Past, present and future methods for assessment of chondrocytes’ viability: A new parameter for determination of the postmortem interval?

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**Refereed papers**


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**Paper presentations**


2007-09-29 The acute injury of the surgical sutures on the human articular cartilage. 7th World congress of the international cartilage repair society ICRS. Warsaw, Poland.

2007-10-02 Human auricular cartilage - an alternative source of chondrocytes for autologous chondrocyte implantation (ACI). 7th World congress of the international cartilage repair society ICRS. Warsaw, Poland.
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- studies longer than expected
- chondrocytes lived several weeks
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- more resistant - several hours to approximately one day
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- determination of the time of individual's death
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determination of the postmortem interval (PMI)
one of the most important questions in the forensic medicine
Past, present and future methods for assessment of chondrocytes’ viability: A new parameter for determination of the postmortem interval?

**Refereed papers**


Alibegović A. Cartilage: a new parameter for the determination of the postmortem interval?. J Forensic Leg Med. (in press). doi:http://dx.doi.org/10.1016/j.jflm.2014.08.005

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2010-05-13 Instrumental analysis of cartilage in forensic medicine. 19th International meeting on forensic medicine Alpe – Adria – Panonia. Tavagnacco-Udine, Italy. (oral communication)

2010-09-27 The optimal temperature for the long term ex/vivo cartilage storage: 11°C is superior to 4°C, 23°C, and 35°C. 9th World congress of the international cartilage repair society. Barcelona, Spain. (oral communication)

2011-06-24 Comparison of cartilage sources and methods for the ex-vivo determination of chondrocyte viability. 4th Symposium of the Osteuropa-Verein. Targu Mures, Romania. (oral communication)

2011-06-01 Estimation of the post-mortem time interval according to the articular chondrocyte viability curves at different temperature levels in vitro. 20th International Meeting on Forensic Medicine Alpe-Adria-Pannonia. 2011 Jun 1-4. Bratislava, Slovakia. (poster)

2011-07-05 Cartilage as the tissue for determination of postmortem interval. International Symposium of Sports Medicine. Las Palmas, Spain. (oral communication)

2013-06-07 CVA or CLSM for estimation of PMI?. 22nd International Meeting on Forensic Medicine Alpe-Adria-Pannonia. Kraków, Poland. (oral communication)
Past, present and future methods for assessment of chondrocytes' viability: A new parameter for determination of the postmortem interval?

- doubts and conclusions
- cartilage as a compartment
- determination of PMI
- particular attributes of cartilage - support this idea
Past, present and future methods for assessment of chondrocytes' viability: A new parameter for determination of the postmortem interval?

Accepted Manuscript

Cartilage: A new parameter for the determination of the postmortem interval?
Armin Alibegović, MD, PhD, Specialist in Forensic and Legal Medicine

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### Substrate Attributes

<table>
<thead>
<tr>
<th>Substrate</th>
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</tr>
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<tbody>
<tr>
<td>Articular cartilage</td>
<td>distance from the torso – the source of the saprophytic microorganisms tissue without vessels and nerves</td>
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</tr>
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<td>pores with 6 nm average diameter obstruct the spread of microorganisms highly hydrophilic</td>
</tr>
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<td>60 to 78% of the net weight of the hyaline cartilage storage for gases, nutrients, proteins, lipids, electrolytes diffusion of solutes (gases, nutrients, metabolic waste, ...)</td>
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anatomical, morphological, mechanical, physical and chemical properties of cartilage, in particular of the articular cartilage, are those with which the cartilage could be determined as a compartment
Past, present and future methods for assessment of chondrocytes’ viability: A new parameter for determination of the postmortem interval?

Could cartilage be used for the determination of PMI?
Could cartilage be used for the determination of PMI?

- small number of chondrocytes - modest requirements for nutrients and oxygen
- abundant ECM with different solutes - diffuse to the chondrocytes after clinical death
- allow the survival of the chondrocytes several days after the individual’s death
- ECM provide an extra protection to the chondrocytes against microorganisms - spread postmortem
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- researches on the chondrocytes long-term survival
- mainly chondrocytes’ cultivation or conservation
- for clinical use - especially for articular cartilage transplantation

- observation of the difference in the proportion of chondrocytes
- survived during the time and at different temperatures

- human allografts kept in the tissue banks at 4 °C and under optimal conditions
- ~ 70 % of the chondrocytes survived for one month
- ~ 35 % of the chondrocytes survived for two months
Could cartilage be used for the determination of PMI?

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**gradual decline in the proportion of chondrocytes that survive clearly demonstrated that chondrocytes viability was a function of time and temperature, which could be useful for the determination of PMI**
Past methods

Comparison of cartilage sources and methods for the ex-vivo determination of chondrocyte viability

Armin Alibegović, Danijel Petrović, Nevenka Kregar Velikonja, Jože Balazić, Matej Drobnic
Past, present and future methods for assessment of chondrocytes’ viability: A new parameter for determination of the postmortem interval?

- aim - best combination of sampling source and measuring method

- one donor

- three sources of cartilage biopsy
  - knee
  - ankle
  - ear

- three chondrocyte viability protocols
  - manual counting under a microscope (MCM)
  - cell viability analyzer (CVA)
  - flow cytometer (FCM)

- two storage temperatures
  - at 4 °C
  - at RT

- two corpse status
  - early postmortem status - no putrefaction
  - late postmortem status - putrefaction

- more time points: Day 0, Day 3, Day 6, Day 13
Past, present and future methods for assessment of chondrocytes’ viability: A new parameter for determination of the postmortem interval?

**MCM**
- trypan blue vital dye - 5 min at RT
- dead and living cells were manually counted using a counting chamber and a light microscope

**CVA**
- trypan blue vital dye included in the kit for automatic dyeing
- size limits for cell capturing were set between 8 to 20 µm
- automatic counting using the trypan blue vital dye exclusion method

- trypan blue vital dye
  - viable cells resist the dye passage through the membrane
  - dead cells are marked intensively blue

**FCM**
- cells were concentrated by centrifugation at 580 g for 5 min
- re-suspended in a buffer solution
- isolated cells were stained with propidium iodide
  - fluorescent dye - penetrates the impaired cytoplasmic membrane of dead or injured cells
  - binds to their nuclear DNA

- each examined tissue sample was analyzed with three parallel aliquots
Conclusions

- CVA provided the most reliable measurements and the best cost-time performance

- study confirmed the time-dependent chondrocyte viability decline
- not affected by the ambient temperature - caused by?
  - the small sample size - single donor
  - large aseptic void volume of the storage container

- Samples taken from the left auricle - microbial infection after three days of body decay at RT → useless in forensics - cartilaginous tissue of the head, neck, and torso

- tangential cutting with a scalpel blade inappropriate
  - blade repeatedly tends to slide over the surface or stop at wavy subchondral bone
  - cartilage shell was of an uneven shape and thickness
  - consisted predominantly of chondrocytes of the superficial and intermediate zones

→ standardized instrument to reach the whole thickness of the cartilage

- does enzymatic tissue digestion prior to the cell analysis cause additional destruction of cells?

→ comparative analysis with the cells in situ was necessary to evaluate possible artifacts
Past, present and future methods for assessment of chondrocytes’ viability: A new parameter for determination of the postmortem interval?

TECHNICAL NOTE

PATHOLOGY/BIOLOGY

Armin Alibegović,¹ M.D., Ph.D.; Jože Balažič,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
Past, present and future methods for assessment of chondrocytes' viability: A new parameter for determination of the postmortem interval?

- aim - compare CVA to method that enables viability analysis on the cells in situ

- Live/Dead probes - confocal laser scanning microscope (CLSM)

- three donors

- knee and ankle

- two temperature conditions

- 1 month after the donor's death

- osteochondral cylinders - stored in a tightly closed tubes filled to the top with the cell preservation media

- approximate the conditions in dead bodies
  - limited amount of media and nutrients that were diffused to the cells
  - media was not exchanged
  - oxygen was limited and was not added
  - waste materials were not removed
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CLSM

fluorogenic reagents:
- calcein-AM
  - membrane permeable nonfluorescent esterase substrate
  - passively diffuses into cytoplasm
  - after intracellular enzymatic hydrolysis remaining calcein is impermeable
  - trapped by intact cell membranes - emits a green fluorescence when excited
  → cell has an intact membrane - considered viable

- ethidium homodimer-1
  - impermeable to intact cell membranes
  - able to diffuse through the porous membranes of dying or dead cells
  - high affinity to nucleic acids and emits a bright red when excited
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CLSM

- 3 of 6 stained slices were randomly selected for the analysis with CLSM

- chosen locations on each slice ~ 40 μm deep into the specimen + 400 μm away from the slice margins

- confocal micrographs with green and red colored spots, live and dead cells

- cells were counted manually
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CLSM

- automatic software-based protocols for the image analysis of Live/Dead chondrocytes
- used in previous studies - faster and observer-independent analysis
- thresholding could induce a systematic error

- our study
- high magnification
- manual cell counting
- groups of green fragments without a red signal
- membrane particles of a dead cells
- fragments - could easily be mistaken for viable cells when using automatic counting (false-positive results)
Conclusions

- cartilage from the knee joint demonstrated less variability
- knee joint was easier to access for sample procurement
- knee has a larger and thicker articular surface
- during harvesting of the ankle cartilage - coring instruments often destroyed - harder subchondral bone

<table>
<thead>
<tr>
<th></th>
<th>CVA</th>
<th>CLSM</th>
</tr>
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<tbody>
<tr>
<td><strong>Isolation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Material costs (€)</td>
<td>9.6</td>
<td>0.7</td>
</tr>
<tr>
<td>(S)</td>
<td>13.5</td>
<td>1</td>
</tr>
<tr>
<td>Time for a single sample (min)</td>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td>Time for 15 samples (h)</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td><strong>Analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Material costs (€)</td>
<td>5.6</td>
<td>0.1</td>
</tr>
<tr>
<td>(S)</td>
<td>7.9</td>
<td>0.14</td>
</tr>
<tr>
<td>Apparatus rental price (€/h)</td>
<td>12</td>
<td>77.4</td>
</tr>
<tr>
<td>(S/h)</td>
<td>16.9</td>
<td>109.1</td>
</tr>
<tr>
<td>Time for a single sample (min)</td>
<td>10</td>
<td>28</td>
</tr>
<tr>
<td>Time for 15 samples (h)</td>
<td>1</td>
<td>7</td>
</tr>
</tbody>
</table>

- CLSM in combination with Live/Dead staining provided slightly superior reliability over the CVA
- technical and cost-time issues
  - CLSM - should be reserved for basic studies
  - CVA - automatic cell counting - more user-friendly
    - high level of reliability
    - could be used in routine work
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Present methods

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Viability of Human Articular Chondrocytes Harvested Postmortem: Changes with Time and Temperature of In Vitro Culture Conditions*
- aim – to acquire 2-month *in vitro* viability curves at different temperatures

- 16 male donors aged from 20 to 47 years
  - died of a sudden natural or violent death at the known time
    - sudden cardiac death
    - head and internal injuries in traffic or other accidents
    - accidental heroin intoxication
    - accidental or suicidal asphyxia

  - no medical history of any knee joint pathology
  - no any record of a systemic illness that could result in cartilage deterioration

- samples were procured and stored in the same way such as in presented pilot studies

- change
  - difference in antibiotics combination and dosis
  - determined after another three pilot studies
  - 5 μg/mL of vancomycin, 100 μg/mL of gentamicin, and 1 μg/mL of amphotericin B
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- procured samples were randomly divided into four groups - stored at
  - 4 °C ± 2 °C
  - 11 °C ± 2 °C
  - 23 °C ± 2 °C
  - 35 °C ± 2 °C

- initial viability analyses - conducted within 36 h after the donor’s death (mean 20 h)
- subsequent analyses at each temperature
  - daily - next 3 days
  - then in 3-day intervals until 63 days after the donor's death

- one sample of every donor was analyzed at each time-temperature point
- obtained 1536 osteochondral cylinders - 16 donors, each with the 96 time-temperature points

- osteochondral cylinders - prepared identically as in previously mentioned pilot studies

- fraction of viable chondrocytes
  - calculated from three separate cell counts - one in each noncalcified cartilage zone
  - to avoid differences in cell shape, density, and metabolic activity

- viability analysis - discontinued when three consecutive samples showed no viable cell

- person unrelated to the research manually counted - viable and nonviable cells from captured micrographs
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- on Day 27 after the donor’s death
- 4 temperatures
- 3 articular cartilage zones (superficial, transitional, radial)
- chondrocytes in the superficial zone were the most resistant
- most of the cells in the transitional and radial zone from the sample stored at 4 °C were dead
- almost all cells from the sample stored at 35 °C were dead
- fraction of viable chondrocytes much higher in the samples stored at 11 °C and 23 °C
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Results
Results

TABLE 2—ANOVA table showing the influence of time and temperature on the knee chondrocytes viability measured using the confocal laser scanning microscope.

<table>
<thead>
<tr>
<th></th>
<th>numDF</th>
<th>denDF</th>
<th>F-Value</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Intercept)</td>
<td>1</td>
<td>1229.00</td>
<td>1890.25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time</td>
<td>23</td>
<td>1229.00</td>
<td>115.60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Temperature</td>
<td>3</td>
<td>45.00</td>
<td>63.30</td>
<td>&lt;0.001</td>
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- ANOVA - significant influence of time and temperature on the fraction of viable cells
Past, present and future methods for assessment of chondrocytes' viability: A new parameter for determination of the postmortem interval?

Results

- all pairwise comparisons between different temperatures showed significant differences

- also viability of the cells at 11 °C and 23 °C was significantly different but the magnitude of the difference was too small for practical importance
Past, present and future methods for assessment of chondrocytes' viability: A new parameter for determination of the postmortem interval?

Results

- pairwise comparisons between different time points was not significantly different for the viability of the cells from Day 0 to Day 9

- on Day 9 the viability was significantly lower than on Day 0

- same trend continued further
Past, present and future methods for assessment of chondrocytes’ viability: A new parameter for determination of the postmortem interval?

Results

- statistical calculations from the collected data were the basis for the construction of the viability curves
- according to the model that best fitted the data for each temperature
- predicted values (solid lines) + prediction intervals with 95 % accuracy (dotted lines) of chondrocytes' viability
- level of 100 % viability is marked (dotted line)
Results

- all estimated curves have a sigmoid shape
- each of them begins with a plateau - 10, 15, 12.5, and 7.5 days long at 4 °C, 11 °C, 23 °C, and 35 °C
- plateaus gradually change into a more or less linear part with different inclinations
  - steeper at 35 °C and 4 °C than at 23 °C and 11 °C
Results

- later all curves become less steep - slowly approach null viability - especially below the level of 5 %
  - margins of the prediction intervals of survived cells below 5 % level
    - at 4 °C → 50-56 days after the first measurement
    - at 11 °C was above 30 % at the last measurement
    - at 23 °C viability curve reached the 5 % level just after Day 63
    - at 35 °C → 34-38 days after the first measurement
Results

- viability curves corresponding to the ambient temperatures when plotted on the same scale
Past, present and future methods for assessment of chondrocytes' viability: A new parameter for determination of the postmortem interval?

Results

- estimated curve models could be applied to the retrograde determination of the first measurement time (theoretically representing the time of death) for the cartilage sample stored in vitro.
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Results

- plateau inable usage of this model - 7.5- to 15-day-long - depending on the ambient temperature
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Results

- at later time points - variability in the data is smaller - prediction intervals are narrower
- allow us to predict the time of the first measurement (time of individual death) more accurately
Past, present and future methods for assessment of chondrocytes’ viability: A new parameter for determination of the postmortem interval?

Results

- below the 5 % level of the surviving cells - viability curves become relatively flat
- determination of the first measurement from these parts of the curves is useless, despite a narrow prediction interval
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Results

- margins of the prediction intervals for the viability curves of the samples stored at:
  - 4 °C reached the 5 % level 50-56 days after the first measurement
  - 35 °C reached the 5 % level 34-38 days after the first measurement
  - 23 °C reached the 5 % level during the last measurement
    - following the trend of the viability curve and the prediction intervals
    - estimation that the 5 % level could be reached 62-68 days after the first measurement
  - 11 °C were around 30 % at the last measurement
    - curve trend suggests
    - that 5 % level could be reached 80-90 days after the first measurement
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**Results**

- presented model is useful after the proportion of surviving cells falls below 64%-69% (upper limit), depending on the ambient temperature, to the level of 5 % (lower limit)

<table>
<thead>
<tr>
<th>Temperature</th>
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<th>Upper Limit Day</th>
<th>Lower Limit Fraction</th>
<th>Lower Limit Day</th>
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<td>4°C</td>
<td>0.67</td>
<td>10–26</td>
<td>0.05</td>
<td>50–56*</td>
</tr>
<tr>
<td>11°C</td>
<td>0.64</td>
<td>15–53</td>
<td>0.05</td>
<td>80–90†</td>
</tr>
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<td>23°C</td>
<td>0.65</td>
<td>12.5–42.5</td>
<td>0.05</td>
<td>62–68†</td>
</tr>
<tr>
<td>35°C</td>
<td>0.69</td>
<td>7.5–20.5</td>
<td>0.05</td>
<td>34–38*</td>
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*Values set from the graphs.
†Values estimated on the basis of the curves’ trend.
Results

- practical usage of this model could present next example

- measured 40 % chondrocyte viability showed prediction intervals with the 95 % accuracy that the first measurement, representing the time of the donor’s death, from the samples stored at:
  - 4 °C was before 29±5 days
  - 11 °C was before 56±10 days
  - 23 °C was before 44±7 days
  - 35 °C was before 22±3 days
Conclusions

- obtained results confirmed that the chondrocytes' viability in the cartilage of a human corpse might be used for the determination of the time since death as it decreases constantly with time and under the influence of the ambient temperature

- main weakness is the *in vitro* setup of the study, but it was necessary
  - to put the chondrocytes from different samples in the same conditions
  - to exclude the influence of the internal and external, ante-mortem and postmortem unpredictable factors
  - such as different types and amounts of bacteria during body decomposition

- presented *in vitro* estimated curves serve only as foundations for the coming *in corpore* study which
  - should be extensive
  - would include, beside ambient temperature, also the other factors in the natural environment

- in the manuscript were discussed numerous other problems and statements
Future methods

- cartilage could be determined as a compartment
- cartilage could be used for the determination of the PMI

- chondrocytes’ viability decreases gradually and steadily as a function of time and temperature
- objective determination of the time of an individual’s death several weeks after the moment of dying
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However, broader study of cartilage as a possible new parameter for PMI determination in forensic medicine has not been performed.
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- objective determination of the time of an individual’s death several weeks after the moment of dying

However, broader study of cartilage as a possible new parameter for PMI determination in forensic medicine has not been performed.

Since the corpse is exposed to a wide variety of environmental conditions that cannot be controlled, it was necessary to perform a study in supervised in vitro conditions before an extensive in corpore study.
Past, present and future methods for assessment of chondrocytes’ viability: A new parameter for determination of the postmortem interval?

Past, present and future methods for assessment of chondrocytes’ viability (a new parameter for determination of the postmortem interval?)

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