INTRODUCTION
Peripheral nerve injuries occur in 1.64 percent of trauma patients.¹ The injuries cause a loss of motor and sensory function in the nervous tissue. The peripheral nervous system consists of all the nerves outside of the brain and spinal cord. Despite how widespread this type of injury is in trauma patients, only about 50% of them recover function in their injured nervous tissue.²³

The current clinical gold standard of treatment for this peripheral nerve repair is the nerve autograft. The autograft is limited by the available nerve tissue for the transplant, and there are complications associated with the procedure.⁴ There are commercially available nerve guides but these fail to heal nerve tissue with the injury greater than 2.5 centimeters wide.⁵ The Adipose Stem Cell Center has developed a polymer nerve conduit that targets the wider nerve gaps.⁶ The polymer conduits have shown evidence of their ability to heal these gaps in nerve tissue.⁷

An adipose-derived stem cell-suspension in keratin hydrogel can be injected into the lumen of the nerve conduit may be a biomaterial to provide support to the regenerating peripheral nerve. Keratin must be able to form a gel at a concentration that maintains its gel state in physiological conditions; 37 degree Celsius and within the ionic environment of extracellular fluid.¹¹

The gelation procedure for keratin gel was optimized, and adipose stem cells was seeded on the gel to test cell compatibility. This leads to results that indicate whether keratin gel is biocompatible or not.

OBJECTIVE
The objectives of this research are to first establish a keratin protein extraction protocol. The second objective is to determine if keratin can be used to form a gel in physiological conditions. The third objective is to determine whether adipose stem cells will survive in the gel.

HYPOTHESIS/SUCCESS CRITERIA
An adipose-derived stem cell-suspension in keratin hydrogel that can be injected into the lumen of a nerve conduit may be a biomaterial to provide adequate support to a regenerating peripheral nerve. One of the success criteria is that keratin protein forms a gel in physiological conditions. The other is that adipose stem cells can survive in the gel as indicated by a cell viability assay.

METHOD
Keratin was extracted from human hair and lyophilized into a powder. A Labconco Free Zone 2.5, Kansas City, MO freeze dryer was used. Lyophilized keratin was dissolved in phosphate buffered saline (PBS) at room temperature. Concentrations of 20%, 22% and 25% by weight percentage of keratin protein are compared for time to gelation and ability to maintain structure when submerged in PBS at 37°C. To test whether the keratin gel was fully formed, the vial in which it was made was inverted. If the gel is formed it maintains its form at the bottom of the vial when upside-down.

Keratin gel and collagen gel were plated separately on glass-bottomed dishes at a plating density of 5,000 cells per square centimeter. ASCs were seeded on top of the gels. The ASC media consisted of DMEM and serum as well as antifungal and antibacterial agents. The ASC media was replaced every 2 or 3 days for 7 days. On day 7 the ASCs were labeled with calcein AM (stains living cells) and ethD-1 (stains dead cells). These were imaged using fluorescence microscopy and cell viability assessed. The microscope used was the Zeiss Axiovert 25, Jena, Germany.

ASCs were seeded on top of keratin gel or seeded directly in a well plate as a positive control at normal cell culture conditions. The cells were proliferated for 4 days without a media change. On day 4 the ASCs were labeled with calcein AM and ethD-1. Fluorescence was quantified in a Tecan Infinite M200 Pro plate reader, Maennedorf, Switzerland. The percentage of live and dead cells was calculated for both experimental groups (Equations 1 and 2), having already subtracted the noise from keratin autofluorescence that was determined with a negative control. A two-tailed, independent t-test was used to determine statistical difference between experimental groups.

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\% \text{ Live Cells} = \frac{100 \times (F(530)_{\text{experimental}} - F(530)_{\text{min}})}{(F(530)_{\text{max}} - F(530)_{\text{min}})} \\
\% \text{ Dead Cells} = \frac{100 \times (F(645)_{\text{experimental}} - F(645)_{\text{min}})}{(F(645)_{\text{max}} - F(645)_{\text{min}})}
\]

RESULTS
A 25% lyophilized keratin solution in PBS was studied due to its optimal gelation properties at physiological conditions highlighted in Figure 1. The 25% solution formed a gel within 30 minutes at both room temperature and at 37°C. The two solutions at lower concentrations did not form a gel at all. Temperature within this range does not seem to affect gelation time.

Fluorescent microscopy indicated qualitatively that ASCs are viable within keratin gel over 7 days. The majority of the fluorescence in the keratin experimental group was green, indicating live staining with calcein AM. These results are shown in Figure 1.

Figure 1. A) ASCs in keratin. B) ASCs in collagen.
Fluorescence was quantified after a four-day experiment of cells grown on keratin gel. No significant difference was observed in the percentage of live cells on keratin compared to live cells grown directly on the well plate \( (p= 0.1528, \alpha=0.005) \). There is a significant increase in the percentage of dead cells in keratin compared to the percentage of dead cells grown directly on the well plate \( (p= 0.0002158, \alpha=0.001) \). These results are shown in Figure 2.

**DISCUSSION**

Once the keratin gelation procedure was optimized, it was used in all of the experiments to determine how the substance affects the cells plated within. It is important to choose the gel that shows the correct properties at the lowest concentration to minimize issues with biocompatibility. The other concentrations tested did not gel, and concentrations higher than 25% may or may not have gelled at physiological conditions, indicating that this 25% gel is optimal.

The mechanical properties of the gel have not been quantified, but qualitative analysis gives evidence that this is an ideal gel to apply to nervous tissue regeneration. The gel is at a viscosity (even in its gelled state) that it can be injected into a nerve conduit with a syringe or 1000 µL pipet, yet remains inside the conduit once placed.

A next step would be to do a quantitative degradation study that models the convective and diffusive forces of an in vivo environment. Quantitative rheological testing is a logical next step, as the ideal consistency for a hydrogel will allow the nervous tissue to grow through the conduit rather than act as a barrier to motion.

In the qualitative study testing cell viability within keratin hydrogel, ASCs survive for at least 7 days. It would be ideal to culture the cells longer, but the ASC media starts to dissolve the gel and refreshing the media becomes impossible without disturbing the cells plated in it.

The importance of the qualitative study of cell viability in the gel was the development of a method for visualizing the cells within the keratin gel. Glass dishes are ideal for imaging as the fluorescence is not distorted. The process of imaging cells within keratin gel was not optimized before this study. Seeding the cells on top of the gel allow for visualization when stained fluorescently. If the cells are seeded within the gel visibility is limited with the normally vibrant fluorescent staining.

The quantification of cell viability gave positive results, as the keratin was not toxic to the ASCs since there is no difference in the number of live cells in keratin compared to no keratin. There were significantly more dead cells in the keratin compared to the collagen group, and this may be because dead cells were not rinsed immediately from the keratin when they were plated. This experiment shows the importance of determining a method of changing media for cells that are seeded on keratin gel because in this experiment it was not changed to not disturb the gel and allow for accurate results. Further experimentation using an MTT assay for cell metabolism would determine whether cells are more active in co-culture than if grown separately.

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**REFERENCES**