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Authors
Karimi, Koohyar
Protsenko, Dimitry
Wu, Edward C
et al.

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Comparison of Bend Angle Measurements in Fresh Cryopreserved Cartilage Specimens after Electromechanical Reshaping

Koohyar Karimi[a], Dmitry Protsenko[a], Edward C. Wu[a], Allen Foulad[a], Cyrus T. Manuel[a], Amanda Lim[a], Brian JF Wong[a,b,c]
[a]Beckman Laser Institute, 1002 Health Sciences Road, Irvine, CA 92612.
[b]Department of Otolaryngology- Head and Neck Surgery, University of California Irvine, 101 The City Drive Bld. 25 Rt. 81, Orange, CA 92868.
[c]Department of Biomedical Engineering Rockwell Engineering Center 204, University of California Irvine, Irvine, CA 92612.

ABSTRACT

Cryopreservation of cartilage has been investigated for decades and is currently an established protocol. However, the reliability and applicability of cartilage cryopreservation for the use in electromechanical reshaping (EMR) has not been studied exclusively. A system to cryopreserve large numbers of tissue specimens provides a steady source of cartilage of similar quality for experimentation at later dates. This will reduce error that may arise from different cartilage stock, and has the potential to maximize efficiency under time constraints. Our study utilizes a unique methodology to cryopreserve septal cartilage for use in EMR studies. Rabbit septal cartilage specimens were harvested and standardized to 20 x 8 x 1 mm, and placed in one of three solutions (normal saline, PBS, 10% DMSO in PBS) for four hours in a cold storage room at 4 degrees Celsius. Then, each cartilage specimen was vacuumed and sealed in an anti-frost plastic bag and stored in a freezer at -80 degrees Celsius for 1 to 3 weeks duration. EMR was performed using 2 and 6 volts for 2 minutes application time. Bend angle measurements of the cryopreserved cartilage specimens were compared to bend angles of fresh cartilage which underwent EMR using the same parameters. Results demonstrate that normal saline, phosphate buffered saline (PBS), and PBS with DMSO were effective in cryopreservation, and indicated no significant differences in bend angle measurements when compared to no cryopreservation. Our methodology to cryopreserve cartilage specimens provides a successful approach for use in conducting large-scale EMR studies.

Keywords: electromechanical reshaping, cryopreservation, septal cartilage, voltage application, EMR, bend angle

1. INTRODUCTION

Cryopreservation of cartilage has been an established protocol in the literature and has considerable importance in medical and research fields. For example, reconstructive surgery performed in the osteochondral, chondral, meniscal, and cricoid regions currently depend on the availability of cartilage allografts. Methods to preserve these grafts would naturally increase the resources available for performing these procedures.[1-3] Many synthetic biocompatible materials commonly used in surgical procedures also require cartilage preservation.[1-3, 8] Likewise, cartilage reshaping modalities such as enzymatic digestion, radiofrequency reshaping, laser cartilage reshaping, and most recently, electromechanical reshaping (EMR) of cartilage are important areas that depend on a substantial amount of cartilage for data analysis.[1-4]
Cryopreservation is a unique process that has been investigated for well over thirty years. It has long been known that viable cells from cartilage tissue can survive at sub-zero temperatures, typically 190 K or −80°C. [5-7] By preserving tissue at low temperatures, most biological activity responsible for tissue decay and cell death would be effectively limited. However, cartilage specimens under cryopreservation can sustain other injuries in this state. Phenomena which can cause damage to cells during cryopreservation include chemical damage due to preservation in solution, extracellular and

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intracellular ice formation, and also dehydration.[5] Many of these effects can be reduced by the concomitant use of cryoprotectants. Generally, sugar compounds such as glucose are used as cryoprotectants to reduce freeze-related tissue injury. In addition, glycerol and DMSO have been prominently used to specifically reduce ice formation and are effectively used to cryopreserve cartilage.[5-6]

Electromechanical reshaping (EMR) is a novel technique developed by our research group that effects conformational change in cartilage structure by application of direct current through areas of stress concentration in tissue.[1-4] Previous studies have found evidence suggesting that EMR uses an oxidation-reduction mechanism within the proteoglycan cellular matrix of cartilage tissue to cause permanent changes in shape and biomechanical properties. The oxidation-reduction reactions across the tissue are influenced by multiple factors such as electrode geometry, electrode type, and most importantly, voltage and application time. Shape change can be quantified by measurement of cartilage bend angle. The importance of EMR lies in its potential clinical use in facial reconstructive surgery and otolaryngology to noninvasively reshape cartilage to a desired conformation (i.e., angle change, folding),

The reliability and application of cryopreservation of rabbit septal cartilage for use in EMR has not been studied exclusively. This is of immense value particularly to investigations that depend on large numbers of tissue specimens for analysis. In addition, as cartilage tissue naturally deteriorates over time, arresting cell necrosis through cryopreservation may preclude the need to procure fresh samples repeatedly. This study aims to 1) create a unique methodology to cryopreserve cartilage and 2) determine whether the methodology is feasible for EMR studies.

2. MATERIALS AND METHODS

Fresh rabbit septal cartilage was extracted from the crania of New Zealand White Rabbits obtained from a local abattoir. The nasal mucosa and perichondral tissues were not removed during the cryopreservation process to maintain the cartilage specimens in its native state and limit possible tissue deterioration. Each specimen was placed into 15 ml test tubes containing solutions of 0.9% normal saline, phosphate buffered saline (PBS), or 10% dimethyl sulfoxide (DMSO) by volume in PBS for approximately 2 hours in room temperature. The specimens were then stored in a cold room at 4 degrees Celsius for approximately 4 hours in order to minimize crystallization of the tissue matrix and to allow adequate diffusion of the cryoprotectant into the cellular matrix.[5-7] A commercially available food vacuum sealer system (FoodSaver Game Saver Sport Plus®, Sunbean Products, Boca Raton, FL) was used to vacuum and seal the cartilage specimens in anti-frost vacuum-sealed plastic bags. The vacuumed bags containing the specimens were placed in a freezer at -80 degrees Celsius for one, two, or three weeks.

Before EMR, the anti-frost plastic bags containing specimens were immediately thawed and placed in a warm water bath (37°C) for approximately 10 minutes. All specimens remained in a sealed vacuum during thawing. Then, they were removed from the bags and placed in PBS solution and prepared for EMR. Each specimen was cut into rectangular specimens (20 ± 1 x 12 ± 0.5 x 0.8 ± 0.1 mm) and the intact mucosa and perichondrial tissue were removed.

Previous studies have shown that the use of small platinum needle (0.3 mm diameter) electrodes directly penetrating cartilage tissue can be used to adequately sustain a voltage source to produce the molecular changes necessary for shape change.[2,4] Electrodes were inserted into custom jigs designed to deform cartilage at a 90° angle (UC Irvine Machine Shop, Irvine CA) and a selected voltage (2 V or 6 V) was applied for a desired length of time (2 minutes). The electrode geometry (i.e., number of electrodes, location of insertion sites, designation of anode and cathode) were identical to a previous study using fresh cartilage with no cryopreservation.[2] The specimens from this prior study were utilized as the control group in order to compare any bend angle differences with cryopreserved cartilage.

Voltage and application time parameters were selected based on thresholds of significant shape change found in a previous study. [2] In one set of experiments, thawed cartilage underwent EMR at 2 V for 2 minutes, while another set of experiments evaluated EMR at 6 V for 2 minutes. Cryopreserved specimens were rehydrated immediately after EMR in PBS for approximately 15 minutes and specimens were photographed using a digital camera (Rebel XT, 100 mm Macro Lens, Cannons USA, Lake Success, NY) that was mounted in a fixed position. Bend angle measurements were analyzed with a Java-based open source image processing program (NIH ImageJ, Bethesda, MD). ANOVA was performed to compare experimental group results (bend angle measurements) with the control.

3. RESULTS

Results comparing bend angles for fresh samples (control) with cryopreserved samples at 2 and 6 volts for 2 minutes application time in PBS, normal saline, and 10% DMSO by volume in PBS are shown in Figures 1 and 2. Each figure also denotes the time from cryopreservation to thawing (1, 2, or 3 weeks). Table 1 and 2 indicate ANOVA *p*-values for all sets of experiments. Figure 3 and 4 show photographs of cartilage specimens.

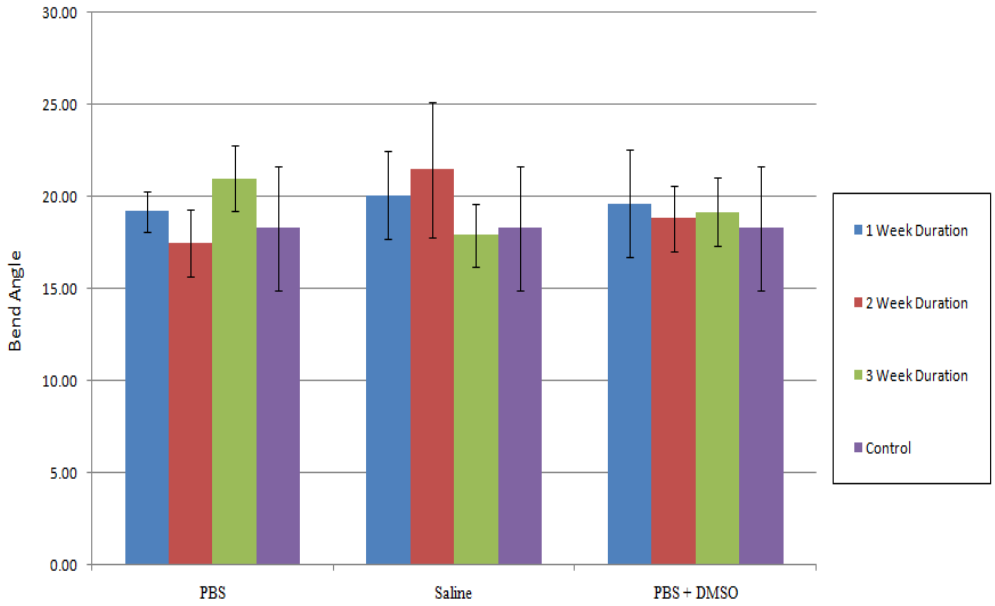

Figure 1.
(2 V, 2 Min)

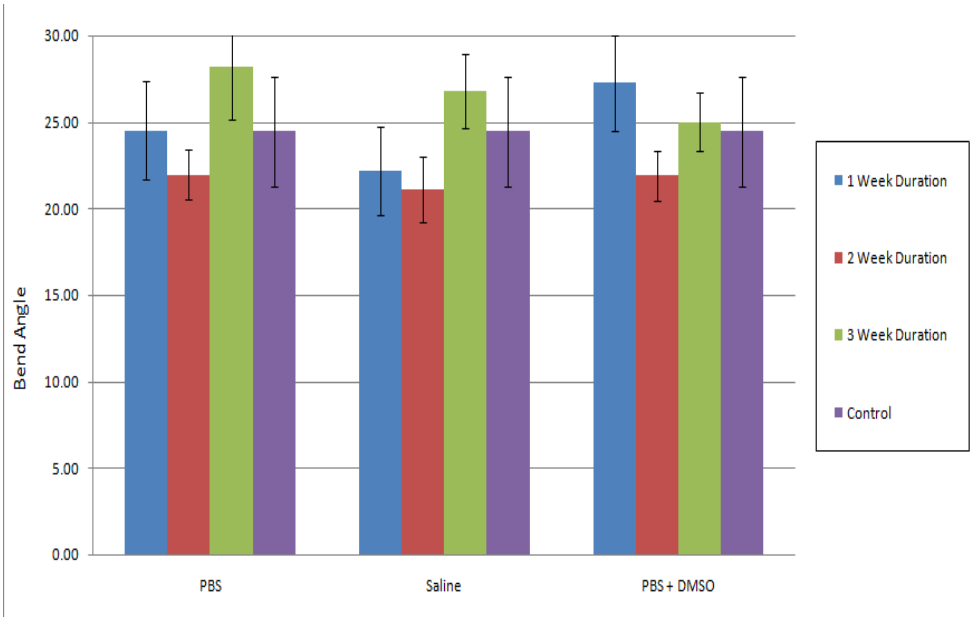

Figure 2.
(6 V, 2 Min)

Table 1
ANOVA *p* values for 2 Volts, 2 Minutes

Week Duration	*p* value
1	0.952
2	0.5741
3	0.6907

Table 2
ANOVA *p* values for 6 Volts, 2 Minutes

Week Duration	*p* value
1	0.4916
2	0.9196
3	0.4235

Table 3
2 Volts, 2 Minutes

		180°- Bend Angle	**Stdev**	**n**	**Std Error**	**Std Error/Avg**
Week 1						
	PBS	19.19	3.32	10	1.106662	5.77
	Saline	20.09	7.19	10	2.397571	11.94
	PBS + DMSO	19.62	7.76	8	2.933115	14.95
Week 2	PBS	17.48	5.78	11	1.83	10.46
	Saline	21.47	9.67	8	3.66	17.03
	PBS + DMSO	18.81	5.06	9	1.79	9.52
Week 3	PBS	20.99	5.32	10	1.771986	8.44
	Saline	17.89	5.48	11	1.73381	9.69
	PBS+ DMSO	19.18	6.13	12	1.848322	9.64
Control	PBS	18.30	8.26	7	3.37	18.42

Table 4
6 Volts, 2 Minutes

		180° - Bend Angle	Stdev	n	Std Error	Std Error/Avg
Week 1						
	PBS	24.54	8.10	9	2.87	11.68
	Saline	22.20	7.77	10	2.59	11.66
	PBS + DMSO	27.30	7.80	9	2.76	10.10
Week 2	PBS	21.98	4.16	9	1.47	6.69
	Saline	21.11	4.65	7	1.90	9.00
	PBS + DMSO	21.94	3.83	8	1.45	6.60
Week 3	PBS	28.24	9.28	10	3.09	10.95
	Saline	26.81	6.08	9	2.15	8.02
	PBS+ DMSO	25.02	5.34	11	1.69	6.75
Control	PBS	24.47	9.47	10.00	3.16	12.90

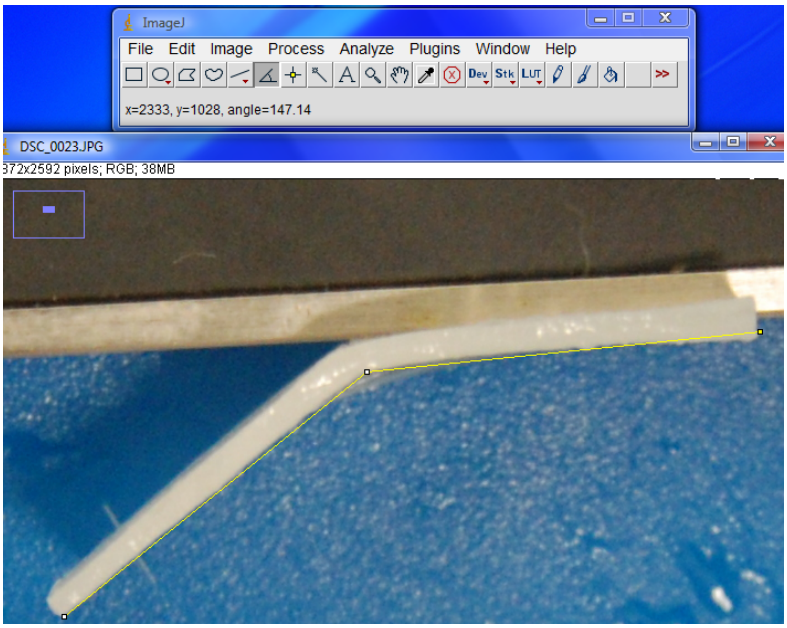

Figure 3.
Photograph of cartilage specimen measured with Image J software.

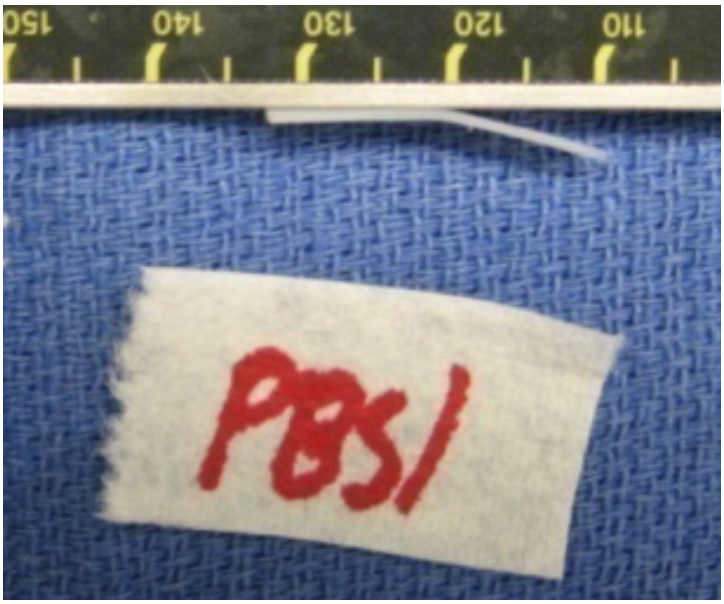

Figure 4.
Photograph of cartilage specimen.

4. DISCUSSION

EMR is a new technique to reshape cartilage with significant clinical value for patients undergoing reconstructive surgery, particularly in the fields of otolaryngology – head and neck surgery and facial plastic surgery. EMR research studies have generally depended on the procurement of a large number of cartilage specimens, all of which naturally deteriorate and become nonviable for experimentation very quickly, typically within two days. The option to preserve cartilage can thus be of enormous value in this area of research. Cryopreservation of cartilage allows for a substantial amount of samples to be procured and used for a later date, making research more efficient and enhancing the power of these studies.

4.1 Solution composition

Prior experiments have indicated that EMR may alter the fixed charge distribution of proteoglycans in the collagen matrix of the tissue.[1-3] This effect, as well as hydrolysis reactions produced by an electrolytic reaction in the cartilage, results in changes to tissue mechanical properties and thus permanent shape change.[2] Thus, the choice of solution used to preserve cartilage specimens may have a role in influencing cartilage biomechanical properties. In prior studies, fresh rabbit septal cartilage has been hydrated with either normal saline or PBS solution and has yielded promising results.[1-3] However, with regards to cryopreservation of cartilage, the literature has supported the use of 10% DMSO by volume in PBS to reduce freezing of the proteoglycan cellular matrix of cartilage.[5-7] Consequently, in this pilot study, we proposed to test three different types of solution for cryopreservation of cartilage (normal saline, PBS, and 10 % DMSO by volume in PBS) in order to effectively determine the most ideal solution used in the cryopreservation process of cartilage.

4.2 Correlation between degree of bend angle and solution used

Compared to the fresh cartilage, there was no significant difference between the three different types of solutions used for cryopreservation. This can allude to the fact that the main molecular composition of each solution tested had a large

concentration of sodium chloride, which maintains a relatively constant ionic composition within the solution. It is also likely that DMSO, being a polar solvent, has an effect on the structural integrity of the cartilage matrix as well as on the ions participating in the redox reactions during the application of voltage. Overall, results have indicated no significant difference in solution type with regards to bend angle within all weeks. Future studies on solution pH may elucidate differences in efficacy among the three solutions.

4.3 Voltage application

Two major parameters in EMR are voltage and application time. In our pilot study, we compared bend angle measurements by varying the voltage between 2 and 6 V while maintaining a constant application time of 2 minutes. By keeping time constant, we were able to isolate the effects of voltage on cryopreserved cartilage and fresh cartilage.

As voltage and/or application time is increased, the bend angle increases in direct proportion. [1-3] When 2 volts was applied for 2 minutes to the cryopreserved cartilage for one to three week duration; bend angle measurements yielded similar results to that of fresh cartilage specimens undergoing same voltage and time parameters. Likewise, when voltage was increased to 6 volts, bend angles measured in cryopreserved specimens were greater than those observed in cartilage cryopreserved specimens treated with 2 volts, as well as yielded bend angle measurements similar to fresh cartilage specimens reshaped at 6 volts. These results suggest that the voltage dependence of EMR is not altered following cryopreservation, though evaluation of time dependence is necessary in future studies.

4.4 Preservation Time

The selection of one, two, and three weeks as the duration of cryopreservation was practical. In prior studies, it was found that EMR may be performed on approximately thirty specimens over the course of five to six hours without ample downtime. In experiments involving a large number of samples, performing EMR repeatedly becomes extremely physically taxing. Drawing out the time frame of EMR experimentation to three weeks is more reasonable, generously allowing for commitment to other activities and experimentation.

4.5 Future work

It is important to note that the salient goal of this pilot study was to verify and validate the cryopreservation methodology for EMR studies. Having more specimens tested along with a range of different voltages would be beneficial to further develop and substantiate this methodology. It is important to systematically evaluate voltage and time dependence given specific needle electrode geometric configurations with EMR in cryopreserved cartilage. Finally, though the solutions in the study showed no drastic differences in cartilage bend angle measurements, determining the pH of the solution, particularly PBS and DMSO would be useful for future EMR studies utilizing cryopreserved cartilage.

5. CONCLUSION

Cryopreservation of septal cartilage for EMR studies is a useful tool for accurate and fast data accrual. The mechanical deformation of septal cartilage caused by voltage application yielded comparable results for cryopreserved and fresh septal cartilage, therefore confirming the feasibility of using cryopreserved specimens in EMR studies. Furthermore, PBS, 10% DMSO by volume in PBS, and saline had no significant differences regarding their affect on shape change, enabling the use of a wide variety of solvents for cryopreservation.

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