The affect of altering calcium ion concentrations in alginate scaffolds on chondrocyte phenotype

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For our experiment, we decided to investigate what affect differing calcium ion concentrations in alginate scaffolds would have on chondrocyte culture phenotype. We created alginate scaffolds using two different calcium concentrations: 50 and 1000 mM. We used FMC Biopolymer, Protanal LF 10/60 as our alginate type. We chose it for its high G/M acid ratio, 70/30.

**Background**

 Chondrocytes are differentiated cartilage cells. However, chrondrocytes are prone to dedifferentiate into fibroblasts. When cultured in monolayer, cells lose their chondrocytic phenotype, and change their expression from chondrocyte-specific genes to that of genes that are normally expressed in fibroblasts. For example, Collagen II is only expressed in the differentiated chondrocyte cell phenotype, and therefore is a good marker to distinguish between the two cell phenotypes.

In order to mediate this problem, chondrocytes are grown in 3-D cultures. Alginate scaffolds are often used. Alginate is a seaweed derived polysaccharide which is composed of M and G acids. G-block polymer chains can be crosslinked by calcium ions, providing the gel its stability. As such, high concentrations of calcium ions would increase the number of crosslinks and the stiffness of the gel, while low concentrations would make the gel less stiff. We hypothesized that because chondrocytes lose their phenotype in monolayer, increased calcium concentrations would support the differentiated phenotype as calcium ions help form the 3-D gel.

**Results**

*Live/dead fluorescence assay*

After one week, we used a live/dead fluorescence assay to test cell viability of our alginate constructs. (Figure 3) Live cells show up green while dead cells show up red. We removed three alginate beads from each sample for observation. We saw clusters of live cells from the 50 mM [Ca ion] sample. However, we saw no cells in the 1000 mM [Ca ion] sample. We saw no dead cells, and live cells were very small and circular.

*Cell count*

We counted cells after roughly 1 week’s growth. For the 50 mM [Ca ion] sample, we got a concentration of 1.6 x 10^5 cells/mL while the 1000 mM [Ca ion] sample had a concentration of 7.8 x 10^5 cells/mL.

*RT-PCR*

Collagen II is a useful marker to differentiate between the differentiated and dedifferentiated phenotypes, while Collagen I should be constant. The alginate beads were removed from the medium and liquefied using EDTA. After isolating RNA from the cell lysates, we measured the OD260 and OD280 of our samples diluted 1:100. For our 50 mM sample, we got .019/.018(A260/A280) and for our 1000 mM sample, we got .013/.015. Using the OD260 values, we calculated a final RNA concentration of 76 µg/mL for the 50 mM sample and 52 µg/mL for the 1000 mM sample. However, the ratio of A260/A280 was roughly one, which indicates that our sample was not pure RNA (ratios >2 indicate purity). Knowing this, we decided to target for 300 ng of RNA for our RT-PCR reaction targeting collagen I and II.

*Gel of RT-PCR products*

We then ran an agarose gel of our PCR products (Figure 1). Lane 1 was a 100 bp ladder, lane 2 was our 50 mM sample targeting collagen I, lane 3 was our 1000 mM sample targeting collagen I, lane 4 was our 50 mM sample targeting collagen II, and lane 5 was our 1000 mM sample targeting collagen II. GAPDH was used as an internal control as levels should be constant. First band in lanes 2 and 3 is Collagen I, while the first band in lanes 4 and 5 is Collagen II. Second band of each lane if GAPDH.

Our gel showed a significant increase the concentration of GAPDH in samples targeting collagen II versus samples targeting collagen I. As such, it is difficult to draw any strong conclusions from the gel. Even so, we attempted to analyze the data. We used ImageJ, a program provided by NIH, to measure band intensities from the gel. Values were normalized by dividing by GAPDH band values. Bands for collagen II from both our samples were ~1, with no significant difference between the two.

*ELISA*

We used ELISA as a second method for determining relative concentrations of Collagen I and II. The reaction involves a calorimetric reaction which turns the sample yellow in relation to the amount of protein present. Along with duplicates of our cell lysates, we loaded PBS as a control, and duplicate protein samples at known concentrations in order to create a standard. We then read the plates at 420 nM. Background values (PBS samples) were first subtracted from the data. Using the standards, we graphed OD values vs. protein concentrations to create a trendline and attempted to match our sample OD values with their protein concentrations. For our standards, we got a linear trendline with R squared values of roughly 0.9. (Figure 2)

For Collagen I, we got background corrected OD420 values of 0.045 for our 50 mM [Ca] sample and 0.002 for our 1000 mM [Ca] sample. This gave us a Collagen I concentration of ~0.05 µg/mL for our 50 mM sample and ~0 for our 1000mM sample. For Collagen II, we got OD420 values of .002 for the 50mM sample and .04 for the 1000 mM sample. This gave us a Collagen II concentration of ~0 for the 50mM sample, and ~0.03 for the 1000mM sample. This increase in Collagen II concentration for our 1000mM sample seems to indicate that scaffolds created with a higher concentration of Ca ions help maintain the chondrocyte phenotype better than scaffolds created at a lower concentration of Ca ions.

**Discussion**

From our ELISA data, it seems as if scaffolds made with higher Ca concentrations help maintain the chondrocyte phenotype. However, there are many discrepancies in our experiments that should be noted when thinking of the validity of this result.

When we first checked our cells on our alginate beads using the live/dead assay, we observed no cells on our 1000 mM sample, but we saw clusters on our 50mM sample. This led us to believe that the 1000mM scaffold was too stiff and not porous enough to allow cell proliferation, and that our cells weren’t able to grow on the scaffold surface. However, we were able to get Collagen I and II values from our lysates, suggesting that there were cells on the beads for our 1000 mM sample. While it is possible that we unluckily picked the three beads on which no cells grew, while all the other beads had cells on their surface, it is unlikely.

The gel of our RT-PCR products is also unusual. GAPDH, our internal control, was supposed to remain constant for all our samples. However, we got an increase in GAPDH levels for our PCR products targeting Collagen II. This specificity led us to believe that this was a result of human error during the PCR reaction, which was the only step in which Collagen I and II samples were separated. Due to this control error, we were not able to draw any conclusive results from the gel. Our ELISA results were also questionable. Collagen I was not supposed to be affected by our experiment, and as such, the difference in Collagen I concentrations between our two samples sheds doubt on any conclusions we can draw from ELISA. Also, our values were below our standards and close to background, which didn’t give us any significant values to work with. The small values were probably due to a low cell count in our samples.

**Conclusion**

Overall, we were able to show that scaffolds made with differing calcium concentrations can have an effect on chondrocyte phenotype. However, due to errors in our experiments, we are unable to conclusively say that higher calcium concentrations, and therefore a stiffer scaffold help maintain chondrocyte phenotype. Further experimentation is needed to support this claim. However, we have shown that calcium concentrations should be taken into account when making alginate scaffolds for cartilage tissue engineering.