted microscope equipped with a 100 W Hg-lamp (Leica, Wetzlar, Germany). The confocal micrographs were scanned with a TCS SP2 CLSM equipped with a 488 nm argon/krypton laser line (Leica). The scans were taken at a 512 × 512 pixel resolution with the pinhole set at 1 Airy unit. Arbitrarily chosen locations on each slice were *c*. 40 µm deep into the specimen and 400 µm away from the slice margins to avoid any artifacts. The chosen location was captured by seven images placed one above the other with the 10 µm in-between interval, giving an optical slice volume of 0.009 mm^3 (field size of 387.5 × 387.5 µm and depth size of 60.1 µm) (Fig. 2). Confocal micrographs with green and red colored spots, live and dead cells, respectively, were arranged by using LAS AF software (Leica). The cells were counted manually. The calculated costs and time consumption for the CLSM analysis are given in Table 1.

Data Presentation and Statistical Analysis

All the results of chondrocyte viability are expressed as percentages of viable cells. For each time–temperature measurement point, average values of all donors were calculated. The intra-class correlation coefficient (ICC), within-subject coefficient of variation (WSCV), and the coefficient of variation (CV) were calculated to determine the most appropriate combination of cartilage source and chondrocyte viability assay. Shoukri et al. (10) demonstrated that in the case when two instruments are used to measure the same set of subjects, and each subject is repeatedly measured by the same instrument, the WSCV index is more appropriate than the ICC or CV. High values of ICC and CV, but low values of WSCV, mean better reproducibility. After this, we evaluated the cartilage cell viability dependence on time and temperature with the two-way repeated measures analysis of variance (ANOVA). The difference to the first measurement was analyzed with contrast analysis. The *p*-values for the contrasts were adjusted with the Benjamini and Hochberg (11) method to control the false discovery rate. A *p*-value of <5% was considered statistically significant in all analyses. All analyses were performed with R language for statistical computing (version R 2.8.1) (12).

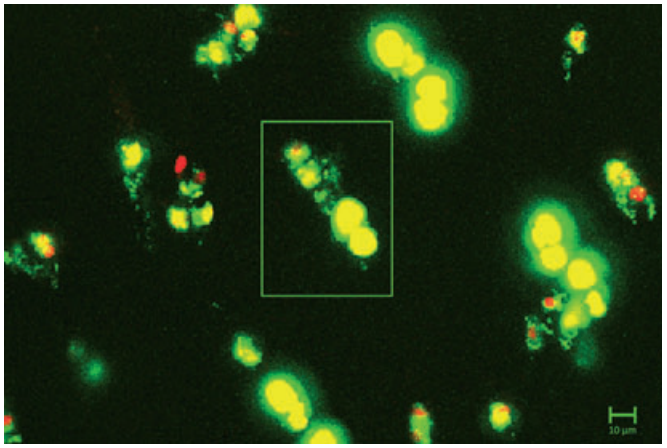

FIG. 2—*Micrograph of the knee cartilage sample after 4 weeks of storage at 4 ± 2°C, stained with a Live/Dead Viability/Cytotoxicity kit (calcein-AM and ethidium homodimer-1) and captured by the confocal laser scanning microscope. In the center of the figure (inside the rectangle) is a group of green and red spots, live and dead cells, respectively, and fragmented green spots with a size and shape that were counted as dead cells. The criterion in the right hand lower corner gives the distance of 10 µm.*

Results

Viable chondrocytes were detected in all samples. One set of ankle samples from the third donor stored at 4 ± 2°C was lost during the preparation for the CLSM analysis because of technical problems caused by the broken mosaico-plasty coring instruments. The results of chondrocyte viability in the samples for each time–temperature measurement point in both assays from each donor and as an average value with a standard deviation (SD) of the results from three donors are collected in Tables 2 and 3.

The results of the calculated indexes (ICC, WSCV, and CV) for each time–temperature measurement point (two joints at two temperature levels and five time measurement points) in both assays (CVA and CLSM) are given in Table 4. The values of the WSCV for the ankle (CVA: mean 0.11, SD 0.05; CLSM: mean 0.19, SD 0.21) are higher than the WSCV for the knee (CVA: mean 0.07, SD 0.04; CLSM: mean 0.11, SD 0.12), suggesting worse reliability in the ankle. Separate comparison of the CLSM and the CVA for the knee revealed that the WSCV for the CVA varies more across time points than for the CLSM.

Figure 3 shows the chondrocyte viability in the knee joints over 4 weeks at the two temperature levels. The charts review points toward a higher viability at RT than at 4 ± 2°C independently of the used assay. The time impact on the results was more expressed in chondrocyte viability measured by the CLSM than the CVA. However, the repeated measurements of knee chondrocyte viability, measured by the CLSM, in evaluating using the ANOVA method demonstrated the significant influence of time ($p = 0.0002$), but not for temperature ($p = 0.237$) (Table 5). The number of viable cells in the knee cartilage after 4 weeks, measured by the CLSM, was significantly lower than the number of viable cells at the first time point. The viability in other measuring time points was not significantly different than in the first measurement (Table 6).

Discussion

The presented *in vitro* study evaluated chondrocyte viability in the human cadaveric knee and ankle cartilage samples under two temperature conditions over the period of 1 month after donor's death measured by the CVA and the CLSM. The cartilage from the knee joint demonstrated less variability in comparison with the ankle. Analysis of the two viability assays gave a slight advantage to the CLSM conducted on the cells *in situ* and, therefore, should be set as a reference method for further basic studies. However, the

TABLE 2—*Chondrocyte viability in percentages given as average values of multiple measurements with standard deviation (in brackets) from each donor at different postmortem time intervals and temperatures. Some samples from the ankle of the third donor were lost because of the technical problems.*

		Cell Viability Analyzer					Confocal Laser Scanning Microscope				
Donor	Day	1	8	15	22	29	1	8	15	22	29
1	Knee										
	4°C	94.47 (3.41)	79.54 (3.06)	66.72 (4.48)	79.89 (5.57)	51.60 (15.41)	90.88 (1.58)	79.23 (23.03)	94.44 (9.62)	63.89 (17.67)	20.49 (15.14)
	RT	77.58 (3.21)	51.27 (13.57)	78.60 (5.91)	58.73 (6.22)	72.20 (3.21)	95.51 (3.94)	97.77 (3.84)	99.01 (1.69)	90.57 (8.33)	4.45 (2.02)
	Ankle										
	4°C	97.22 (6.80)	90.71 (11.60)	67.06 (5.35)	76.04 (13.40)	58.20 (23.64)	90.79 (5.19)	90.79 (5.19)	61.11 (28.34)	28.83 (11.15)	0 (0)
	RT	83.85 (10.84)	83.35 (7.92)	73.11 (7.36)	77.14 (18.39)	77.29 (9.34)	98.85 (1.99)	96.08 (3.94)	98.44 (2.68)	83.46 (11.67)	18.26 (4.52)
2	Knee										
	4°C	94.71 (2.09)	91.49 (4.34)	95.03 (0.58)	67.59 (8.78)	83.65 (2.02)	90.82 (8.46)	94.79 (9.02)	97.72 (3.93)	73.95 (6.34)	11.80 (11.29)
	RT	95.00 (3.25)	82.75 (2.97)	93.61 (2.85)	93.84 (2.43)	71.98 (3.55)	74.73 (7.09)	90.27 (14.43)	96.24 (3.28)	94.06 (2.21)	75.78 (3.27)
	Ankle										
	4°C	95.62 (9.44)	93.46 (4.46)	92.38 (2.65)	65.53 (10.37)	75.76 (11.70)	67.38 (13.21)	94.33 (6.02)	48.01 (28.02)	72.59 (5.59)	15.35 (8.35)
	RT	93.41 (7.65)	93.53 (6.49)	90.76 (8.27)	86.51 (8.27)	71.73 (9.15)	98.66 (2.30)	91.80 (7.13)	97.97 (1.75)	85.97 (4.46)	37.37 (23.29)
3	Knee										
	4°C	93.20 (5.39)	73.65 (5.27)	88.66 (4.80)	70.56 (4.98)	60.97 (12.75)	84.09 (15.79)	100.00 (0)	96.66 (5.77)	100.00 (0)	48.19 (17.75)
	RT	83.19 (6.56)	77.91 (6.53)	84.33 (1.96)	94.01 (4.32)	78.80 (8.26)	100.00 (0)	100.00 (0)	97.46 (2.22)	85.00 (6.00)	85.65 (1.60)
	Ankle										
	4°C	82.30 (12.39)	82.28 (6.59)	87.50 (2.66)	74.74 (6.44)	70.71 (15.16)	Lost	Lost	Lost	Lost	Lost
	RT	81.59 (11.14)	89.69 (12.37)	84.00 (7.01)	89.31 (8.88)	74.80 (4.94)	95.43 (4.77)	97.84 (3.72)	100.00 (0)	93.93 (10.49)	89.52 (10.72)

RT, room temperature (23 ± 2°C).

TABLE 3—*Chondrocyte viability in percentages given as average values with standard deviation (in brackets) from three donors at different postmortem time intervals and temperatures.*

	Cell Viability Analyzer					Confocal Laser Scanning Microscope				
Day	1	8	15	22	29	1	8	15	22	29
Knee										
4°C	94.13 (0.81)	81.56 (9.08)	83.47 (14.85)	72.68 (6.42)	65.41 (16.47)	88.60 (3.89)	91.34 (10.80)	96.27 (1.67)	79.28 (18.63)	26.83 (19.00)
RT	85.26 (8.89)	70.65 (16.95)	85.51 (7.57)	82.19 (20.32)	74.33 (3.87)	90.08 (13.47)	96.01 (5.09)	97.57 (1.38)	89.87 (4.57)	55.29 (44.30)
Ankle										
4°C	91.71 (8.19)	88.82 (5.82)	82.31 (13.42)	72.10 (5.72)	68.22 (9.04)	79.09* (16.55)	92.56* (2.49)	54.56* (9.25)	50.71* (30.94)	7.67* (10.85)
RT	86.28 (6.27)	88.86 (5.14)	82.62 (8.90)	84.32 (6.37)	74.60 (2.78)	97.65 (1.92)	95.24 (3.10)	98.80 (1.05)	87.79 (5.47)	48.38 (36.88)

RT, room temperature (23 ± 2°C).
*Average values for samples are based on two donors only because of the technical problems.

TABLE 4—*The average results of the intraclass correlation coefficient (ICC), the within-subject coefficient of variation (WSCV), and the coefficient of variation (CV) at each time–temperature measurement point (high values of ICC or CV, and low values of WSCV mean better reproducibility).*

		Cell Viability Analyzer					Confocal Laser Scanning Microscope				
	Day	1	8	15	22	29	1	8	15	22	29
ICC	Knee										
	4°C	0.19	0.97	0.99	0.84	0.90	0.37	0.61	0.18	0.91	0.99
	RT	0.90	0.98	0.95	0.95	0.79	0.97	0.48	0.72	0.68	1.00
	Ankle										
	4°C	0.84	0.73	0.99	0.57	0.62	0.90	0.24	0.38	0.97	0.91
	RT	0.67	0.60	0.87	0.57	0.43	0.79	0.48	0.55	0.47	0.94
WSCV	Knee										
	4°C	0.04	0.05	0.04	0.09	0.21	0.10	0.16	0.06	0.13	0.14
	RT	0.04	0.13	0.05	0.05	0.07	0.04	0.10	0.02	0.06	0.03
	Ankle										
	4°C	0.09	0.10	0.04	0.17	0.24	0.12	0.08	0.37	0.17	0.77
	RT	0.12	0.12	0.10	0.16	0.11	0.02	0.06	0.02	0.11	0.34
CV	Knee										
	4°C	0.05	0.28	0.44	0.24	0.65	0.12	0.26	0.07	0.43	0.23
	RT	0.26	0.60	0.20	0.61	0.14	0.26	0.13	0.03	0.11	1.39
	Ankle										
	4°C	0.24	0.19	0.40	0.26	0.41	0.38	0.10	0.48	1.07	2.57
	RT	0.22	0.18	0.28	0.25	0.14	0.04	0.08	0.02	0.16	1.36

RT, room temperature (23 ± 2°C).

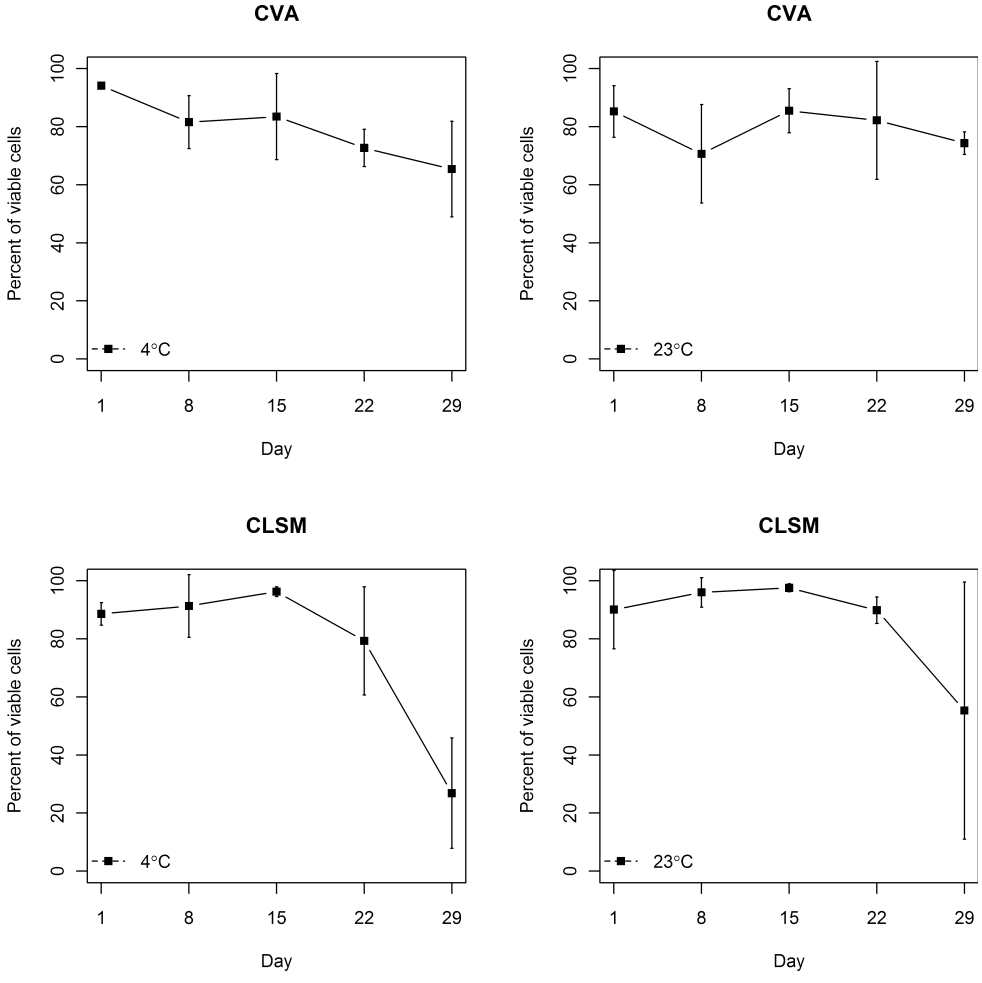

FIG. 3—*Graphs of chondrocyte viability in knee cartilage measured by the cell viability analyzer (CVA) and the confocal laser scanning microscope (CLSM): the first measurement 24–36 h after death, other measurements in weekly intervals at 4 ± 2°C and room temperature (23 ± 2°C) as average values from three donors in percentages with standard deviations (±SD).*

sophisticated equipment requirements for the CLSM, together with a worse time–cost performance, support the CVA on cells isolated from the knee joint to be used in routine work.

The long-term survival of chondrocytes has been well documented in studies on osteochondral allografts, but to the authors' knowledge, only one study tested the chondrocyte viability in

TABLE 5—*ANOVA table demonstrates the influence of time and temperature on knee chondrocyte viability measured by a confocal laser scanning microscope.*

	numDF	denDF	*F*-value	*p*-value
(intercept)	1	16.0000	586.2942	0.0000
Temperature	1	4.0000	1.9284	0.2373
Time	4	16.0000	10.6205	0.0002
Temperature–time	4	16.0000	0.6546	0.6321

TABLE 6—*Contrasts of cells viability in knee cartilage measured by a confocal laser scanning microscope in measuring time points.*

	Estimate	SE	*t*-value	Pr (>|*t*|)
Day 8 vs. Day 1	4.3381	9.9232	0.4372	0.6678
Day 15 vs. Day 1	7.5864	9.9232	0.7645	0.6678
Day 22 vs. Day 1	−4.7623	9.9232	−0.4799	0.6678
Day 29 vs. Day 1	−48.2767	9.9232	−4.8650	0.0007

human corpses (3). Time since death is routinely determined by early and late postmortem changes such as body cooling, lividity, *rigor mortis*, muscle excitability, chemical changing in fluid compartments, and decomposition of the corpse. These parameters are useful in the early postmortem period (13,14), especially when the temperature-based nomogram method is integrated with other non-temperature-based methods, like in the compound method (15–17). The reliability and accuracy of parameters and methods are progressively decreased after a longer time after death (18–21). Owing to the long survival time, the ratio between viable and nonviable chondrocytes may further improve the estimation of time since death, especially in the late postmortem period.

The main goal of our study was to determine the best combination of articular cartilage source and cell viability assay. The results indicated that combination of the CLSM with knee cartilage gave the most reliable performance. Moreover, the knee joint is also easier to access for sample procurement, and it has a larger and thicker articular surface to be harvested for analysis. Its only disadvantage over the ankle is a higher incidence of cartilage degeneration (22). The degenerated cartilage may express a different viability decline than the healthy one, but this will have to be addressed elsewhere. During harvesting of the ankle cartilage, the coring instruments were often destroyed because of a harder talar subchondral bone. Osteochondral cylinders were stored in a tightly closed tubes filled to the top with the cell preservation media, which was not changed throughout the experiment. These conditions (a limited amount of media and nutrients that were diffused to the cells, the media was not exchanged, oxygen was limited and was not added, and the waste materials were not removed) tried to approximate the conditions in dead bodies. Although the CLSM in combination with Live/Dead staining provided the most reliable measurements, the technical difficulties (laser microscope scanning, manual cell counting) and the costs do not support the CLSM usage in routine forensic work. The CVA with automatic cell counting is more user-friendly and reproducible, and it still offers a high level of reliability.

Different manual and automatic software-based protocols for the image analysis of Live/Dead chondrocytes were used in previous studies (1,3,4). Automatic protocols provide faster and observer-independent analysis, but they induce a systematic error because of thresholding. In our study, confocal images with a high magnification were included, and with a consequently low object number, manual cell counting was employed. During manual counting, we also localized groups of green fragments without a red signal (Fig. 2). We concluded that these fragments represent membrane particles of a decaying cell, and we counted them as dead cells. Such fragments are of different sizes and could easily be mistaken for viable cells when using automatic counting (false-positive results).

Our study also reconfirmed previously documented chondrocyte viability decline over time, but it failed to demonstrate the influence of temperature (1,3,4). The main reason for the former is most likely hidden in the insufficient sample size.

Conclusions

The presented *in vitro* study, which simulated the conditions in a dead body, demonstrated that changing the chondrocytes' viability for forensic needs (determination of the PMI) would be most useful on samples from knee cartilage. The CLSM provided slightly superior reliability over the CVA, but because of the technical and cost–time issues, this method should be reserved for basic studies and the CVA could be used in routine work.

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Additional information and reprint requests:
Armin Alibegović, M.D.
Institute of Forensic Medicine
Medical Faculty, University of Ljubljana
Korytkova 2
1000 Ljubljana
Slovenia
E-mail: armin.alibegovic@mf.uni-lj.si