INTRODUCTION
The anterior cruciate ligament (ACL) is the major structural ligament located at the front of the knee. It prevents the tibia from sliding out in front of the femur and provides rotational stability for the knee. Injuries to the ACL are amongst the most common in orthopaedic medicine, with approximately 200,000 cases of reported ACL injuries in the United States each year [1].

Since the ACL is typically not a self-healing tissue, major tears and injuries are often treated with complete ACL reconstruction. Reconstruction is a procedure in which the injured tissue is removed from the knee, and replaced with tissue grafts from elsewhere in the patient’s body or from a cadaver donor. While this procedure is traditional, it also leads to several long-term complications. After 10-30% of ACL reconstructions, there is a high risk of either reinjuring the reconstructed ligament, or injuring the ligament in the opposite knee due to the increased load it bears to compensate for the healing knee [2]. Additionally, there is little evidence that ACL reconstruction helps prevent the osteoarthritis that is associated with ACL rupture [3].

In order to address these complications, current research has shifted its focus to healing injured ACLs instead of replacing them. The Musculoskeletal Research Center (MSRC) at the University of Pittsburgh has designed a magnesium-based ring device that can aid in the healing process. The ring, placed around the injury site on the ACL and held in place by screws on the upper and lower legs, bears load in the knee while the injured ligament is healing and is coated with biological agents that assist the healing. Over time, as the ligament heals and bears more load on its own, the magnesium ring degrades and is reabsorbed harmlessly into the body [4].

Magnesium is a favorable material choice for the ring device due to its mechanical properties, biocompatibility, and biodegradability [5]. In a preliminary study run the MSRC, it was found that goat ACL fibroblasts cultured in extracts of AZ31, (the Mg material used to make the initial ring device) had “high viability.” This reading was hypothesized to be the result of one of two scenarios; magnesium was increasing the cell proliferation of the fibroblasts or magnesium was increasing the cellular output of collagen. A higher proliferation rate would translate to a faster healing time in the ACL. Likewise, more collagen production would allow for better remodeling of the injured ACL.

OBJECTIVE
The objective of this study is to determine how extracts of various dilutions (0%, 6%, 12.5%, 25%, 50%, and 100%) of AZ31 magnesium alloy affect the cell proliferation of goat ACL fibroblasts in an in vitro cell culture.

HYPOTHESIS
Based on a study performed by Wei et al. which used magnesium to enhance bone healing and a study performed by Sternberg et al which found that certain concentrations of magnesium increased the proliferation of human coronary artery endothelial cells, it was hypothesized that magnesium extracts diluted to 50% or less of the total cell media would increase the cell proliferation in goat ACL fibroblasts [6, 7]. It was also hypothesized that higher concentrations of magnesium would be cytotoxic and kill the fibroblasts, likely changes in pH caused reactions of magnesium.

METHODS
To test the effect of various magnesium concentrations on the proliferation of goat ACL fibroblasts, magnesium extracts needed to be first prepared according to ISO standard 10993 (“Biological Evaluation of Medical Devices Part 1: Evaluation and Testing”). Using AZ31, a an allow of magnesium, aluminum, and zinc, cylinders were machined with a 5mm diameter and 5mm thickness, and then polished, cleaned, and sterilized using UV radiation. These cylinders were dissolved in α-MEM cell culture media. The extract solutions (100% extract) were serially-diluted in α-MEM to also produce solutions of 50%, 25%, 12.5%, and 6% Mg.

Goat ACL fibroblasts were seeded, 5000 cells/well, in a 96-well plate and incubated for 24 hours with 200µL α-MEM to allow for cell attachment. After attachment, the cell culture media was removed and replaced with the 200µL Mg extract dilutions. In addition to the experimental samples, a control of cells cultured in 0% Mg (strictly α-MEM) was included. Each concentration was used in 3-5 wells to allow for a better representation of the data. The cells were then incubated for 72 hours before the cell proliferation assay.

The cell proliferation assay (Click-iT EdU, Molecular Probes, Inc., Eugene, OR) will was performed using the manufacturer’s protocol. After 72 hours of incubation, the EdU label was added to each well. The label, an analog of thymidine incorporates into newly synthesized DNA when it is formed during proliferation. The rest of the assay was performed 24
hours after adding the label. A microplate reader (Glomax, Promega., Madison, WI) was used to measure the fluorescence of green light (585 nm) emitted by antibodies that had found the label added the day before. The microplate reader assigned a relative fluorescence number (RFU) to each well, allowing for quantification of the amount of proliferation that occurred. Each data point was compared to the control of cells cultured in 0% Mg. A positive response was one in which the proliferation of goat ACL fibroblasts increased compared to the control. A negative response was one in which the proliferation was decreased. A neutral response was one which showed little difference in proliferation compared to the control.

RESULTS

The reported RFU values are the mean of 10 assays for each concentration of magnesium extract. Results were not obtained for the cells cultured in 100% extract because of a limitation in the available materials. Due the small sample size, no statistical analysis was performed on the data. The data represents a trend that was initially observed during preliminary experimentation.

| Table 1: RFU of goat ACL fibroblasts cultured in AZ31 extracts |
|-----------------|-----|-----|-----|-----|-----|
|                 | 0%  | 6%  | 12.5% | 25%  | 50%  |
| Mean RFU (n=10) | 26731 | 36056 | 32590 | 36551 | 28022 |

As seen in Figure 1, cells cultured in 6%, 12.5% and 25% Mg extract had substantially higher RFU than the control (0% Mg), indicating that these concentrations of extract elicited a positive proliferative response. Cells cultured in 50% Mg extract did not show a substantial deviance in RFU compared to the control. Thus, 50% Mg extract elicited a neutral response, causing insignificant changes in the proliferation of the goat ACL fibroblasts.

Figure 1: RFU of goat ACL fibroblasts cultured in various dilutions of AZ31 extract. Data points indicate the average RFU of 10 samples at each concentration. The dashed line represents the baseline RFU of the control (0% Mg).

DISCUSSION

The preliminary results partially support the initial hypothesis. As expected, proliferation was increased in cells cultured in 6%, 12.5%, and 25% Mg extract. However, cells cultured in 50% Mg extract did not have a positive proliferative response as expected, and instead showed similar amounts of similar proliferation to the control. Higher concentrations of magnesium were not tested, so their cytotoxicity was neither confirmed nor disputed.

The initial data suggest that there is range of Mg extract concentrations that promotes proliferation in goat ACL fibroblasts. Further testing with the AZ31 alloy must be completed to obtain statistically significant data. With this data, a well-defined range of extract concentrations that elicits a positive response can be determined and further investigated for clinical applications.

One significant limitation in this study is the translation of its results back to the clinical application. The in vivo conditions of the Mg ring used on a healing ACL are difficult to replicate accurately in vitro. It is difficult to know the exact concentration of Mg that fibroblasts in the ACL will experience when the ring is introduced.

CONCLUSION

In future studies, various parameter changes in the procedure will be explored. Incubation periods of 24 hours and 48 hours will be tested and compared to those of 72 hours to examine how the results of each incubation time differ. Additionally, various magnesium materials, such as single-crystal Mg and additional Mg alloys will be tested. Ultimately, the results of all of these studies will be used to further understand and improve the Mg ring design, and choose the best one for clinical applications.

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REFERENCES