PROCESSING OF COLLAGEN GELS USING NON-ENZYMATIC GLYCATION

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INTRODUCTION

Collagen gels have been widely used as scaffolds for tissue engineering [1-3]. A variety of techniques have been to control the properties of collagen gels. Glutaraldehyde and thermal processing have been used to crosslink scaffolds and thus increase the mechanical properties of the gels [4]. Non-enzymatic glycation, the process by which sugars act as a reducing agent to crosslink proteins such as collagen, has been studied primarily as a phenomena related to diabetes [5]. Studies examining the effects of non-enzymatic glycation on native tissues have shown a change in the mechanical and biochemical properties of the tissues [6]. However, due to their cytotoxicity, these methods cannot be used on seeded gels.

The use of glycation in tissue engineering by incubating collagen scaffolds with reducing sugars for several days has been shown to improve the mechanical properties of tissue and has also been shown to decrease the rate of degradation of the scaffold [7,8]. This study documents the use of two different methods of non-enzymatic glycation to condition collagen gels, and the effects of these processes on cell-mediated gel contraction.

METHODS

Cell culture

NIH 3T3 fibroblasts were cultured in DMEM, 1% antibiotics and 10% FBS. Cells were passaged twice per week at 70% confluency using trypsin-EDTA.

Post Glycated Gels

Acid soluble Type I rat tail collagen (SIGMA) was solublized in 0.1% acetic acid at a concentration of 3mg/mL. Gels were mixed with PBS to obtain a final concentration of 1.5 mg/mL collagen and then 300µL of 1M NaOH with 10x DMEM was added to form gels. 750µL of the solution was placed in wells of a 24 well plate and were incubated at 37°C for one hour. The gels were then incubated with various concentrations of ribose solutions for 5 days.

Pre-Glycated Collagen Gels

Acid soluble Type I rat tail collagen (SIGMA) was solublized in .1% acetic acid at a concentration of 3mg/mL. The collagen was then mixed with various concentrations of ribose in .1% acetic acid. The collagen/ribose solutions were then incubated at 4°C for 5 days, then mixed with 300µL 1M NaOH and 10x DMEM. 750µL of the solution was pipetted into wells in a 24 well plate and incubated at 37°C for half an hour. Gels were washed 3 times with PBS and then 1mL of DMEM with 10% FBS and 1% antibiotics were then placed over each gel. After 24 hours the gels were washed and used for contraction or fluorescence.

Contraction studies

Gels were washed with PBS and allowed to equilibrate with media overnight. The gels were then seeded with 100µL of a solution of 5million cells per mL of media. Cells were incubated for 2 hrs to allow for attachment and then 1mL of media was added to each well. The gels were then detached from the bottom and sides of the wells with a pipette tip, and the gels were transferred to 6 well plates. Pictures were taken of the gels at different time points with NIH image and then the area at each time point was measured with NIH image to determine change in area.

Fluorescence

Fluorescence measurements were taken to quantify the formation advanced glycation endproduct (AGE) [9]. Fluorescence was measured with. The collagen gels were digested with .0025% papain buffer in PBS and fluorescence was measured at 360/440 nm with a Perkin Elmer Bioassay Reader HTS 7000.

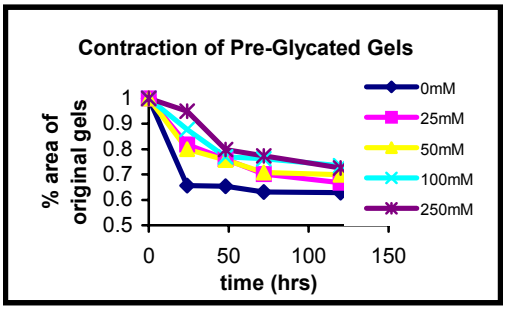
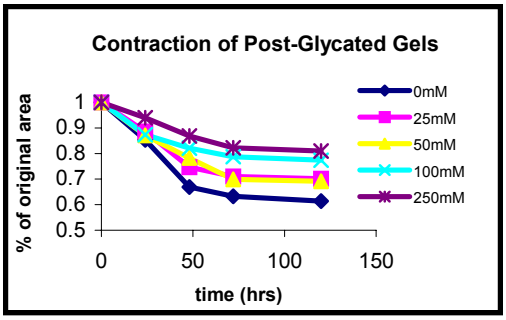


Figure 1

RESULTS

Gels that were pre-glycated with 250mM ribose, when seeded with fibroblasts, contracted to about 72% of their original

Figure 2



area, while the gels that were cultured with 0mM ribose contracted to about 65% of their original area (Figure 1).

This complements the contraction that is seen in gels that are post glycated (Figure 2). The post glycated gels cultured with 0mM ribose also contract to about 65% of their original area, while the gels cultured in 250mM ribose contracted to about only 85% of their original area (Figure 2).

Flourescence measurements of the digested gels showed an accumulation of AGE's (Figure 3). The gels that were pre-glycated with ribose showed higher fluorescence per microgram of collagen, while the gels that were post-glycated had lower fluorescence throughout (Figure 3).

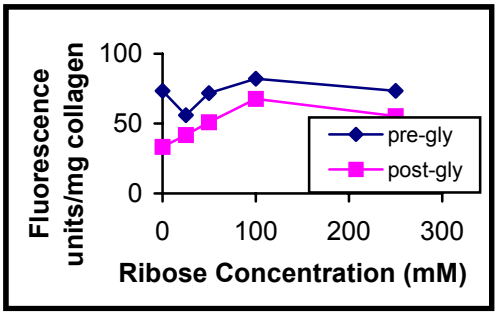


Figure 3

DISCUSSION

This study shows two different methods of glycation, one where the collagen is exposed to ribose in solution under acidic conditions, and one where the gel is exposed to ribose at physiologic temperatures. Using low temperatures and a low pH to condition the collagen with ribose drives the intermediates of the glycation reaction and allows for a buildup of reversible amadori products, which may lead to an increase in AGE's and crosslinks in the collagen after gel formation. Using this method to glycate the collagen also keeps the viscosity of the solution low, which makes it a useful method of conditioning for injection techniques and injection molding techniques. The advantages to pre-glycating the collagen include the ability to culture cells without exposing them to high osmolarities as well as improving cell delivery techniques for tissue engineering, because it allows cells to be incorporated into the gels while the collagen is still in solution.

The contraction data in this study correlates with other studies that show similar results [10,11]. This data also shows that the degree of glycation in not necessarily indicative of contraction inhibition (Figure 4). At the same concentrations of ribose, depending on the conditions of exposure to ribose the inhibition of contraction is different.

It is interesting to note that in this study we found that the rates of contraction are not the same for the various concentrations of glycation solutions. At lower levels of glycation, the contraction was almost complete at 24 hours while the contraction equilibrium when using higher ribose concentrations took longer. This may be due to cells' sensing differences in the material and mechanical properties of the gels and altering their behavior. We also see a difference in the rates of contraction between the post and pre-glycated gels at 0mM which may be due to the difference in culture temperature and processing. It is thought that temperature may play an important role in the crosslinking and fibril formation of collagen, and that physiologic temperatures are conducive to fibril formation [12].

The florescence of the gels show that the pre-glycated gels have a higher fluorescence indicating a higher accumulation of AGE's vs. the post-glycated gels, but it is important to realize that the accumulation of AGE's may not be linked to inhibition of contraction. The increase in AGE's may show an increase in the amount of crosslinks that form in the gels, however it remains unclear whether these crosslinks are inter vs. intra fibrillar crosslinks. It is conceivable that intrafibrillar crosslinks are forming in the pre-glycated gels, while the collagen is in solution, and these crosslinks prevent as many fibrils being formed during gellation.

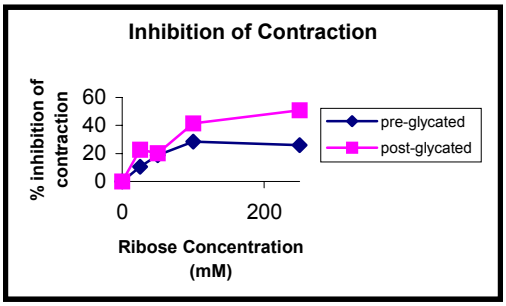


Figure 4

The use of pre-glycated collagen may be important for tissue engineering techniques in that it provides a method to condition scaffolds that is not toxic to cells. Previous methods to glycated collagen gels or sponges have included agents such as glutaraldehyde, which makes it impossible to culture cells and crosslink the collagen at the same time. These methods of glycating collagen gels are also important as they serve as a way of altering the mechanical properties of the gels, while studying the cell behavior in reaction to differences in the mechanics and biochemical nature of the extracellular matrix.

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