Viability of Human Chondrocytes in an *ex Vivo* Model in Relation to Temperature and Cartilage Depth

(chondrocyte viability / human model / cartilage zones / temperature influence)

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Abstract. Chondrocytes in human articular cartilage remain viable post-mortem. It has however not been established yet how the storage temperature affects their survival, which is essential information when post-mortem cartilage is used for toxicologic studies. Our aim was to construct a simple model of explanted knee cartilage and to test the influences of time and temperature on the viability of chondrocytes in the *ex vivo* conditions. Osteochondral cylinders were procured from the cadaveric femoral condyles. The cylinders were embedded in water-tight rubber tubes, which formed separate chondral and osteal compartments. Tubes were filled with normal saline, without additives, to keep chondrocytes under close-to-normal conditions. The samples were divided into two groups stored at 4°C and 35°C, respectively. Three samples of each of these two groups were analysed at the time of removal, and then three and nine days later. Images of Live-Dead staining were scanned by a confocal laser microscope. Count of viable chondrocytes in four regions, from surface to bone, was obtained using image analysis software. The regression model revealed that the number of viable chondrocytes decreased every day by 19% and that an increase in temperature by 1°C decreased their viability by 5.8%. The temperature effect fell by 0.2 percentage points for every 100 μm from the surface to the bone. Herein we demonstrate that chondrocytes remain viable in the *ex vivo* model of human knee cartilage long enough to be able to serve as a model for toxicologic studies. Their viability is, however, significantly influenced by time and temperature.

Received April 1, 2005. Accepted April 28, 2005.

The work was supported by the Ministry of Education, Science and Sports of the Republic of Slovenia under the Junior Investigator's Grant to M. Drobnič (No. S3-312-020/21500/2000) and part of the Research Grant to Z. Grubič (No. P3-0043).

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Healthy articular cartilage is essential for normal pain-free movements of human joints. In adults, it consists of chondrocytes, ellipsoidally shaped cells of low density, and extracellular matrix formed by them. Chondrocytes depend primarily on anaerobic metabolism and are predominantly supplied with nutrients by diffusion from the synovial fluid. Four depth zones: superficial, transitional, radial, and deep, are distinguished in healthy cartilage, each with its own biological, biochemical, and biomechanical characteristics (Buckwalter and Mankin, 1998). One of the main problems of articular cartilage is an inadequate repair after trauma or disease, leading to the premature joint degeneration (Buckwalter, 2002). Many medical and surgical procedures have been developed to increase the restitution of compromised cartilage surfaces (for review see Hunziker, 2002). The results are promising, but are still limited by our poor understanding of chondrocyte response to various environmental stimuli. Further research in this field would be necessary and an appropriate experimental model to allow studies of such response would be of great benefit.

Most of the present evidence concerning chondrocytes derives from the studies on cell cultures and animal models (Hunziker, 2003; Reinholz et al., 2004), while a reliable and simple model of human cartilage has not been introduced yet. One possible approach to study human chondrocytes is the use of viable *ex vivo* human cartilage, stored under controlled laboratory conditions. The advantage of such model over the cultured cells would be preservation of the interactions of chondrocytes with the extracellular matrix (van der Kraan et al., 2002). These interactions, which are lacking in the culture models, are important for chondrocyte behaviour. The proposed model would also overcome disadvantages of the animal models that originate from the well-documented species-specific differences in cartilage thickness and cell density (Stockwell, 1971). These differences are serious obstacles to the extrapolation

of the findings obtained on other species to humans (Reinholz et al., 2004). The aim of our study was to construct a simple model of explanted human cartilage and to test the effects of time and temperature on the chondrocyte viability in different cartilage zones in this model. An important support to this *ex vivo* model is high post-mortem viability of chondrocytes, which is most likely due to their low density and abundant extracellular matrix protecting them from environmental changes. A study on human cadavers revealed that nearly 60% of chondrocytes in the human knee survived six days under appropriate conditions (Lasczkowski et al., 2002). Even one and a half month after death nearly 10% were still viable. Studies on human osteochondral allografts confirmed 54% to 83% viable chondrocytes one month after the procurement, if stored in special media and physical environment (Csonge et al., 2002; Williams et al., 2003; Ball et al., 2004; Pearsall et al., 2004). On the basis of these data we hypothesized that chondrocytes may survive for a significant period of time also in an *ex vivo* model of human cartilage under controlled laboratory conditions.

Material and Methods

Sample procurement and storage

The experiments followed the requirements of the ethical approval (No. 74/12/01) issued by the Ethical Commission at the Ministry of Health of the Republic of Slovenia. Fifteen osteochondral cylinders (Ø 6 mm, depth 20 mm) were removed with the mosaico-plasty coring instruments without drilling (Helipro, Lesce, Slovenia) from femoral condyles of a 60-year-old male cadaver. There was no medical history of any knee joint pathology, neither was there any record of a systemic disease that could result in cartilage deterioration. The person died from cardiac arrest and was kept at 4˚C from the second post-mortem hour on. Osteochondral cylinders from the femoral condyles were procured 16 h after death. They were kept moist throughout the initial manipulation, which was carried out at the room temperature (20˚C). Macroscopically, the cartilage was without any detectable degeneration and was classified as grade 0 by the ICRS criteria (ICRS General Committee, 2000). The cylinders were tightly embedded in 30 mm long rubber tubes. At the level of cartilage surface, additional constrictive wire secured the water-tightness. The cartilage surface and tube's inner wall formed a separate container filled with sterile saline (0.9% NaCl). The osteal part of the cylinder protruded into a saline bath at the other end of the tube (Fig. 1). Three samples were analysed for viability immediately after removal (Day 0). Six samples were then stored at 4˚C and other six at 35˚C. Three samples from each of these two groups were analysed after three (Day 3) and nine days (Day 9). Procurement and storage were conducted in aseptic conditions.

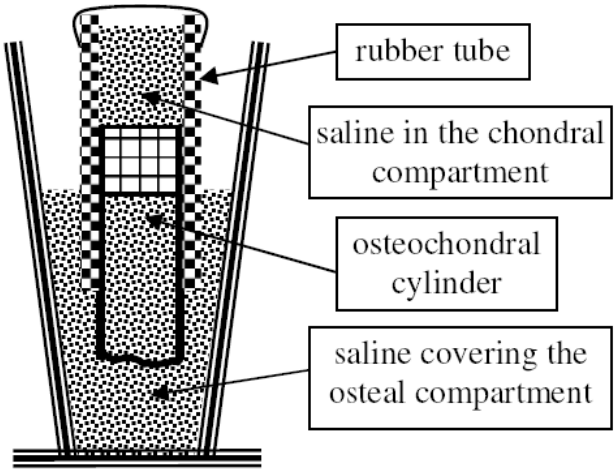

Fig. 1. Schematic presentation of the cartilage *ex vivo* model. Osteochondral cylinder was embeded in a water-tight rubber tube. Sterile normal saline covered separate chondral and osteal compartments.

Viability assessment and image analysis

After the cylinders were removed from the tubes, their chondral parts were separated from the bone at the level of subchondral plate. The chondral samples were split into two halves with a central perpendicular cut and immediately incubated for 30 min with the solution of fluorescent Live-Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR). One component of the kit, calcein AM, stains viable cells and produces intensive green fluorescence of their cytoplasm, whereas

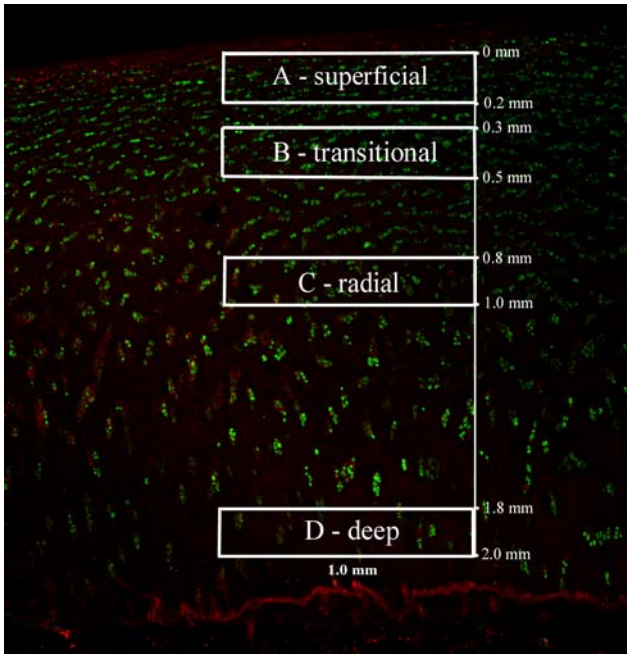

Fig. 2. Demonstration of the image analysis on the Sample 1 of Day 0. The selected rectangle regions (size 0.2 x 1.0 mm; optical slice thickness 51 μm) approximately match cartilage zones.

$$\log(viable\_chondrocytes) = const - 0.190 * time - 0.058 * temp - 0.104 * depth + 0.002 * temp * depth$$

(-6.5) (-4.9) (-4.0) (1.8)

$R^2_{adj} = 0.66$

the other component, ethidium homodimer-1, produces bright red fluorescence of the dead cells' nuclei. After the incubation, samples were scanned throughout the whole depth with a confocal laser microscope Zeiss LSM 510 (Zeiss, Jena, Germany).

The distance from cartilage surface to the tide-mark, representing cartilage thickness, was first measured in the central part of the acquired images. Each image (size 2.6 × 2.6 mm, optical slice thickness 51 μm) was further sampled with four rectangular regions, which approximately corresponded to the cartilage zonal distribution (Fig. 2). They were positioned at the following depths: A (superficial) – 0 to 0.2 mm; B (transitional) – 0.3 to 0.5 mm; C (radial) – 0.8 to 1.0 mm; D (deep) – 1.8 to 2.0 mm. The rectangular regions (depth 0.2, width 1.0 mm) were selected in the central part of the sample, at least 0.5 mm from each lateral margin, to avoid possible tissue disturbances due to the previous direct contact with the rubber tube. Dispersion of the red fluorescent signals caused by the nuclear disintegration enabled us to count more homogeneous green fluorescent signals of viable chondrocytes' cytoplasm only. Automatic count of viable chondrocytes in each selected rectangular region was performed using Object Count function of the ImageTool 3.00 (UTHSCSA, San Antonio, TX).

Statistical analysis

The viable chondrocyte counts acquired by the image analysis (*viable_chondrocytes*) were first analyzed by ANOVA. Three parameters: temperature (*temp*), time (*time*), and depth of the rectangular region (*depth*), including their interactions, were taken into account. The effects of significant factors were afterwards quantified by the corresponding regression model. Statistical analysis was performed using the Stata Statistical Software (StataCorp LP, College Station, TX).

Results

The average cartilage thickness in the central part of the sample was 2.33 mm (SD 0.14). All samples confirmed the presence of viable and non-viable chondrocytes in the experimental model (Fig. 3). The details on viable chondrocyte counts in each group of samples are presented in Table 1. One sample of the day 9 at the temperature of 4°C was destroyed during staining and therefore the results at this time-temperature point are based on two samples only.

Analysis of variance revealed that all factors and interaction between depth and temperature contributed significantly to the variance of *viable_chondrocytes* numbers (at the level $P < 0.1$). Therefore, a regression model was estimated, as shown in the box above.

Coefficients of explanatory variables, fit and (in the brackets) t-statistics are given. Obviously, the regression model and the coefficients of three studied factors were highly significant (at the level $P < 0.001$); only significance of the coefficient at temperature and depth

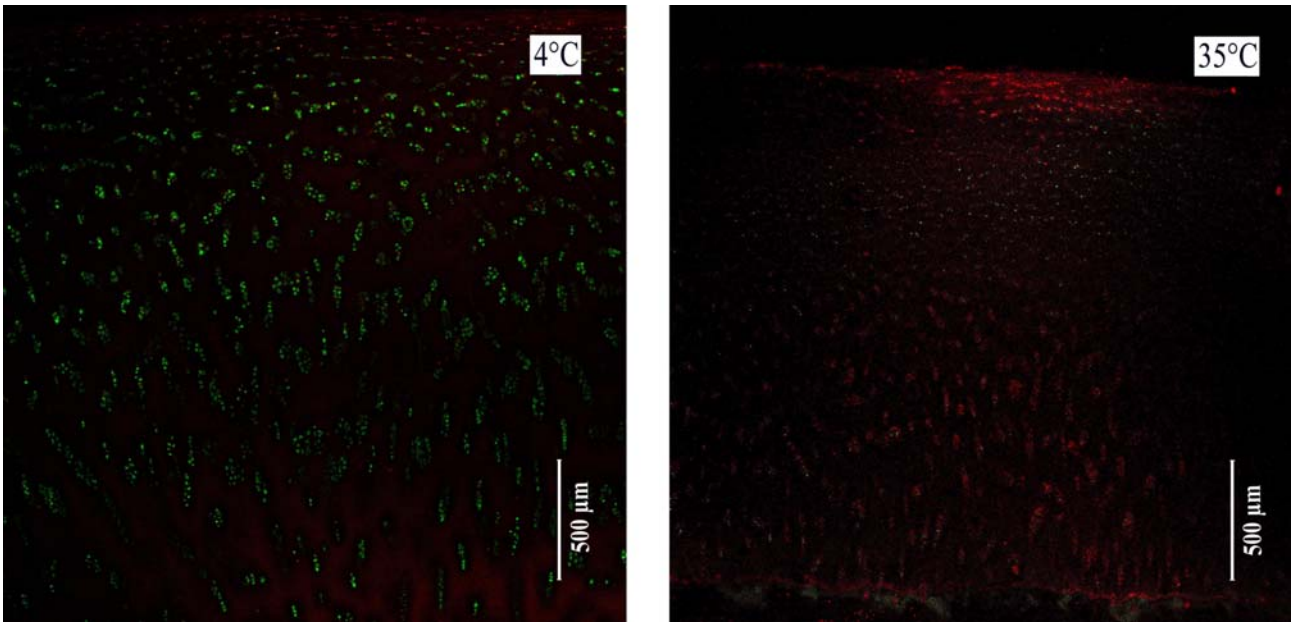

Fig. 3. Viable (green fluorescent cytoplasm) and non-viable (red fluorescent nucleus) chondrocytes in the *ex vivo* model after nine days at the temperatures of 4°C (left image) and 35°C (right image).

Table 1. Viable chondrocytes at different cartilage depths

Time	Temperature	Cartilage depth regions [a]				Average [b]
	(°C)	Superficial	Transitional	Radial	Deep	%
Day 0	20	79.7 (5.5)	90.7 (0.6)	48.0 (5.6)	21.3 (8.1)	100 (0)
Day 3	4	70.0 (15.1)	49.7 (6.7)	33.7 (3.1)	15.7 (12.1)	72 (14)
Day 3	35	12.7 (8.7)	23.7 (4.0)	20.7 (17.9)	8.3 (8.5)	31 (12)
Day 9	4	40.0 (17.0)	39.0 (0.0)	30.0 (2.8)	14.0 (23.3)	55 (11)
Day 9	35	2.0 (3.5)	3.0 (4.4)	2.0 (2.0)	2.3 (1.2)	5 (4)

[a] Mean number (SD) of viable chondrocytes in each region. Region size: 0.2 × 1.0 mm. Optical slice thickness: 51 µm

[b] Average (SD) viable chondrocyte number compared to the zero sample (Day 0)

interaction (P = 0.07) was lower. The model showed that every day the number of viable cells decreased by 19% (at the same temperature and depth). It also showed that an increase in temperature by 1˚C decreased the number of viable cells by 5.8% (at the same time and depth). The effect decreased from the surface to the interior; it fell by 0.2 percentage points for every 100 µm. Significant (at P < 0.001) was also the coefficient at depth variable, which quantifies the effect of chondrocytes' zonal distribution.

Discussion

Many experimental models have been introduced in the past to study the biology of chondrocytes and their response to various environmental factors. Most of the present knowledge on this subject derives from the experiments on cultured chondrocytes and animal models (Hunziker, 2003; Reinholz et al., 2004), while a simple human model in which the normal chondrocyte environment is reproduced has not been described yet. Here we describe a model of explanted human articular cartilage that fulfils this requirement relatively well. The viability of chondrocytes, which is crucial for this model to be used, was thoroughly and systematically analysed. We confirmed the presence of viable chondrocytes at three and nine days under our experimental conditions. As expected, a significant influence of time and temperature was established; however, the effect of temperature decreased by the distance from the surface. A higher number of viable cells was found at the lower temperature.

The number of viable chondrocytes observed in our experiments was lower than the viabilities reported in previous studies for various animal cartilage specimens (Amiel et al., 1989; Rohde et al., 2004; Williams et al., 2004) and human osteochondral allografts (Williams et al., 2003; Ball et al., 2004; Pearsall et al., 2004). More than 60% viability reported in these studies after one month of storage most probably reflects better controlled removal and storage conditions. The post-mortem interval, from death to the procurement, of our samples matched the intervals of the aforementioned studies on human allografts, ranging between 5 to 72 h (Csonge et al., 2002; Williams et al., 2003; Ball et al., 2004; Pearsall et al., 2004). However, while donors were kept under controlled environment all the time in these reports, it was not possible in the current work to control climate conditions during the first two hours after death, during which the damage of an unpredictable number of cells might have occurred (Lasczkowski et al., 2002). Differences in storage conditions were probably even more critical as far as chondrocyte survival is concerned. Donor allografts are normally stored in special preservation media containing basic cell nutrients, which surround the whole graft (Csonge et al., 2002; Williams et al., 2003; Pearsall et al., 2004). As confirmed by Ball et al. (2004), these media are superior to the lactated Ringer's solution; however, various components of the preservation buffers and nutrients might interact with the cartilage and introduce unwanted interactions in the future usage of this cartilage model. To exclude any possible artifacts that might emerge due to the non-natural distribution of nutrient delivery from the cartilage surface to its deeper regions in the *ex vivo* conditions, we sacrificed the longer chondrocyte viability and decided to use normal saline instead. The decline in the number of viable chondrocytes in our study was comparable to Bujia et al. (1994), who reported a 32–43% viability of human chondrocytes in nasal cartilage after ten days stored in normal saline at 4˚C.

Recent studies on aortic allografts (Baric et al., 2004) and human cadaveric skin (Robb et al., 2001) demonstrated improved tissue viability when stored at higher, i.e. 37˚C and room temperature, respectively, in comparison to the temperature of 4˚C. Our results do not support these findings. Rather, they confirm the observations of Lasczkowski et al. (2002) on human cadavers. Namely, we found higher chondrocyte viability at the temperatures around 4˚C in comparison to the physiological knee temperature range above 30˚C (Oosterveld et al., 1992; Warren et al., 2004). Our results support the usual practice for osteochondral allograft storage, which takes place at 4˚C (Aubin et al., 2001). We speculate that controlled hypothermia decreases chondrocyte metabolism and the resulting energy demand, so that the cell nutrient supply offered

under such conditions covers metabolic needs. At the same time, this temperature does not induce any significant chondrocyte injury, as reported after freezing (Ohlendorf et al., 1996; Csonge et al., 2002).

Our demonstration that surface layers are more affected by the environmental factors are in accord with the previous reports on human osteochondral allografts (Ball et al., 2004). We tried to divide the images for chondrocyte analysis so that they would match the cartilage zones described by other authors (Mitrovic et al., 1983; Ball et al., 2004). This appeared, however, to be very difficult to achieve, especially in the superficial zone. In our experience, the exact criteria proposed for zonal distribution cannot be precisely implemented in practice, simply because there are significant differences among individuals in this respect (Eckstein et al., 2001). Our model supported a well-known fact of higher chondrocyte density closer to the surface (Mitrovic et al., 1983; Buckwalter and Mankin, 1998), but it also showed that the temperature effect decreased with depth. We therefore speculate that densely populated areas of chondrocytes, under an increased cell metabolism at higher temperatures, deplete the nutrient reservoir in the matrix faster than their low-density counterparts closer to the bone.

It is acknowledged that the automatic, computerized analysis of confocal microscope images used in this study has its limitations. Better information on the absolute count of viable and non-viable chondrocytes could be gathered by more established methods, like flow cytometry (Pearsall et al., 2004; Williams et al., 2004). However, the disadvantage of this approach is that it requires the chondrocytes to be released from the matrix, resulting in a loss of information about the cartilage depth. Due to the dispersed non-viable signals, only the viable chondrocyte counts were acquired. As the differences in cartilage thickness were small, and all the samples were procured with a standardized technique from adjacent locations of the same knee joint, we assumed the total number of chondrocytes in each zone remained constant across different samples. In any case, our cell count can be considered to be only an estimation of the exact chondrocyte number. However, although a certain degree of error is introduced in this way, it does not seem to be critical. Namely, it is the relative and not the absolute count of cells that is important in most of the studies (Lasczkowski et al., 2002; Williams et al., 2003; Pearsall et al., 2004; Williams et al., 2004).

In summary, we find the *ex vivo* experimental model proposed herein suitable to study the toxicologic effects of various medications. The examined substances can simply be added to the saline in the chondral compartment, where human chondrocytes are located at their normal *in vivo* positions and are kept in their natural matrix environement. A drawback of the model is poor cell stability at the physiological knee joint temperatures, which makes it more useful for the studies in which keeping chondrocytes at the physiological temperature is not necessary.

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