Stored Human Septal Chondrocyte Viability Analyzed by Confocal Microscopy

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Objectives: To analyze the effects of prolonged storage time, at warm and cold temperatures, on the viability of human nasal septal chondrocytes and to understand the implications for tissue engineering of septal cartilage.

Design: Basic science.

Subjects: Septal cartilage was obtained from 10 patients and placed in bacteriostatic isotonic sodium chloride solution. Four specimens were kept at 23°C, and 4 were kept at 4°C. The viability of the chondrocytes within the cartilage was assessed using confocal laser scanning microscopy every 5 days. The 2 other specimens were assessed for viability on the day of harvest.

Results: Viability on the day of harvest was 96%, implying minimal cell death from surgical trauma. After 1 week, cell survival in all specimens was essentially unchanged from the day of harvest. At 23°C, the majority (54%) of cells were alive after 20 days. At 4°C, 70% of cells survived 1 month and 38% were alive at 2 months. Qualitatively, chondrocytes died in a topographically uniform distribution in warm specimens, whereas cold specimens displayed a more irregular pattern of cell death.

Conclusion: Septal chondrocytes remain viable for prolonged periods when stored in simple bacteriostatic isotonic sodium chloride solution, and such survival is enhanced by cold storage.

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Tissue engineering has emerged as one of the most promising techniques of generating new, functional tissue in recent years. Within the field of otolaryngology, tissue engineering of cartilage holds particular potential, given the frequent use of this tissue in plastic and reconstructive surgery. In general, tissue engineering of cartilage involves harvesting such tissue from a donor, digesting the extracellular matrix, and multiplying the isolated chondrocytes in vitro. These cells are then cultured in 3-dimensional configurations and induced to deposit new extracellular matrix, theoretically forming neocartilage with similar biomechanical properties to native cartilage. This neocartilage can be used to reconstruct surgical or traumatic defects or improve the appearance and function of structures altered by aging or inadequate anatomy. There are several potential advantages of tissue-engineered cartilage autografts, such as a limitless supply of neocartilage, the ability to mold neocartilage into any desired size and shape, and minimized immune rejection and disease transmission.

Chondrocytes from multiple anatomic sources have been evaluated for tissue engineering potential. Research by Benya and Shaffer involved the harvest of rabbit articular chondrocytes. Early successes were also realized by Vacanti et al., who worked with chondrocytes isolated from newborn calf shoulders. Costal, auricular, and nasal septal sources have also been considered. There is reason to conclude, however, that among these choices, nasal septal cartilage represents the ideal source for tissue-engineered cartilage constructs. Septal cartilage is firm and nonmalleable and has superior supportive properties to resist deformity by the contraction of skin and scarring during the healing process. Harvesting septal cartilage also entails less morbidity than harvesting costal or auricular cartilage. Septal chondrocytes might therefore be best to generate neocartilage that replicates the advantages of native cartilage. The only significant disadvantage to using septal cartilage is its small size and solitary presence.

Although multiple methods to improve the growth of neocartilage have been studied, little investigation has been under-
Human septal specimens were obtained from 10 patients (mean age, 37.9 years; range, 22-57 years) undergoing septrhinoplasty at the University of California San Diego Medical Center or the San Diego Veterans Healthcare System. Informed consent was obtained from all donors, and tissue was collected with the approval of the human subjects committees formed at each institution.

**METHODS**

Human septal specimens were obtained from 10 patients (mean age, 37.9 years; range, 22-57 years) undergoing septrhinoplasty at the University of California San Diego Medical Center or the San Diego Veterans Healthcare System. Informed consent was obtained from all donors, and tissue was collected with the approval of the human subjects committees of both institutions.

**SPECIMEN STORAGE**

At the time of surgical harvest, specimens were placed in bacteriostatic isotonic sodium chloride (saline) solution. Four septal specimens (mean age, 38.5 years; range, 24-54 years) were designated for the “warm” study group and kept at room temperature (approximately 23°C). A second group of 4 specimens (mean age, 38.3 years; range, 22-57 years) was designated the “cold” study group and immediately placed in a refrigerator (4°C). These warm and cold groups were then transported to the laboratory and analyzed for long-term chondrocyte survival. The remaining 2 specimens (mean age, 36 years; range, 31-41 years) were examined for the effects of surgical trauma on immediate chondrocyte survival. After transport to the laboratory, these were kept at room temperature and protected from light. After 20 minutes, each specimen was rinsed twice in 1 mL of phosphate-buffered saline (10 minutes per rinse) and again protected from light.

**ASSESSMENT OF VIABILITY**

After staining, cartilage slices were analyzed for cellular viability using a CLSM (Bio-Rad Laboratories Inc, Hercules, Calif) attached to a Nikon Diaphot 300 inverted microscope (Nikon USA, Melville, NY) with a plan fluor objective lens (original magnification ×20) with a 0.5 numerical aperture. The CLSM was equipped with a krypton-argon laser, and filters were chosen to permit detection of red and green emitted light for simultaneous recording on 2 photomultiplier tubes (PMT). The 2 stained cartilage slices from each donor were transferred to a glass slide (Corning Glassworks, Corning, NY) and covered with a coverslip secured by tape. Slides were inverted and placed on the microscope stage. Two central regions of each slice were located and focused using fluorescent light microscopy (at least 50 µm deep into the specimen, away from the upper surface) so that edge effects were excluded. Once located, each region was imaged using the CLSM. Settings of the CLSM were consistent for every analysis, with the gain settings for PMT 1 (red, or dead/dying cells) and PMT 2 (green, or viable cells) adjusted so the emissions of approximately 10% of cells were saturated. Differences in the PMT 1 and PMT 2 were then merged into 1 image, which was saved to the computer hard drive. Thus, 4 images were collected for every donor on each day of analysis.

Counting of live (green) and dead (red) cells began by individually marking cells using Adobe Photoshop 5.0 software (Adobe Systems, San Jose, Calif). Separate photographic layers were used for green and red marks, and layers were saved as individual computer files. To avoid double counting or undercounting, marks were then quantified using Image J software (National Institutes of Health, Bethesda, Md) so the total numbers of live and dead cells from each image were obtained. These results for all donors, on each day of analysis, were averaged and plotted as a function of days in storage.

**SPECIMEN PREPARATION**

On the day of analysis, each specimen was removed from the saline solution, and all edges were trimmed using a handheld scalpel. Two thin slices, each approximately 0.5-mm thick, were taken from the center of each specimen. Slices were then stained using the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes Inc, Eugene, Ore), which includes 2 dyes, calcein-AM and ethidium homodimer-1. Calcein-AM is a membrane permeable esterase substrate that passively diffuses into cytoplasm. After 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 when excited by blue light at 494 nm, indicating that the cell has an intact membrane and esterase activity and is therefore viable. Ethidium homodimer-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 (617-nm) light when excited at 528 nm. Therefore, dying or dead cells appear red when viewed by epifluorescence or confocal laser scanning microscopy (CLSM).

Calcein-AM and ethidium homodimer-1 dyes were prepared according to package instructions to ×100 concentration, and then further diluted by combining 5 µL of each stain with 500 µL of phosphate-buffered saline in individual wells of a 48-well plate (Becton Dickinson Labware, Franklin Lakes, NJ). One specimen slice was added to each well at room temperature and protected from light. After 20 minutes, each specimen was rinsed twice in 1 mL of phosphate-buffered saline (10 minutes per rinse) and again protected from light.
STATISTICAL ANALYSIS

Multivariate repeated measures analysis was used to determine the effect of storage temperature on overall viability. The Dunnett test of multiple comparisons against a single control was used to compare viability at days 5 and later with day 1 for each storage temperature. P<.05 was considered significant.

RESULTS

Plots of long-term chondrocyte viability at each temperature, as a function of days in storage, are presented in Figure 1. On the first day after harvest of warm specimens, 93% of chondrocytes were viable, and on day 5, survival was essentially unchanged (92%). By 20 days, the majority (54%) of cells were still alive. After 1 month of storage, however, only 16% of warm chondrocytes were viable. Statistical analysis of the decrease in viability using the Dunnett test showed a trend toward significance at day 15 (P=.07 compared with day 1). Viability became significantly decreased compared with day 1 at days 20, 25, and 30 (P<.001 for each time point).

In contrast, cold specimens displayed a higher overall viability during the first month (P=.003) compared with warm samples (Figure 1). Of the cold cells, 98% were alive on postharvest day 1 and viability was essentially unchanged in 95% after 15 days. After 1 month, as many as 70% were alive. Even after 2 months of storage in a refrigerator, 38% of chondrocytes were viable. A trend toward significant cell death was demonstrated at day 25 (P=.07 compared with day 1), and by day 30, viability was significantly decreased (P=.03 at day 30 compared with day 1, and P<.002 for all later time points).

For both storage conditions, the total number of cells counted remained fairly constant throughout the storage period (Figure 2). This suggests that when a cell dies, it remains red and is countable at all later time points. Viability was therefore not being overestimated as the storage time increased.

It was interesting to note that viability on the day after harvest was not 100% in either group. To determine if cells were dying as a result of the process of surgical harvest, 2 specimens were stored at warm temperature and analyzed for viability immediately after harvest and again on postharvest day 1. Mean viability was 96% immediately after harvest, and destroyed chondrocytes were evenly distributed throughout the specimens. Viability of these specimens on postharvest day 1 was also 96%, which was similar to results of postharvest day 1 presented in Figure 1 for the warm and cold specimens.

Qualitative analysis of chondrocyte death during the first month of storage reveals similar patterns between the warm and cold specimens. At warm temperatures, cells died in a relatively even and consistent distribution throughout each specimen. This process is summarized by the images obtained from CLSM and displayed in Figure 3A-D. From postharvest days 1 through 20 (Figure 3A-C), the increasing number of red (dead) cells were interspersed with green (alive) cells in an even arrangement. By day 30 (Figure 3D), most cells were dead. Specimens stored at cold temperatures displayed a similarly even distribution of dead cells (Figure 3E-H). By day 30, the majority of cold specimen chondrocytes were still viable.

During the second month of cold storage, however, the pattern of death changed slightly. In all cold specimens, cell death began to proceed in an uneven, patchy distribution during this period. Some confocal fields showed mainly dead cells, while other fields showed both live and dead chondrocytes. Figure 4 presents an example of this finding. These images are derived from 1 cold specimen at day 55 of storage and represent an entire cross-section of the specimen. The left peripheral (Figure 4A) and central (Figure 4B) fields reveal mostly dead cells, while the right peripheral field (Figure 4C) displays a patch of viable chondrocytes interspersed with dead cells. All of these fields were used in quantifying viability as presented in Figure 1.

COMMENT

This study represents the first attempt at analyzing the effects of storage time and temperature on viability of human nasal septal chondrocytes over a prolonged period.
Though specimens were stored in bacteriostatic saline with no additives, chondrocytes remained viable for relatively prolonged periods. At room temperature, 92% of cells were alive after 5 days, and a majority were alive after 20 days. Survival was prolonged by reducing the storage temperature: 95% of refrigerated cells were alive after 15 days and the majority were alive after 45 days. Even after 2 months in storage, refrigerated specimens contained more than one third of viable cells. Regarding patterns of cell death, this study demonstrates a uniform progression with specimens stored at warm temperatures compared with the patchy distribution of dead cells in cold specimens.

This study also shows that not all septal chondrocytes are viable on the day of harvest. Viability of 2 specimens immediately after harvest was 96%, suggesting either that some cells are destroyed through the surgical process or that some turnover of chondrocytes occurs in vivo. This level of cell survival was similar to that of other specimens, both warm and cold, analyzed on postharvest day 1.

Bacteriostatic saline was the only storage medium used in this study, and it is possible that chondrocyte survival would be higher in other media. However, our goal was to assess survival in a basic and simple environment. Saline was selected because it is perhaps the most accessible and convenient storage medium available, especially in a clinical surgical setting. Furthermore, the saline was not changed, so specimens were kept in a constant environment for up to 2 months. Survival was still high, even in this limited setting with no nutrients available.

Procedures were used to avoid analyzing areas that were affected by manipulation of our specimens. Contact with forceps and scalpel blades leads to cell death that would not otherwise occur in whole specimens used

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**Figure 3.** Confocal laser scanning microscopic images of septal specimens stored at warm (A-D) and cold (E-H) temperatures. Cells are stained for live (green) and dead (red) cells. Progressive days in storage are represented (day 1, A and E; day 15, B and F; day 20, C and G; and day 30, D and H) (calcein-AM and ethidium homodimer-1 staining, original magnification ×20).

**Figure 4.** Confocal laser scanning microscopic images of 1 cold specimen taken on postharvest day 55. These images represent a complete cross-section of the specimen, including the left (A), middle (B), and right (C) fields (calcein-AM and ethidium homodimer-1 staining, original magnification ×20).
for tissue engineering. Therefore, any specimen’s edge that was exposed to sectioning for the purposes of this study was excluded from cell counting. As a result, the number of viable cells determined in this study most closely reflects the cells that would actually be available for tissue engineering of whole cartilage specimens.

The results of this study have several implications for tissue engineering. They suggest that harvested septal tissue can be stored for prolonged periods prior to expansion of cell number. Thus, when tissue engineering of septal cartilage becomes feasible, some flexibility is possible in transporting specimens from the clinical setting to the laboratory. In addition, storage media do not necessarily require nutrients such as Dulbecco minimal essential medium, serum, or growth factors to maintain viability, at least for periods up to 2 months. Further studies will be needed to demonstrate the ability of these stored specimens to yield chondrocytes that can multiply and produce extracellular matrix. We are hopeful, however, that these functions will remain intact after storage in saline solution. Because staining with calcein dye may indicate intact cell membranes and esterase activity, it may also imply the preservation of other cellular functions. However, these assumptions must be confirmed by further research.

There is evidence that nasal septal specimens, stored for prolonged periods in bacteriostatic saline solution, effectively serve as homografts and autografts for reconstructive surgery. To the extent that such chondrocytes can maintain graft bulk and stability long after implantation, it is reasonable to assume that stored chondrocytes possess the physiologic mechanisms to expand in monolayer cell number after storage. Few other storage protocols for septal cartilage have been evaluated. Cryopreservation has been proposed as an alternative to storage in saline solution or other media. However, such freezing can adversely affect cell viability and function. Bujia et al showed nasal septal chondrocyte viability to be 4% after freezing, even when using dimethyl sulfoxide as a cryopreservative. Moreover, only 1% of cells were able to grow in monolayer after cryopreservation. In a separate study, Bujia et al demonstrated that previously cryopreserved nasal chondrocytes expand in monolayer at a slower rate than cells from fresh donors.

The variance in patterns of cell death between warm and cold specimens was prominent. Though reasons for this finding are unclear, one hypothesis relates to the interaction between chondrocytes and surrounding collagen. It has been suggested that chondrocyte survival is enhanced by contact of cell surface receptors with extracellular matrix components, particularly type II collagen. It is therefore likely that in unfavorable environments, chondrocyte loss leads to collagen breakdown, which contributes to the progression of cell death. As chemical reactions occur more quickly at higher temperatures, this cycle would proceed more rapidly at room temperature. However, at refrigerated temperatures, the process would be slowed so regional differences could become manifest and lead to a patchy distribution of dead cells. Future research will be able to better elucidate the factors that initiate cellular loss in septal cartilage and will therefore lead to methods of prolonging survival. With articular cartilage, medium additives such as autologous serum, interleukin 1β, pyrrolidine dithiocarbamate, insulinlike growth factor 1, and caspase inhibitors have been shown to reduce apoptosis and could therefore be beneficial to septal cartilage as well.

Confocal laser scanning microscopy was used in this study as a method of evaluating native septal chondrocyte viability. With CLSM, laser light of 1 or more wavelengths is passed through a fluorescently labeled specimen, and emitted light is filtered to produce a digital image of excellent clarity. A significant benefit is the ability to clearly image thin layers of specimens that are not actually sectioned and to do so in a reproducible manner. We suggest that this technique is an ideal method for assessing viability because it is nondestructive to the tissue and provides information about the cells in their local environments. Several methods have been used in the past, including the trypan blue exclusion test, flow cytometry, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), and fluorescent staining for light microscopy. However, limitations with these techniques led many authors to adopt CLSM for assessing the viability of a wide variety of tissues, including bacteria, microbes in food products, and brain. Confocal laser scanning microscopy has already been used effectively to assess the viability of cultured human nasal septal chondrocytes seeded onto polymer fleeces.

Another interesting use of the CLSM involved estimating postmortem intervals of cadavers by examining the viability of chondrocytes from knee cartilage. Similar advances in forensic sciences could be derived using nasal septal chondrocytes. By applying the relationships between temperature, time, and viability, analysis of septal specimens could also predict postmortem intervals.

In conclusion, the present study demonstrates the ability of human nasal septal chondrocytes to survive prolonged storage periods in bacteriostatic saline solution. This viability is further increased by storage at cooler temperatures. The patterns of cell death for warm specimens differ from that of cold specimens, as the latter demonstrate a variable distribution of cell death. Finally, this study suggests the use of CLSM as an excellent means of studying septal chondrocyte viability. Future studies will examine the ability of septal chondrocytes, stored for prolonged periods, to multiply and produce extracellular matrix.

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Author Contributions: Dr Hicks had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Hicks, Sage, Schumacher, Jadin, Sah, and Watson. Acquisition of data: Hicks, Sage, Agustin, and Watson. Analysis and interpretation of data: Hicks, Sage, Schumacher, Sah, and Watson. Drafting of the manuscript: Hicks. Critical revision of the manuscript for important intellectual content: Hicks, Sage, Schumacher, Jadin, Agustin, Sah, and Watson. Statistical analysis: Hicks and Sah. Obtained funding: Watson. Administrative, techni-
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REFERENCES