# Osteochondral Transplant Delivery System

Final Report

Biomedical Engineering Design 200/300
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## Abstract

Current osteochondral graft surgical procedures repair articular cartilage defects on the femoral condyle using impaction for insertion of the replacement graft. Impaction negatively impacts chondrocyte viability within the cartilage layer and can cause long-term failure of the surgical procedure, requiring subsequent surgeries. Thus, a design must be developed to reduce mechanical forces exerted on the cartilage layer during insertion of the graft in order to obtain higher postoperative chondrocyte viability and increase the success rate of the osteochondral allograft procedure. Threading the graft and recipient site is a novel technique that has not seen an extensive amount of previous research. Threaded grafts maintains bone's mechanical properties while decreasing compressive forces required to insert it into a hole. Six bone plugs were obtained for grafting. Three were inserted using the thread technique and three were inserted using impaction. The cartilage was removed from each plug and were subjected to a live/dead assay. The cartilage was sectioned and imaged using a fluorescence microscope to obtain cell counts and later analyzed for cell viability percentages. The live/dead obtained a p-value = 0.89, showing statistically insignificant data, a need for more consistent standardized testing, as well as more extensive research.

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# Introduction

### *Motivation*

Articular cartilage defects in the knee are commonly detected in roughly 60% of patients undergoing knee arthroscopies [1]. Disease or blunt trauma to the knee induces a degenerative cascade of articular cartilage that can ultimately result in osteoarthritis (*Figure 1*). Symptoms experienced by individuals with osteoarthritis include varying degrees of pain and loss of anatomical movement or altered function of the diseased bone. Current treatment for osteoarthritis defects involve preventative care and surgical procedures that aim to replace the diseased cartilage, such as osteochondral allografts. However, osteochondral allograft surgeries exhibit a 30% failure rate on account of current methods used to deliver the graft [2]. Current methods have detrimental effects on the long term survival of the cartilage due to the mechanical loads associated with graft delivery [3]. As of now, there are no clinical procedures that are consistent and reliable in regenerating cartilage. Hence, there is a need for a novel procedure that will minimize the magnitude of mechanical loads required for proper delivery of the graft to reduce adverse effects in transplanted cartilage.

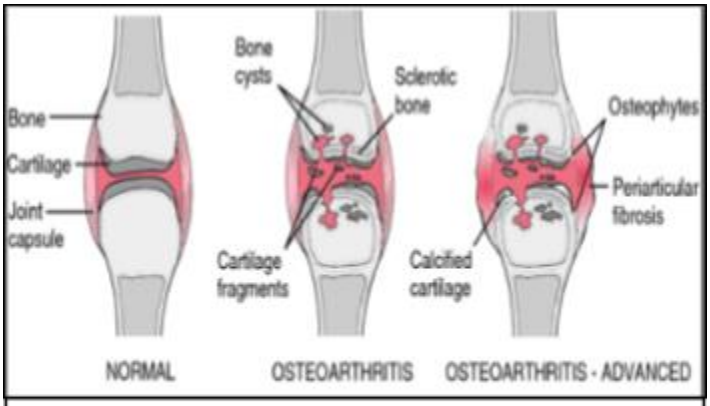


**Figure 1:** A diagram depicting differences between healthy bone and various cases of osteoarthritis [4]

### *Existing Devices/ Current Methods*

Current osteochondral allograft procedures begin with a sizing cylinder placed perpendicular to the defect. A guide pin is drilled through the cylinder deep into the defect [5]. The size block is removed and a reamer is used in conjunction with the guide pin to remove the defective cartilage and damaged bone [5]. The reamer fits directly onto the guide pin, and its trajectory is dependent upon the angle of insertion of the guide pin. Subsequently, measurements of depth are taken from the four quadrants of the cylindrical recipient site for creation of the replacement bone plug. The replacement bone plug is harvested from the donor tissue using a hollow bore reamer or a similar cylindrical harvesting device. The plug is then sized appropriately according to depth and diameter dimensions of the recipient site using a sagittal saw. A calibrated dilator [6] or bevel of the bone in the recipient site [7] can be utilized to obtain

a press fit by hand, but often impaction is required to obtain a tight fit and appropriate seating into the recipient site [6]. The cartilage surface of the graft is impacted until it is flush with the articular surface surrounding the recipient site.

The impaction required to insert the graft frequently causes a decrease in the viability of the chondrocytes, which are the cells in cartilaginous tissue. This can ultimately cause associated cartilage disorders due to the lack of viable tissue. For example, fibrocartilage can form during implantation. Fibrocartilage is a scar tissue that forms due to the limited regenerative capabilities of cartilage. It also has a higher coefficient of friction, which can lead to early degeneration of the tissue [8]. In order to limit these effects, the implanted tissue should contain greater than 70% chondrocyte viability postoperatively [9]. However, this is an estimated value, and an acceptable procedure should allow for the long term success of the graft by reducing the mechanical stimuli necessary for graft insertion to limit cell death that could induce associated complications such as fibrocartilage. If after transplantation the graft is not inserted so that it is flush with the articular surface the surgeon must drill into the plug, remove it from the recipient site and make sizing adjustments of the recipient site or graft as necessary. This causes further damage to the cartilage layer as well and decreases cell viability, and is an inherent risk associated with this procedure.

### *Problem Statement*

Osteochondral allografting is a common procedure performed on patients that have a defect in cartilage and bone tissue. Current methods of implantation require the application of mechanical forces that have a detrimental effect on the live chondrocytes present on the graft tissue. Maximizing the amount of viable tissue during and after the surgery is a crucial factor for the success of the procedure. Hence, the client requests a delivery system that will reduce the amount of mechanical forces required to securely place the implant into the recipient site.

## Background

### *Client Information*

Dr. Brian Walczak, an orthopedic surgeon, was seeking a novel delivery system for osteochondral allografts that would reduce the compressive loads and in theory increase success rates of the surgical procedure.

### *Relevant Biology & Physiology*

Unlike the majority of other connective tissue found in the human body, articular cartilage is composed of a single cell type, the chondrocyte [10]. Although they are the only cell types found in cartilage, they are present at relatively low densities. Chondrocytes are responsible for producing the collagenous extracellular matrix that makes up the avascular and aneural network of cartilage. The lack of blood supply in combination with the natural sparse presence of chondrocytes contribute to the poor regenerative properties of articular cartilage. Consequently, any significant damage done to the chondral surface of a knee will experience little to no regeneration. The injury could cause sustained cartilage degeneration from imposing detrimental stresses on the defect or following fibrocartilage formation and eventually lead to osteoarthritis.

The poor regenerative properties of articular cartilage emphasize the significance of minimizing the loss of viable chondrocytes during osteochondral allografting procedures. Given that these cells are responsible for producing and maintaining the extracellular matrix of articular cartilage, any damage done to them can jeopardize the success of a procedure that is intended to replace diseased cartilage. The relevance of chondrocytes in these procedures is further investigated in a study that explored the effect of donor chondrocyte viability in the overall success of osteochondral allografts performed in the femoral condyle of canines [9]. A successful procedure was defined as one that demonstrated graft integration and lacked cartilage disorder six months after implantation. The study found that successful grafts had at least 70% of viable donor chondrocytes at the time of transplantation. Grafts with less than 70% chondrocyte viability were unsuccessful following the six-month period and experienced infiltration from fibroblasts in the surrounding host tissue. Similarly, the quantity of viable chondrocytes in human osteochondral allografts has an integral role in the overall success of the procedure.

### *Product Design Specifications*

This design must meet specific standards in order for it to be a valuable option for use in osteochondral allograft procedures. The most important criterion must be an increase in the percent chondrocyte viability from the current impaction method, which is approximately 70% mentioned by Cook et al. [9], as well as the client. This will prospectively be achieved through a reduction of mechanical forces on the articular cartilage during insertion. Another criterion is that the device will not cause any chipping or fragmentation of the bone plug during the procedure by remaining under the stress limitations of trabecular bone. These two specifications along with proper fitting of the graft into the recipient site will ensure success of the implantation with proper graft integration into native tissue, properly maintained hyaline cartilage, lack of associated cartilage disorder, and lack of significant fissuring, fibrillation, or fibrous tissue infiltration [9]. These should improve upon the current success rate mentioned by the client of 75-80%.

Additionally, the device should be intuitive and simplistic for use in the operating room as well as easily sterilizable. Any necessary plastic or polymeric components used in the design will be designated for single use only, and all metals will be sterilizable and available for multiple uses if desired by the surgeon. The device should comply with FDA standards for surgical devices [11]. The sizing of the device will vary depending on the extent of the defect, but should be capable of creating a recipient site and a plug at mm increments ranging from 5mm-20mm. This device should also allow for easy insertion of the graft, and should not require more time in the operating room than the current standard of 5 hours mentioned by the client. Compliance of these specifications should be made with an estimated budget of $300. For the full project design specifications see Appendix I.

## Preliminary Designs

Three preliminary design ideas were created prior to testing. These designs were a bone screw using tines, a bone screw using suction, and a bone plug inserted into a hollow threaded hydroxyapatite casing. All aforementioned designs implemented a screwing mechanism. No other methodology capable of decreasing compressive loads while allowing for a flush fit into the recipient hole could be determined. The screwing mechanism would be created using a tap and die. The hole and plug sizes range from 5mm to 20mm depending on the size of the defect.

Metric tap and die sizes would be appropriate, sized from 5M through 20M using a standard coarse pitch thread of 1.5mm.

***Design Alternative 1***

The tine screw design, *Figure 2*, consists of a threaded bone plug that inserts into a threaded recipient hole using tines inserted into the top cartilage surface to generate the torsional forces required for implantation via screwing. This design would be hand powered to reduce possible sources of user error such as exceeding torsional limits and improper thread alignment, and decrease parts required for sterilization. This would also allow for a disposable tool or interchangeable parts. *Figure 2* shows a design using one tine. Similar to a flat-head screwdriver, it would be inserted through the cartilage into a small groove machined into the bone to create a stronger contact surface and prevent slippage and subsequent damage to the cartilage. *Figure 2 also* shows a design using two tines creating a larger area of turning power while attempting to reduce the size of the damaged cartilage. This design could have up to four tines to attempt to maximize torsional forces while minimizing cartilage damage. Furthermore, per the client's request, the tines must be no larger than 1/16 inches in diameter to minimize damage of cartilage.

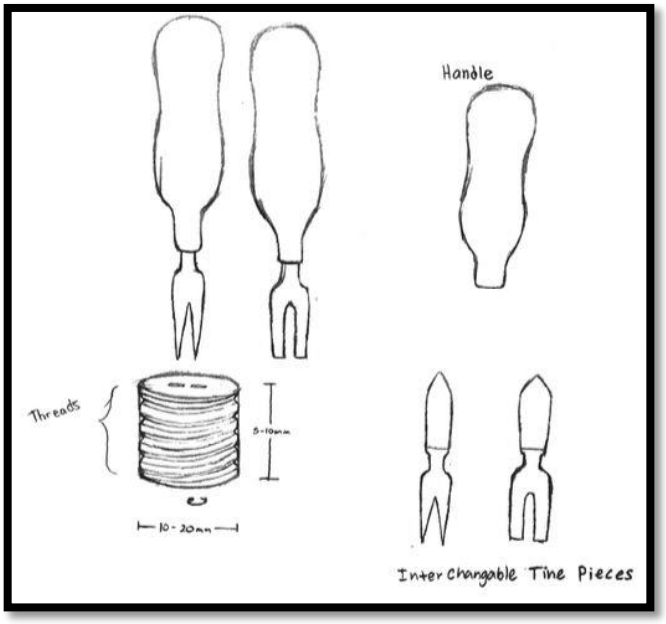


**Figure 2:** The tine screw design, where two tines would be used to screw the threaded graft into place

***Design Alternative 2***

The suction screw design, *Figure 3*, consisted of a threaded bone plug that would be inserted into a threaded recipient hole using a suction cup with a vacuum to generate the torsional forces required for implantation. The suction force would be provided by a suction cup with diameters ranging from 5 to 20mm. The force would be increased by use of a vacuum generated by systems currently inside operating rooms. *Figure 3* shows the design attaching to a vacuum tube allowing for strong suction forces. Furthermore, the device must be suited for the correct diameter tubing, as the largest tubing available in the operating room would be able to provide a greater flow rate [3].

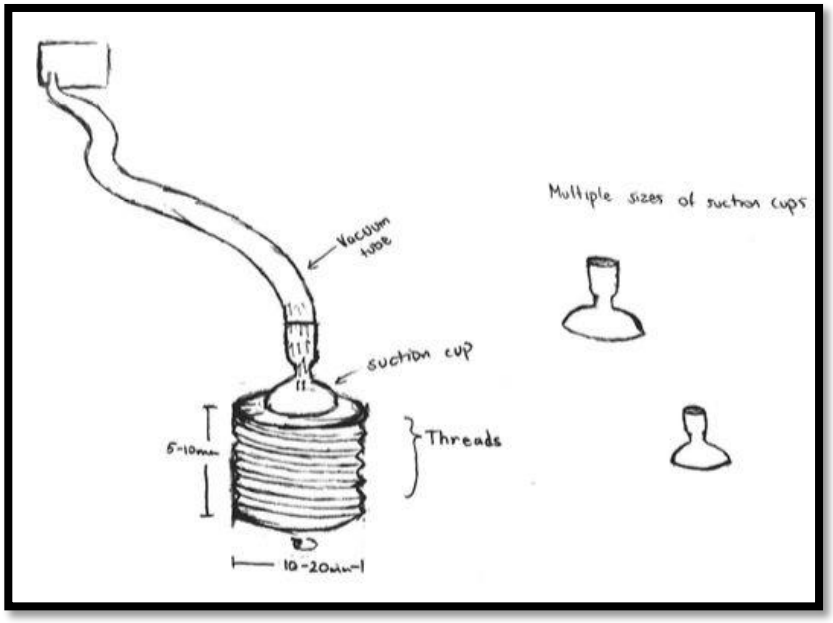


**Figure 3:** The suction screw design where a small suction cup is attached to the cartilage surface and suction provides the torque to turn the threaded graft into place

***Design Alternative 3***

Finally, the threaded synthetic casing design, *Figure 4*, involved the use of a hydroxyapatite casing produced prior to surgery. This design implemented a hydroxyapatite plug that would be threaded and hollowed. This hollow plug would encase the graft. The graft would be adhered to the casing via bone glue and would be pre-installed prior to surgery. This hydroxyapatite casing would also come pre manufactured with handles that could be broken or sliced off upon completion of surgery and allow for a simple insertion procedure into the threaded recipient hole. Furthermore, hydroxyapatite would reintegrate into the bone and allow the area to function naturally.

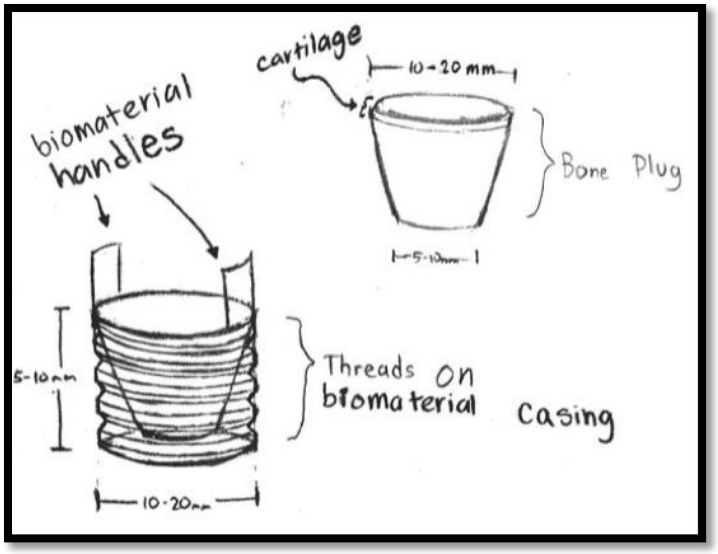


**Figure 4:** The synthetic casing design, in which a normal graft is removed but is then placed within a threaded hydroxyapatite scaffold which would then be screwed into the recipient site

## Preliminary Design Evaluation

| **Criteria** | Design 1: Tine Screw | | Design 2: Suction Screw | | Design 3: Synthetic Casing | |
|---|---|---|---|---|---|---|
| Potential for Chondrocyte Damage (25) | 2/5 | 10 | 4/5 | 20 | 4/5 | 20 |
| Procedure Length (20) | 3/5 | 12 | 3/5 | 12 | 4/5 | 16 |
| Ease of Use (18) | 3/5 | 10.8 | 3/5 | 10.8 | 3/5 | 10.8 |
| Sterilizability (15) | 5/5 | 15 | 5/5 | 15 | 2/5 | 6 |
| Adjustability (12) | 3/5 | 7.2 | 5/5 | 12 | 2/5 | 4.8 |
| Cost (10) | 5/5 | 10 | 4/5 | 8 | 2/5 | 4 |
| Total (100) | | 63 | | 77.8 | | 61.6 |

**Table 1:** The design matrix used to determine the prototype to be developed. Three designs were evaluated, and the suction screw received the highest score.

The design matrix criteria (*Table 1)* were chosen and weighted based on importance from the criteria in the PDS. The goal was to reduce chondrocyte damage by reducing forces applied to the cartilage layer of the donor graft. Thus, potential for chondrocyte damage was weighted at 25. Since no mechanical testing was performed, they were weighted based on a relative scale from estimated force magnitudes for implantation or imposed defects. The suction screw and synthetic casing were ranked 4/5 as both designs involved small forces for insertion, thus reducing potential damage, although both designs required some changes to the bone underneath the cartilage for implant. The tine screw would however kill all chondrocytes at the site of insertion and was thus ranked 2/5.

Procedure length was weighted at 20 as it was a critical design specification specified by the client. Devices that decrease the time spent in the operating room would increase the safety of patient and reduce costs. Both the tine screw and suction screw were ranked at 3/5 due to the necessity of threading both the graft and insertion site. Synthetic casing was ranked 4/5 on account it being created prior to the procedure, however the bone would still need to be altered to fit into the casing.

Ease of use was weighted at 18, on account of the limited resources, time constraints and differences in surgeon abilities. The tine screw and suction screw were ranked equally at 3/5 as they require manual labor for threading. The synthetic casing was also ranked at 3/5 as it would require placement of the bone plug into the casing as a way to allow the bone plug to bond with the synthetic casing. All three designs would then be screwed into the site, a procedure which would be simple and familiar.

Sterilizability was rated at 15, as all surgical tools and materials must be sterilized prior to use in the operating room. The creation of the synthetic casing would require a sterile environment with sterile materials, as well as transportation without compromising sterility. These difficulties lead to the synthetic casing being ranked a 2/5. The suction screw ranked a 5/5 as all suction components could be disposable and therefore be sterilized and thrown out after use. The tine screw was also ranked 5/5 on account of its components being metal which would be easily autoclaved.

Adjustability of the design was weighted at 12 because osteochondral defects vary in size. The suction screw was given 5/5 as multiple suction cup sizes could be used. The tine screw was given 4/5 as the tines would have to be a similar size for all grafts causing relatively more damage to smaller grafts. Finally the synthetic casing ranked 2/5 as the design would have to be machined in a different size to match the predicted defect size. A unique casing would need to be made for each person, and modification of the dimensions of the casing during procedure would be near impossible.

Finally cost was weighted at 10 on account of its low importance for the surgical procedure. While the budget is $300 for the creation of the design, actual implementation in an operating room would not have such a small budget. The tine screw was ranked 5/5 because it would use altered existing tools to test and finalize the design. The suction screw was ranked similarly at 4/5 as suction cups would not be of high cost, although they would need to be disposable. Lastly, the synthetic casing was ranked at 2/5 on account of the costs of purchasing and manufacturing the necessary biomaterial.

### *Proposed Final Design*

The preliminary design chosen was the suction screw (*Figure 3*) on account of its overall score on the design matrix. This design was the most simplistic and realizable while meeting the criteria given by the client.

After performing preliminary threading tests with bovine knuckles, it was discovered that finger tightening of the allograft was sufficient to seat the plug flush to the cartilage layer. The plug, if threaded cleanly, is exceptionally easy to twist into place. We no longer put efforts into developing a specialized tool for this process. Because a surgeon can turn the plug manually, there is no need to create a novel device. Future efforts were directed toward evaluating the efficacy of threaded allografts versus impacted allografts.

## Fabrication/Development Process

### *Materials*

Fabrication of the bone plugs and insertion of them into recipient sites required a variety of materials from many different sources (*Figure 5).* An animal model was needed, and bovine was chosen due to the sizable knee area and ease of access. Four cow knuckles were obtained from 2 different local butchers. For the creation of the recipient site, threading of the site and the plug, and sawing of the bone, tools were obtained from the Student Shop. These included a cordless power drill, drill bits, tap and die, hacksaw, a vice, and a mallet. For the removal and insertion of the plugs, the client donated an osteochondral autograft system. This included 2 harvesters, a core extruder, a graft driver for impaction, a tamp/sizer for measuring, a graft delivery tube, and surgical lube.

**Figure 5:** From left to right: graft driver, tamp/sizer, hand saw, mallet, harvester, die, tap, cordless power drill and bit

To test the aforementioned plugs for cell viability, materials were obtained from the BME department. To keep the femurs from the butcher fresh and moist, 1X PBS was used. MEM-C media, a 24 well plate, and 2 μM Calcein AM and 4 μM Ethidium Homodimer were used to culture and stain the cartilage disks from the bone plugs. Sterile scalpels, 3 microscope slides, and a fluorescent microscope were the materials necessary for imaging the stained cartilage disks. Detailed protocols can be found in Appendix III.

***Methods***

The distal end of a bovine femur was acquired within the same day that the animal was slaughtered. The lower extremity of the femur was removed and placed into a Ziploc bag containing 1X PBS solution within a cooler containing an ice bath. Bovine tissue was selected on account of its accessibility and the large condyle surface area that allowed for extraction of multiple grafts. The lower extremity of the femur was transported to the Engineering Centers building where working surfaces and tools were thoroughly washed with 70% ethanol. This was the only form of sanitizing used for tools and working surfaces due to budget constraints and because many of the tools were incompatible to use with more efficient sterilizing techniques (e.g. autoclaves or ethylene oxide gas). Working surfaces were covered with laboratory countertop paper and the condyle was placed into an industrial vice, also covered with laboratory countertop paper. Using a hacksaw, the knuckle was cut into two halves, separating the condyles from the patellar surface. The half containing the patellar surface was placed back into the 1X PBS solution in the cooler while the condyle was used for the extraction of bone plugs. The patellar surface would later be used to acquire the remaining bone plugs.

To obtain the bone plugs, the condyle portion of bone was placed into the vice with the knuckles facing upright. Using manual compression, the osteochondral autograft harvester--10

mm in diameter-- was wedged through the chondral layer into the cortical bone to a depth of about 1.5 mm. Following this initial insertion, the mallet was used to apply axial compressive loads directly to the surface of the harvester as a means to drive it deeper into the bone. This was done in frequent, continuous successions until the ruler on the side of tool indicated that a 20 mm mark was reached. Since the harvester could not be manually extracted after reaching the desired depth, a hacksaw was used to saw bone off from underneath and surrounding the harvester until it could be extracted manually. The core extruder push pin was then screwed into the harvester until the plug was pushed out of the harvester. The extracted plug was placed into a 50 mL centrifuge tube that contained approximately 45 mL of MEM-C media. Following extraction of the plug, the harvester was inspected for damage. Additional plugs were obtained in an identical fashion for a total of three acquired from the condyle surface. Furthermore, three plugs were acquired from the patellar surface using the aforementioned procedure. The plug was removed by placing the anterior and posterior surfaces into a vice, and inserting the autograft harvester into the medial aspect of the patellar surface.

**Figure 6:** Non-threaded osteochondral graft inserted into recipient site using guide for impaction method

Three randomly chosen plugs were then placed in a vice with the cartilaginous layer (lateral end) facing outwards and threaded starting from the cartilage layer inwards using the 10mm X 1.5 die. The flat surface that remained after separating the condyles from the patellar surface was utilized for the recipient sites. Here, two recipient holes were created using a 8.43mm drill bit attached to a cordless power drill. These holes were threaded using a 10mm X 1.5 tap. Two additional holes were created using a 10mm drill bit and served as the recipient sites for impaction testing. The threaded bone plug was then manually screwed into the recipient site by turning the plug clockwise into the hole; slight pressure was placed on the cartilage during the final turns when insufficient bone was available for grip. The grafts used for the impaction condition were inserted utilizing the tamp and delivery tube included in the autograft

kit (*Figure 6*). The tamp was impacted with a mallet until the graft was inserted. Using a sterilized scalpel, the cartilage was removed from all threaded and impacted grafts following insertion. These methods are detailed in Appendix III.

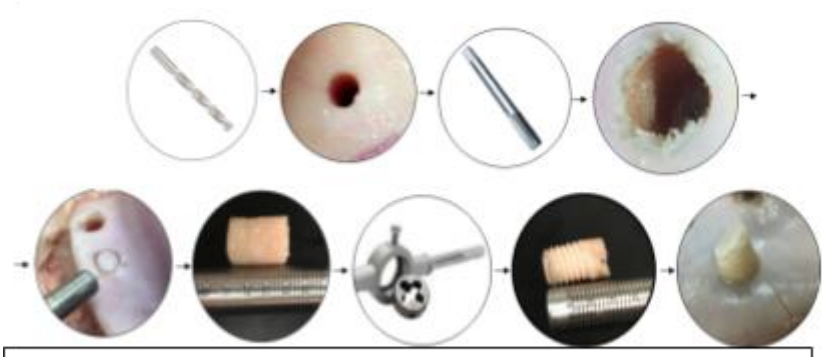

**Figure 7:** Schematic of the basic methods by which a bone plug is created and inserted into the recipient site

***Final Prototype***

The final prototype includes the 10mm bone plug and recipient site. These are created using the methods outlined above and have been tested using the protocols below (*Figures 8-10*).

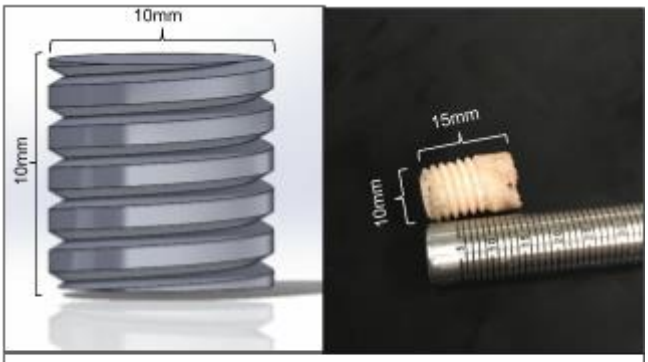


**Figure 8:** On the left, 10mm x 10mm plug representation was created on SolidWorks. On the right is a 10mm x 15mm plug, created from a bovine condyle.

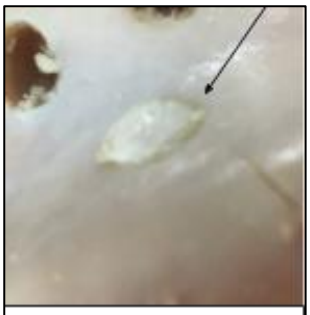

**Figure 10:** The threaded plug with intact cartilage properly screwed into the recipient site


**Figure 9:** On the left is a SolidWorks model of the threaded recipient site, and on the right is the threaded recipient site in the femoral condyle of the cow femur

***Testing***

The purpose of testing was to compare chondrocyte viability of the threading and impaction methods. This factor was prioritized due to its correlation to the overall success rate of the procedure. Although testing was limited to a non-sterile environment, the described procedures were conducted under the same conditions that were expected to impose a large variability for both methods. Hence, the maintenance of chondrocyte viability of the threading method greater than or similar to the impaction method would show promise for future testing and better results in more optimized conditions.

Using the methods outlined above, six osteochondral plugs containing fresh cartilage were obtained from bovine condyles in the COE Student Shop area. All materials were then moved to the BME tissue engineering teaching lab and re-sanitized. The cartilage surfaces were removed from each graft using a surgical scalpel and the six cartilage disks were placed into an appropriately labeled 24 well plate and cultured in MEM-C media. Each disk was incubated for one hour. A single time point of one hour was used because only six grafts were harvested due to a limited amount of available tissue acceptable for graft extraction, tool failure, and limited amount of staining solution available. Three biological replicates for the three conditions compared at the same time point were desired to provide an indication of the error predicted by the sample. Thus, one hour was chosen because similar studies incorporated a one-hour time point, and there was significant cell death observed at one hour (21%) following osteochondral allograft insertion [3]. Additionally, it was found that >70% chondrocyte viability at the time of insertion was necessary for the success of the graft. Thus, measurement at one hour will provide an adequate representation of the success of our transplant method. As a control to serve as a baseline for the viable tissue prior to treatment with graft harvesting and the insertion methods, a sterile scalpel was used to sever three sections of tissue directly from the epicondyle that was under storage following graft acquisition and testing. This will serve to demonstrate the extent to which graft harvest and treatment conditions caused cell death; however, these cartilage sections were not incubated in MEM-C for an hour.

Testing was completed using two stains to detect live and dead cells. Live cells were stained green using calcein acetoxymethyl ester (AM) when in the presence of active esterases that converted the calcein AM precursor to calcein. Dead cells were stained red with ethidium homodimer-1 when it penetrated into cells through damaged membranes, which allowed it to bind to nucleic acids [12]. The sections were exposed to the stain for one hour using 1 mL of calcein AM/ethidium homodimer-1 stain solution per section at a concentration of 2μM calcein AM and 4μM ethidium homodimer in 1X PBS. Calcein AM/ethidium homodimer-1 was selected for Live/Dead assay on account of its visibility under a fluorescence microscope. An hour duration was selected for the staining to allow for complete penetration of the stain solution into our tissue slices. The recommended time for cells in culture was 30-45 minutes [12], but for larger tissue slices as long as 2 hours is necessary [13]. The sections were then washed twice for five minutes in 1X PBS to allow for the removal excess stain.

The cartilage sections were then cut along the plane normal to the articular surface to receive two cross-sectional tissue slices. The slices were then placed on a microscope slide with the plane of the cut normal to the fluorescent microscope. This allowed for the visualization of the entire depth of the cartilage tissue. The FITC channel was used to observe the green fluorescence of the live chondrocytes. The TRITC channel was used to observe red fluorescence.

Due to variances in size of the slices, two or three images were taken of each tissue at 4X on both FITC and TRITC. It would have been better to have images taken at 10X, but the

objectives were not aligned to take clear images at higher magnification.

Analysis of the images was completed using ImageJ. The detailed protocol can be found in Appendix IV. First, images were placed in stacks according to their condition and channel. Next, subtract background was used to remove residual staining from auto fluorescence of the tissue. These were then thresholded until only cells could be seen. Particles were then separated using watershed. Analyze Particles was used to count the cells and this data was later used to assess cell viability.

Other factors that were qualitatively considered during testing included procedure length, sterilizability, adjustability, and ease of use. All tools used were sterilizable and would be sterilized in optimal conditions. Procedure length was indeterminate due to tool availability, a high number of grafts necessary for testing, as well as a lack of experience with surgical procedure. This testing would also be possible to include a range of graft sizes and would solely require differently sized tools corresponding to each graft sizing. Furthermore, both threading and impaction had similar ease of use for those performing the procedure.

## Results

Using the cell count data obtained from ImageJ analysis, found in Appendix V, averages were obtained for percent viability from each graft. Percent viability was obtained by dividing the number of live cells by total cells (i.e. the sum of live and dead cells). This value was obtained for each image. Subsequently, all the values of percent viability from the images corresponding to one graft were averaged together to arrive at percent viability for each given plug. Then, an average was obtained for all of the grafts within each condition. A standard deviation was calculated for the samples within each condition, and a standard error was calculated for the mean estimate for each condition sampled by dividing the standard deviation value by the square root of the number of observations in the sample. Results from this analysis are below (*Table 2*). It is important to note that live and dead cell counts that were below a value of 30 were excluded from the statistical analysis. The images that yielded these excluded values were considered insignificant because of the limited signal that was acquired when the image was taken under the fluorescent microscope. The results from the control test were excluded because they yielded values far below that of the two conditions, most likely due to being exposed to different conditions before staining.

In order to test for a difference between the impacted and threaded conditions a null hypothesis was established. The null hypothesis stated that there is no difference in chondrocyte viability between the threaded and impacted samples. To test this null hypothesis a two sample t-test with unequal variances was used. Given that both data samples were independent, an unequal variance was assumed. The results of the t-test was a p-value of 0.89. This p-value indicates that the results fail to reject the null hypothesis because this is much larger than the standard significance level of $p \leq 0.05$. Thus, there was no significant statistical difference between conditions. More importantly, there was no evidence for the alternative hypothesis which stated that the threading technique improved percent viability over the impaction technique.

| | Viability of Threaded Plugs | Viability of Impacted Plugs |
|---|---|---|
| 1 | 59.56 % | 63.50 % |
| 2 | 96.73 % | 83.43 % |
| 3 | 80.54 % | 84.41 % |
| Average | 78.94 % | 77.11 % |
| Standard Deviation | 18. 64 % | 11.80 % |
| Standard Error | 10.76 % | 6. 81 % |

**Table 2:** Summary of raw cell count data collected from the threaded and impacted conditions

## Discussion

The conducted experimentation aimed to demonstrate the proposed delivery system's ability to reduce the amount of compressive loads applied to the cartilage during allograft delivery and to test whether this would indeed minimize chondrocyte death after delivery. While the results of the live/dead assay of chondrocytes on the impaction and threaded conditions were insignificant, it is important to highlight the implications of successful allograft delivery through the proposed threaded mechanism.

Current allograft delivery procedures that rely on impaction for proper delivery of the allograft have been shown to lose up to 21% of viable chondrocytes in the superficial 500 μm layer one hour after implantation [3]. The cylindrical allografts used in this study were 15 mm in diameter and required approximately 10 consecutive impacts, each generating an average 2.4 kN load, for delivery. Furthermore, the articular cartilage experienced a total loss of 47% of chondrocytes in the same superficial layer 48 hours after the procedure with observed chondrocyte death in deeper layers. This increase in cell death was noted as a result of induced apoptotic pathways from the forces applied to the cartilage [14]-[16]. Hence, it is essential to minimize the application of compressive loads to the articular cartilage during graft delivery to avoid triggering these apoptotic pathways. The proposed delivery system proved to accomplish this by eliminating the need for large compressive loads to insert the graft into the recipient site. Rather, the threads on the allograft allowed for manual insertion into the delivery site through the application of relatively small torsional forces. Given previous and ongoing research, the reduction of force applied directly to the articular cartilage surface should in theory reduce loss of chondrocytes through necrosis as well as apoptotic pathways.

The results of the live/dead assay did not show any statistical difference in chondrocyte viability between delivery through impacted and threaded allografts. Nevertheless, the study done by Cook et al. [9] highlights the relevant significance of the threaded delivery system's potential for increasing the success rate of osteochondral allograft transplants. Since the proposed delivery system does not require the application of detrimental compressive mechanical loads to the cartilage surface, it shows promise for increasing the percent viability

immediately after implantation. It is true that our impaction condition did not replicate similar levels of chondrocyte death as in the study done by Borazjani et al [3], but this can be attributed to our inability to experiment on a human condyle and many sources of error. Furthermore, it is worth noting that the threading method did not fare any worse than the impaction method (*Table 2, Figure 11*). Although the impaction method is fairly straightforward and repeatable, the threading technique was crude and with simple modifications could be greatly improved.

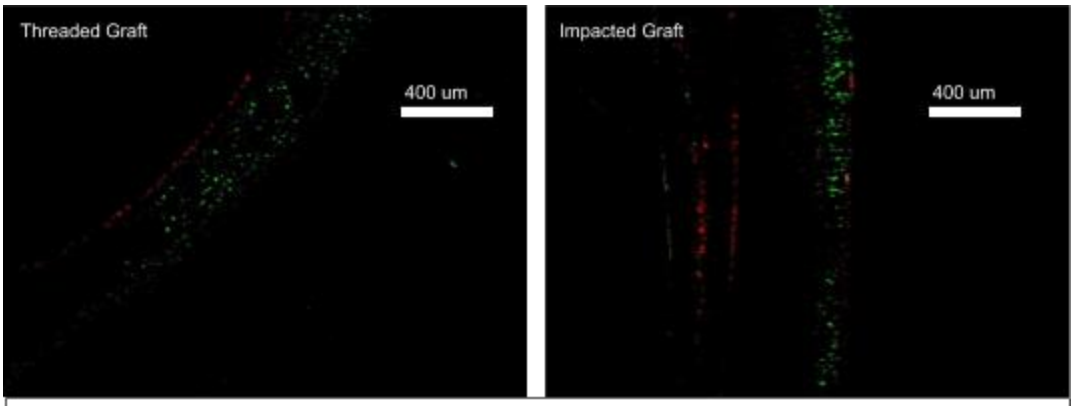


**Figure 11:** The left photo shows live/dead, 4X images of a threaded condition, and the left shows live/dead for an impacted condition

***Sources of error and Proposed Alterations***

None of the procedures performed in our experiments were performed in ideal testing conditions. Many alterations to the current procedure can be executed in future testing. All testing was done in the Engineering Centers Building on the UW-Madison campus. Most of the testing was performed in the tissue engineering teaching lab (Biosafety level 1) with the mechanical work done in the COE Student Shop area. The shop tables utilized are often used for greasy metal work and operation of other industrial machinery. Both environments were not sterile, and were only sanitized using 70% ethanol. However, all tools used, excluding the single use autograft system, allowed for repeated sterilization. Published research surrounding osteochondral allografts is always performed under conditions that meet the standards of an operating room. Future experiments should also be performed in environments compliant with clinical standards to more accurately model these procedures.

According to the PDS (*Appendix I)* all materials used in the procedure should be appropriate for medical use. Many of the tools utilized in this project do not meet medical standards, but have the potential for meeting them. In particular, the hacksaw used in our testing was coated in yellow paint (*Figure 12*). After testing, a substantial amount of the yellow paint had been transferred to the condyles or plugs cut. This is unacceptable and introduces chemicals to the final product. The paint may have affected the cell viability, reacted with the stain, or introduced other forms of error. A medical grade stainless steel oscillating saw would be ideal for future applications.

Another source of error was the vice used for holding the femur and plugs. The vice in the COE Student Shop had large, sharp teeth meant for gripping large metal materials. A smaller, softer, and insulated vice--preferably one from an operating room--would prevent damage to the plugs during threading. The tap and die could also be redesigned for medical use. The tools in

the COE shop have all been repeatedly used for non sterile industrial applications and likely contained contamination. High grade stainless steel tooling would not rust and would be more suitable for tissue. The die should be low profile, as it does not need to cut threads into metal bolts. A low profile die would allow threads to be cut into shallower plugs.

The technique and tooling used in threading and inserting plugs should be improved upon. With practice and testing, the team improved the procedure with the tools available. For example, the threading technique was not even mastered before the final testing. Each of the three plugs was threaded slightly different, until the best method was finally applied to the last plug. In order to maximize efficiency, proper training by an orthopedic surgeon would be beneficial. Published papers regarding osteochondral allografts involve the expertise of senior orthopedic surgeons.

As mentioned before, the osteochondral autograft transfer system used for harvesting the plugs was a single use product. After each extracted plug, the tool became more damaged (Figure *13*). This introduces further error as each plug is extracted with an increasingly damaged tool. For future testing, all of the plugs should be extracted with a freshly opened transfer system or a powered reamer like those used in operating rooms. The latter is the ideal choice for animal testing as the autograft transplant is not designed for harvesting plugs from denser animal condyles. It would also allow for deeper plugs to be harvested, which would simplify the threading process.

Imaging and image analysis can also be improved upon. In our testing, a scalpel was used to cut the cartilage layer off of the plug. We attempted to keep them as similar as we could, but human error can never be overcome. Due to this error, use of a sectioning tool could obtain thinner, more precise slices which could allow for better imaging. Precise section depth would allow one to determine the ideal thickness of cartilage tissue for staining. Our sections were all quite thick, and may have made it difficult for the stain to penetrate. Future experimentation with section thickness and staining should be performed. A different way to solve this issue could be the use of a confocal microscope. Fluorescent microscopes do not have the ability to image different layers of tissue. The use of a confocal would allow the layers of cartilage to be examined. This could allow for a more accurate assessment of chondrocyte viability.

The use of animal models prior to human testing is common practice. Bovine knuckles with intact cartilage were used for the procedures in this report. Porcine models should be used for future work because their bone and cartilage more closely resemble human tissue [15]. While bovine knuckles were quite easy to thread, the same cannot be said about human tissue until proper testing is done. Ideally, *in vivo* animal models should be used for long term testing. Ultimately, human cadavers should be tested to ensure that human bone can withstand the threading process.

The original plan for testing included three control grafts that would be removed using the same methodology except without threading, impaction, or any attempted insertion. Thus, exposing the decrease in cell viability after treatment with an insertion method. However, due to equipment failure, time constraints, and a lack of sites available for adequate graft harvest obtaining these grafts was impossible. The sections were not cultured in MEM-C for one hour, which may have accounted for the observed decrease in chondrocyte viability. Ultimately it was decided to omit these control sections because the lack of culture rendered them incomparable to the other sections. Their percent viability was also significantly decreased compared to the other two conditions, which should not occur if no treatment was imposed on the cartilaginous surface prior to harvest.

Also, only one time point was used due to the limited capabilities of plug extraction. When more plugs can be extracted, more time points can be added with three or more technical replicates. Cell death can occur by necrosis and apoptosis, and the timescale by which cell death will result will vary depending on the its location during the trauma related event i.e. surface or at a significant depth within the tissue [3], and the signals that particular cell receives. Thus, further and later time points are required to provide a greater prediction as to the cell death that is expected to occur in each of the treatment methods in the time following the transplant. Thus, this will give a better inclination as to the success of the graft.

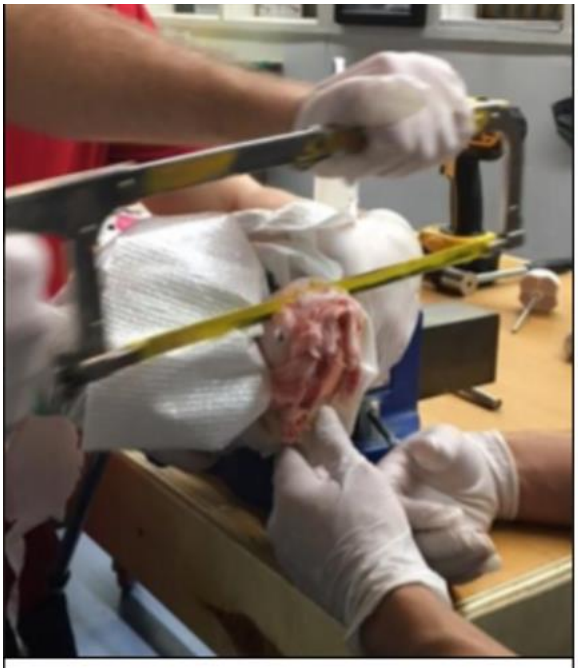

**Figure 12:** Hand saw with yellow paint used to help remove grafts from autograft tool, and remove excess bone on grafts prior to insertion

***Ethical Considerations***

Osteochondral allografts were introduced decades ago and the safety of the procedure is well documented. Multiple clinical studies have shown positive results from fresh osteochondral allograft transplantation [17]. Viral and bacterial infections are rare, but potentially fatal. Prior to the implementation of a screening process, allografts were inserted within 24 hours of harvest and in 2002, the American Association of Immunologists reported 37 bacterial infections associated with allograft procedures. Infection most commonly occurs with donor allografts as bacteria can lie in the donor tissue or bacterial contamination can occur during the procurement and/or processing of condyles. Currently, meticulous screening of the donor is required before the operation. Fresh transplants are no longer used in the USA and the FDA has banned any procedures without proper screening. Most commercial grafts in the USA are used between 15 and 35 days; and are rarely implanted before a 7 to 10-day screening process. Studies have shown good clinical results up to 42 days after harvest although chondrocyte viability decreases with storage time. While the screening process prevents many infectious diseases, clostridium

contamination risk increases with time after donor death [18, 19]. Thus, the screening process must be kept short while remaining effective. In order to keep donor tissue free of disease, there must be emphasis on aseptic harvesting, aseptic processing, and use of antimicrobials at every stage. Safety guidelines established by the American Association of Tissue Banks (AATB) advocate donor screening, serologic, bacterial, and viral testing as well as procurement and storage requirements are required until negative testing results have been received.

A difficulty associated with controlling disease is that sterilizing techniques are often cytotoxic. Every process that the donor tissue undergoes must avoid contamination while also keeping the desired cartilage alive. The procedure is performed to replace damaged cartilage with healthy, mature hyaline cartilage from the donor, so maximizing the cell viability of the donor is important.

Another important aspect of the surgery is the efficacy of the transplant. The patient must report improvements postoperatively and currently osteochondral transplants are effective more often than not. Clinical studies published as far back as 1997 reported an overall success rate as high as 85%. Proper healing in 108/126 knees in 123 patients were successful after a mean check-up time of 7.5 years. The check up times ranged from 2 to 20 years, indicating that these grafts are successful long term. In this case, failure was related ($p \leq 0.05$) with age over 50 years and bone instability. Collapse of the graft by more than 3mm was seen frequently in the failed grafts [20] . This highlights the need for a more stable graft, which may be possible with a threaded technique provided that future testing corroborates this hypothesis. Failure in the osseous portion of the allograft is most common, where subchondral collapse may occur. Some patients also reported low grade pain, which may be attributed to chronic inflammation surrounding the graft. Despite these problems, the International Knee Documentation Committee (IKDC) subjective knee force scores show that the operations are effective overall. On a 0-100 point system, the preoperative mean was 27 and the postoperative mean was 79 [21]. While 100% satisfaction is nearly impossible to attain using threaded techniques may have a positive impact on current procedures. The methods for producing threaded allograft donors have the capability of meeting all standards and can be screened using current techniques. After extensive future testing, threaded techniques may be able to be implemented in clinical studies.

The possible benefits in the operating room of threaded techniques could be greater stability due to the thread engagement, quicker operation times due to threading technique, and overall improved chondrocyte viability because of the lack of impaction required. These benefits must be studied further to have any validity. In addition, MRI techniques are improving, as stated by our client, Dr. Walczak. The possibility of sizing a defect prior to operation is now possible and could prove to be extremely beneficial. This would allow the allograft to be created and threaded prior to surgery. The patient could then be operated upon, the defect drilled out and tapped, and the allograft could be inserted without the defect having to be manually sized. Furthermore, this would decrease procedure length as according to our client, the sizing and depth matching is the most time intensive part of the operation. Creating the plug before the operation would inevitably reduce operating time and limit the open wound exposure to contaminants in the operating room, ultimately increasing the safety of the procedure.

## Conclusions

Current surgical procedure for knee grafts is rudimentary and archaic as it requires brute force to implant living tissue that has poor regenerative abilities. The extent of compressive loads applied to the cartilage throughout the procedure ultimately results in necrotic and apoptotic

chondrocyte death that is detrimental to the overall success of the procedure. A novel technique is required to reduce these compressive forces used for implantation of the grafts that in return increases long-term surgical success. To prevent the loss of chondrocytes after implantation, a new method has been devised that relies on threading the allograft to create a bone screw from donor tissue that can then be implanted into a threaded recipient hole. The delivery of the allograft is accomplished through successful clockwise rotations by hand until it is appropriately flushed. From inspection, delivery through rotational means greatly reduces the amount of compressive loads applied to the cartilage surface. The new delivery system is assessed through live/dead staining to determine if it successfully increased postoperative cell viability. Statistical analysis of cell viability demonstrated no significant difference between the cartilage delivered through threaded allografts and cartilage delivered through impacted allografts. In spite of inconclusive results, this method demonstrated the possibility of creating implantable bone screws and shows promise for healing bone defects through similar means in other parts of the body. Furthermore, bone screws created and used in this study allowed for retrieval of the allograft through counterclockwise retraction. Such a feat was noted to be of particular significance by the client given his emphasis on the current delivery system's inability to retrieve the bone plug without first damaging the articular cartilage surface. The ability to easily retract the allograft is of convenience for the surgeon in the case that the plug is pushed beyond the desired depth. Although there was no significant difference in cell viability between threading and impaction, it is noteworthy that there is much room for improvement on threading techniques. Impaction methods have little room for improvement, yet threading the allografts can easily be improved with proper tools. This provides a positive outlook for the future of threaded allografts, and justification for the continuation of this investigation.

On account of the potential sources of error much future work must be done to test the cell viability in a similar setting to surgical procedure. Extraction tools must be upgraded to those used for surgical allografts instead of tools meant for autografts. Disposable autograft tools deform after use and increase margin of error upon each subsequent use as well as increasing difficulty of extraction of bone plugs. Furthermore, these tools are designed for human bone and receive damage when being used on cow bone with a significantly higher density, as well as almost five times the fracture stress capabilities [22]. This means that while threading bone works in a bovine model, it may not work in human bone due to the differences in density and allowable stresses. Additionally, there are distinct differences in chondrocytes isolated from large animal models compared to those derived from human articular cartilage [23]. These differences demonstrate that a more accurate testing of chondrocytes would use porcine chondrocytes instead of bovine chondrocytes, due to the similarities of cartilage-specific matrix expression and proliferation rate [17]. Furthermore, testing must be done to determine cell viability at multiple time points as well as cell viability at multiple standardized depths of cartilage. These tests would allow for determination of long-term effects of threading when compared to impaction. Impaction is known to trigger apoptotic pathways that take several days to have a significant impact [14, 15, 16] and the possibility that threading could prevent these apoptotic pathways from being triggered would result in comparatively higher long-term cell viability. Future work is required before disregarding a threading procedure and much work will be done in the near future.

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# Appendix

## *I. PDS*

*Osteochondral Graft Delivery System Product Design Specifications*

**Team:** Eduardo Enriquez, Chrissy Kujawa, Rodrigo Umanzor, Robert Weishar, Nicholas Zacharias

**Date:** December 14, 2016

**Function:** Osteochondral allografting is a common procedure performed on patients that require replacement of diseased bone. Current methods of implantation require the application of mechanical forces that have a detrimental effect on the live chondrocytes present on the implant. Maximizing the amount of viable tissue during and after the surgery is a crucial factor for the success of the procedure. Hence, the client requests a delivery system that will reduce the amount of mechanical forces required to securely place the implant into the donor site.

**Client Requirements:**

- Budget: $300
- Donor tissue must be placed into donor site with less force required from surgeons during current methods
- Must securely fit into donor site while keeping chondrocyte cell viability >70% during and after the procedure
- Delivery system must only require the use of sterile tools available in a surgery room, or must be made to to fit surgical standards

**Design Requirements:**

- *Performance Requirements:*
    - Application of bone graft should result in approximately >70% viability of chondrocytes on donor graft
    - Procedure must be simple enough to be done in operating room, within the time period of a surgery that takes about 5 hours
    - Bone graft and vice must be held to lie perpendicular so screws on bone graft remain straight
    - Device must be capable of decreasing the force used to insert the bone graft
    - Forces exerted on the bone by the device should not cause any bone chipping, or fragmentation
- *Safety:*
    - Device should not increase chances of infection, increase chances of graft dislocation, decrease chances of surgical success, or create complications post-op.
    - Our device has failed if, postoperatively, the graft does not exhibit proper integration into the native tissue, if the hyaline cartilage is not properly maintained, if an associated cartilage disorder develops, or if significant fissuring, fibrillation, or fibrous tissue infiltration occurs
- *Accuracy and Reliability:*

    - The device should have a success rate that exceeds that of current devices (75-80% success rate)
    - The device should also allow for successful graft implantation, meaning it makes the procedure able to maintain >70% chondrocyte viability, with no greater risk for post-op complications than what already exists
- *Life in Service*:
    - Expensive or specialized components should be reusable, and easily manufactured components should be one time use. Both should be inherently sterilizable. Length of time to be determined with materials chosen
    - If plastic or biodegradable materials are included in the design, then these components may be one-use only
- *Shelf Life:*
    - The device should be capable of storage at room temperature for 9 months unless sterility is compromised before then. No corrosion should be observed on the device during its life of service and must be compliant with hospital regulations
- *Operating Environment:*
    - Product has to be sterile while in use
    - Should operate in temperatures typical of an operating room (20-23 C), with humidity of 20-60%
    - All pieces will have to withstand the forces exerted on them during operation (tension, rotational, shear, and compression).
    - Must be able to be used in conjunction with other orthopedic tools, including supports, water, bone glue...etc.
    - Must be able to be operated by an orthopedic surgeon
- *Ergonomics:*
    - Device should be able to be used easily by surgeon without damage during operation. Forces placed on the cancellous bone of the graft should not exceed 6.6 MPa from torsional stress, 3-20 MPa in tension, and 1.5-50 in compression [24]. Cortical bone is stronger than cancellous, so force limitations inherently include the cancellous bone. The forces applied to the articular cartilage should not exceed those at which there is <70% viability.
- *Size:*
    - Device will be sized appropriately based on the size of the defect.
    - Range of 5mm-20mm diameter for threading device
    - Depth of at least 10 mm
- *Weight:*
    - Device should weigh 5 lbs or less to be able to be comfortably hand-operated
- *Materials:*
    - Materials should comply with medical standards set out by the FDA [22, 25, 26]
    - Reusable materials must be sterilizable
- *Aesthetics, Appearance, and Finish:*
    - No color or aesthetics
    - Function over form

**Production Characteristics:**

- *Quantity:*

    - 1 final product, preferably multiple prototypes for testing
- *Target Product Cost:*
    - TBD
- *Standards and Specifications:*
    - Implanted allograft should be in compliance with the FDA regulations under Section 361 of the Public Health Service Act as monitored by the Tissue Reference Group [24]. All surgical tools should comply with the code of federal regulations under Title 21 with the FDA [25].

**Characteristics:**

- Device should consist of a tap, die, and a bone screwdriver and vices
- Must be made of surgery-grade material
- Various devices to stabilize tools will be necessary
- *Patient-related concerns:*
    - Completed bone graft must not cause pain
    - Allergies, immune response hemocompatibility, and biocompatibility
    - Device should not release unwanted fragments of bone
    - Required surgery with device should not be more invasive than current procedures
- *Customer:*
    - Orthopedic surgeons implanting an osteochondral graft

## *II. Materials and Expenses*

| Item | Part Number | Supplier | Amount | Cost | Description | Notes |
|---|---|---|---|---|---|---|
| Cow Knuckles | order #20461 | Conscious Carnivore | Two | $37.03 | Cow Knuckles to model the medial and lateral condyles of the femur | Determine possibilities of design ideas as well as forces generated on cartilage and difficulties of working with bone |
| Cordless Drill and drill bits | N/A | Student Shop | | $0 | Drill used on cow femurs to test threading capabilities | Multiple drill bits used with sized tap/die sets. |
| Taps and Dies | N/A | Student Shop | | $0 | Tap/Dies used to thread drilled hole and donor graft | Size 10/1.5 mm |
| Hand Saw | N/A | Student Shop | | $0 | Used to remove section of cow knuckle | |
| Autograft Tool Set | REF AR - 1S81 - 10S | Dr. Walczak | | $0 | Use of reamers to remove donor graft from cow knuckle | Included harvesters, bone lube, impaction, and measuring tools |
| Fresh Cow Knuckles | N/A | Underground Butcher | Two | $9 | Fresh cow knuckles purchased to extract plugs containing live chondrocytes | Rodrigo paid for this |
| 2 uM Calcein AM/4 uM ethidium homodimer | 1/8 Kit | Dr. Puccinelli | 10 mL | $45 | Live/Dead stains were purchased to assess the viability of our bone plugs | We will have to reimburse the BME department for this via a check from our client, it must be submitted to Susan Sauer (#2124 ECB) |
| Uber Ride | N/A | Uber | 1 | $7.24 | Uber was used to return to campus from Butcher after buying cow knuckles | Rodrigo paid for this |
| Poster | Semi-Gloss Instant [ 190 g/m2 42 | Student Print | 1 | $29.54 | | Rodrigo paid for this |
| | | | | | TOTAL: $127.81 | |

## *III. Graft Retrieval and Threading Protocol*

### Preparations Before Threading Procedure (not for viability testing):

Materials:

- Large plastic bin
- Kitchen knife
- 1 cow knuckle
- Hand saw
- Plastic bag

Procedure:

1. Two bone samples were obtained from a butchery and stored in a kitchen freezer
2. 24 hours prior to testing, one of the knuckles was removed from the freezer, placed in a plastic bin and maintained at room temperature
3. After the sample was thawed for 24 hours, excess fat was removed from the surrounding bone using a kitchen knife and a hand saw was used to remove the ligaments of the knee
4. The sample was then placed in a plastic bag and transported for further testing

.

### Threading Protocol

Materials:

- 1 cow knuckle
- 1 rubber mallet
- Drill
- Q size drill bit
- 10mm x 1.5 tap and die
- 70% Ethanol
- Osteochondral autograft donor plug retriever

Procedure:

1. On a relatively flat surface on the knuckle, a drill with a Q sized bit was used to ream a 10 mm hole
2. Bone shavings and all other residue was removed to clear out the cylindrical hole that is meant to serve as the recipient site
3. The tap was used to thread the recipient site
4. On another flat surface, to retrieve a bone plug the autograft tool was sized (10 mm in diameter and 20 mm in height) forced into the bone. Initially, a vertical axial force was applied to initiate the process. Once the device was ~ 1 mm into the bone, a rubber mallet was used to force the remaining 19 mm into the bone
5. Once the device was 20 mm into the bone, a great amount of force was used to extract the device (now containing the desired plug) out of the bone or the hand saw was used to slice perpendicular to the tool, underneath the graft

6. The bone plug was removed from the device
7. The plug was threaded
8. Now the threaded plug could be placed into its recipient site with ease

## *IV. Image Analysis Protocol*

### Protocol for Creating Stacked Images

Materials:

- Images of tissue named in order by tissue sample and picture number, specifying FITC or TRITC channel
- ImageJ

Procedure:

1. Open two stacks of images on ImageJ, one of FITC and one of TRITC for one condition (i.e. threaded condition)
2. Process - Enhance Contrast - Equalize histogram + process all 17 slices - OK
3. Process - Subtract Background - Rolling ball radius: 30 pixels - OK
4. Image - Color - Merge Channels - Red: TRITC, Green : FITC - save as .tif composite
5. Image - Color - Split Channels
6. (For each channel) Image - Adjust - Brightness/Contrast. Put Contrast almost all the way up, and adjust brightness until you see only cells and remove most auto-fluorescence across slices.
7. Image - Color - Merge Channels - Red: TRITC, Green: FITC - save as .tif composite

### Protocol for Calculating Cell Viability

1. Using ImageJ, press File-Import-Image Sequence -(Choose Image Sequence) - (Name either FITC or TRITC to separate filters) - make it 8 bit greyscale
2. Once you have image sequence, go to Process - Subtract Background - (Rolling Ball 100)
3. Go to Image - Adjust - Threshold - Stack Histogram - (Adjust until you see no background on layers with the most cells) - Don't press apply, just exit from the threshold screen
4. Process - Binary - Make Binary
5. Process - Binary - Watershed
6. Analyze Particles - Size (Pixel^2): 0-15.00 - (All other settings the same)
7. Copy and paste 'summary' info into an excel sheet

## *V. Image Analysis Data*

**Raw Data from ImageJ**

| Slice | Count | Area | Av. Size | IntDen | Slice | Count | Area | Av. Size | Int Den. |
|---|---|---|---|---|---|---|---|---|---|
| TL1_1 FITC | 88 | 249 | 2.83 | 721.534 | TL1_1 TRITC | 47 | 140 | 2.979 | 759.574 |
| TL1_2 FITC | 13 | 36 | 2.769 | 706.154 | TL1_2TRITC | 27 | 43 | 1.593 | 406.111 |
| TL2_1 FITC | 124 | 464 | 3.742 | 954.194 | TL2_1 TRITC | 14 | 14 | 1 | 255 |
| TL2_2 FITC | 142 | 655 | 4.613 | 1176.232 | TL2_2 TRITC | 13 | 13 | 1 | 255 |
| TL3_1 FITC | 69 | 249 | 3.609 | 920.217 | TL3_1 TRITC | 48 | 119 | 2.479 | 632.188 |
| TL3_2 FITC | 223 | 953 | 4.274 | 1089.753 | TL3_2 TRITC | 29 | 45 | 1.552 | 395.69 |
| TR1_1 FITC | 40 | 159 | 3.975 | 1013.625 | TR1_1 TRITC | 23 | 25 | 1.087 | 277.174 |
| TR1_2 FITC | 149 | 683 | 4.584 | 1168.893 | TR1_2 TRITC | 149 | 683 | 4.584 | 1168.893 |
| TR2_1 FITC | 239 | 866 | 3.623 | 923.975 | TR2_1 TRITC | 26 | 43 | 1.654 | 421.731 |
| TR3_1 FITC | 224 | 945 | 4.219 | 1075.781 | TR3_1 TRITC | 29 | 67 | 2.31 | 589.138 |
| TR3_2 FITC | 177 | 854 | 4.825 | 1230.339 | TR3_2 TRITC | 24 | 37 | 1.542 | 393.125 |
| TR3_3 FITC | 320 | 1267 | 3.959 | 1009.641 | TR3_3 TRITC | 87 | 260 | 2.989 | 762.069 |
| | | | | | | | | | |
| I1L_1 FITC | 331 | 825 | 2.492 | 635.574 | IL1_1 TRITC | 91 | 293 | 3.22 | 821.044 |
| I2L_1 FITC | 213 | 482 | 2.263 | 577.042 | IL1_2 TRITC | 160 | 539 | 3.369 | 859.031 |
| IL1_2 FITC | 238 | 692 | 2.908 | 741.429 | IL1_3 TRITC | 145 | 636 | 4.386 | 1118.483 |
| IL1_3 FITC | 308 | 604 | 1.961 | 500.065 | IL2_1 TRITC | 49 | 108 | 2.204 | 562.041 |
| IL2_2 FITC | 144 | 327 | 2.271 | 579.062 | IL2_2 TRITC | 44 | 68 | 1.545 | 394.091 |
| IL2_3 FITC | 12 | 12 | 1 | 255 | IL2_3 TRITC | 99 | 382 | 3.859 | 983.939 |
| IL3_1 FITC | 243 | 822 | 3.383 | 862.593 | IL3_1 TRITC | 96 | 282 | 2.938 | 749.062 |
| IL3_2 FITC | 451 | 1511 | 3.35 | 854.335 | IL3_2 TRITC | 106 | 343 | 3.236 | 825.142 |
| IL3_3 FITC | 418 | 1520 | 3.636 | 927.273 | IL3_3 TRITC | 110 | 359 | 3.264 | 832.227 |
| IR1_1 FITC | 16 | 47 | 2.938 | 749.062 | IR1_1 TRITC | 43 | 147 | 3.419 | 871.744 |
| IR1_2 FITC | 5 | 5 | 1 | 255 | IR1_2 TRITC | 28 | 73 | 2.607 | 664.821 |
| IR1_3 FITC | 6 | 6 | 1 | 255 | IR1_3 TRITC | 27 | 82 | 3.037 | 774.444 |
| IR2_1 FITC | 72 | 181 | 2.514 | 641.042 | IR2_1 TRITC | 9 | 9 | 1 | 255 |
| IR2_2 FITC | 142 | 330 | 2.324 | 592.606 | IR2_2 TRITC | 10 | 10 | 1 | 255 |
| IR3_1 FITC | 161 | 879 | 5.46 | 1392.205 | IR3_1 TRITC | 11 | 12 | 1.091 | 278.182 |
| IR3_2 FITC | 266 | 1036 | 3.895 | 993.158 | IR3_2 TRITC | 35 | 71 | 2.029 | 517.286 |
| IR3_3 FITC | 151 | 528 | 3.497 | 891.656 | IR3_3 TRITC | 24 | 34 | 1.417 | 361.25 |
| | | | | | | | | | |
| C1_1 FITC | 97 | 448 | 4.619 | 1177.732 | C1_1 TRITC | 263 | 493 | 1.875 | 478.004 |
| C1_2 FITC | 47 | 106 | 2.255 | 575.106 | C1_2 TRITC | 56 | 58 | 1.036 | 264.107 |
| C1_3 FITC | 95 | 586 | 6.168 | 1572.947 | C1_3 TRITC | 296 | 803 | 2.713 | 691.774 |
| C2_1 FITC | 30 | 59 | 1.967 | 501.5 | C2_1 TRITC | 80 | 81 | 1.012 | 258.188 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| C2_2 FITC | 32 | 79 | 2.469 | 629.531 | C2_2 TRITC | 18 | 18 | 1 | 255 |
| C2_3 FITC | 9 | 14 | 1.556 | 396.667 | C2_3 TRITC | 16 | 16 | 1 | 255 |
| C3_1 FITC | 20 | 75 | 3.75 | 956.25 | C3_1 TRITC | 575 | 2274 | 3.955 | 1008.47 |
| C3_2 FITC | 13 | 28 | 2.154 | 549.231 | C3_2 TRITC | 541 | 2162 | 3.996 | 1019.057 |
| C3_3 FITC | 9 | 12 | 1.333 | 340 | C3_3 TRITC | 307 | 775 | 2.524 | 643.73 |

## Statistical Analysis Data

| Total Cells | Percent Viable | AVG Percent | Stdv | Standard Error |
|---|---|---|---|---|
| 135 | 65.18518519 | T1 Avg: | | |
| 27 | 48.14814815 | 59.55908289 | | |
| 124 | 100 | | | |
| 142 | 100 | T2 Avg: | | |
| 117 | 58.97435897 | 96.72955975 | | |
| 252 | 88.49206349 | | | |
| 63 | 63.49206349 | | | |
| 298 | 50 | T3 Avg: | | |
| 265 | 90.18867925 | 80.5375504 | | |
| 253 | 88.53754941 | | | |
| 201 | 88.05970149 | Total Avg: | | |
| 407 | 78.62407862 | 78.94206435 | 18.6365305 | 10.7598059 |
| | | | | |
| 422 | 78.43601896 | I1 Avg: | | |
| 373 | 57.10455764 | 63.49254125 | | |
| 383 | 62.14099217 | | | |
| 357 | 86.2745098 | I2 Avg: | | |
| 188 | 76.59574468 | 83.42507558 | | |
| 99 | 12.12121212 | | | |
| 339 | 71.68141593 | I3 Avg: | | |
| 557 | 80.96947935 | 84.41256154 | | |
| 528 | 79.16666667 | | | |
| 59 | 27.11864407 | | | |
| 28 | 17.85714286 | | | |
| 27 | 22.22222222 | | | |
| 72 | 100 | | | |
| 142 | 100 | | | |
| 161 | 100 | | | |
| 301 | 88.37209302 | Total Avg: | | |

| | | | | |
|---|---|---|---|---|
| 175 | 86.28571429 | 77.11005946 | 11.80344796 | 6.814723857 |
| | | | | |
| 360 | 26.94444444 | C1 Avg: | | |
| 103 | 45.63106796 | 32.2907292 | | |
| 391 | 24.29667519 | | | |
| 110 | 27.27272727 | C2 Avg: | | |
| 50 | 64 | 42.42424242 | | |
| 25 | 36 | | | |
| 595 | 3.361344538 | C3 Avg: | | |
| 554 | 2.346570397 | 2.8520054 | | |
| 316 | 2.848101266 | Total Avg: | 20.55597436 | 11.86799733 |
| | | 25.85565901 | | |

| | % Viable Cells (Threaded) | % Viable Cells (Impaction) | % Viable Cells (Control) |
|---|---|---|---|
| | 59.55908289 | 63.49254125 | 32.29 |
| | 96.72955975 | 83.42507558 | 42.42 |
| | 80.5375504 | 84.41256154 | 2.85 |
| | | | |
| T-Test (Threaded vs. Impaction) | 0.893777874 | | |