EFFECTS OF SIMULATED MICROGRAVITY ON NITRIC OXIDE PRODUCTION AND PROTEOGLYCAN SYNTHESIS BY CHONDROCYTES ENCAPSULATED IN 3D PEG HYDROGELS

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INTRODUCTION

Adaptation to microgravity during spaceflight can adversely affect daily joint function of astronauts on their return to Earth's gravity (Marshall et al., 2005). However, the basic mechanisms underlying orthostatic intolerance are not fully understood. Recent evidence points to an up-regulation of nitric oxide (NO) production in vascular and avascular tissues (Ma et al., 2003). NO, an intra- and inter-cellular molecule, has been found to alter chondrocyte response and adversely affect cartilage maintenance and tissue growth (Bryant et al., 2004) .

The NASA Rotating Wall Vessel bioreactor (RWV) is a model system used for culturing cells and tissues in conditions that emulate certain aspects of spaceflight. Although this device does not simulate microgravity, it produces a culture environment that creates a multidirectional gravitational force. Previous studies have shown that cartilage tissue cultured in the RWV has similar responses to microgravity conditions in spaceflight (Marshall et al., 2005). Therefore, this system may be used to elucidate intracellular signaling pathways involved in cartilage degradation due to spaceflight.

To isolate chondrocyte response within simulated microgravity culture, a neutral poly(ethylene glycol) (PEG) based hydrogel was chosen as a model for 3D chondrocyte culture. Hydrogels are crosslinked networks where the crosslinking density (ρ_x) can be controlled via changes in the gel processing conditions to tailor the equilibrium water content and the diffusion of nutrients within the gel structure. This study aimed to elucidate the pathways involved in chondrocyte response under simulated microgravity culture and as a function of gel crosslinking density. Chondrocyte response was measured by nitric oxide and glycosaminoglycan (GAG) production within the RWV and compared to cultures under normal gravity within spinner flasks (SF) and free swelling (FS) conditions.

Material and Methods

Bovine articular chondrocytes were isolated from full depth cartilage slices of the metacarpal-phalangeal joint (front feet) of 2-3 year old adult steers within hours after slaughter (Bartow Meats, Georgia, USA). Hydrogels were fabricated by dissolving the poly(ethylene glycol) dimethacrylate (3000 MW, PEGDM) macromer in phosphate buffered saline (PBS) (pH 7.4) or deionized-H2O at concentrations of 10 or 20% (w/w) with the photoinitiator (1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one) at a final concentration of 0.05% (w/w) or 0.0125% (w/w), respectively. Isolated chondrocytes were suspended in the prehydrogel solution at a concentration of $50x10^6$ cells/mL and exposed to 365 nm light (~2 mW/cm^2) for 10 min to encapsulate chondrocytes within PEG hydrogels. Cylindrical hydrogels (5 mm in diameter and 3 mm in height) were fabricated.

Several rotating wall vessel (Synthecon, Houston, TX) reactors with a 10 mL capacity High Aspect Ratio Vessel were used. A maximum of 4 hydrogel scaffolds were placed in each vessel to prevent aggregation of the gels and improve movement of samples within the RWV. A spinner flask was used as a control for normal gravity. A constant media/construct ratio of 2.5 and an average rotational speed of 22 rpm was used for the RVW and the spinner flask. Free swelling cultures were used as static controls. The chondrocyte seeded PEG gels were incubated at 37^oC in 5% CO_2 for 2 and 5 days of culture time.

Cell constructs for each crosslinking density (total sample size/experiment=10) in the RWV were lyophilized, digested for 16 hours at 60°C in an enzymatic solution containing papain. Total nitric oxide (nitrate + nitrite), was measured in the post-loading digests using the Colorimetric Total Nitric Oxide Assay Kit (Cayman Chemicals). Hoeschst 33258 (Polysciences, Inc.) fluorescence assay was used to quantify total DNA content. Total GAG content was quantified using the dimethylmethylene blue dye method. The amount of NO in digests, DNA and GAG content was normalized with respect to DNA content in each construct.

Results and Discussion

An increase in PEGDM macromer concentration from 10 to 20 wt% led to a decrease in the overall equilibrium water content and lower mesh sizes. The mesh size directly affects the diffusion of nutrients through the gel [4].

Table 1. PEG hydrogel properties.

PEGDM %	Mesh size, nm	% Equilibrium Water
10	14.9 ± 1.0	92 ± 0.03
20	7.1 ± 0.2	83 ± 0.04

A simple ANOVA analysis was used to compare the effects of culture condition and crosslinking density to cell response. Total nitric oxide (NOx) production was assessed by measuring the stable end products, nitrite and nitrate. NO production by chondrocytes encapsulated in the 10% PEGDM gels was similar in the RWV and SF, but was significantly higher in the free swelling conditions ($p<0.001$, Figure 1). A similar trend was seen for the 20% PEGDM. Interestingly, at early 2 day culture

times NOx was higher with increased gel crosslinking density in the RWV and spinner flask. However, with increased culture time, no significant differences were observed for total NOx at increasing ρ_x.

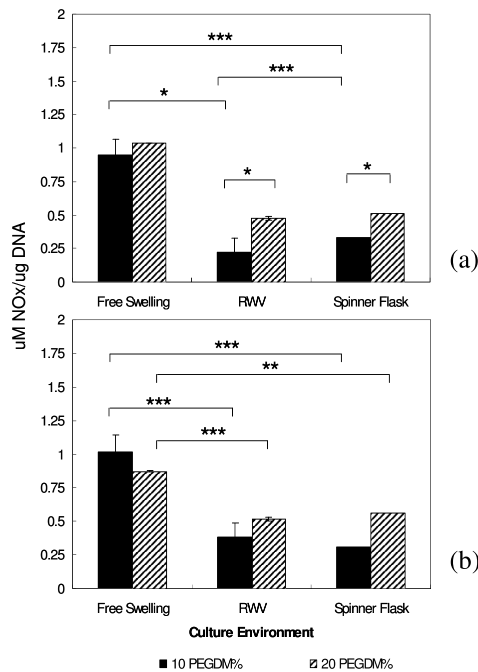

Figure 1. *Total NOx production by chondrocytes encapsulated in 10 or 20% PEGDM gels and cultured under free swelling, simulated microgravity in a rotating wall vessel or within a spinner flask for 2 days (a) or 5 days (b). * p<0.05; ** p<0.01; ***p<0.001.*

GAG production was significantly higher in the free swelling conditions compared to the RWV and SF cultures. In the RWV and SF cultures, GAG production increased with increasing culture time for the 10 PEGDM % gels (Figure 2). With a higher crosslinked gel, GAG production increased significantly in the RWV with longer culture time. For each bioreactor environment and at each culture time, there were no significant differences with increasing crosslinking density.

Interestingly, at short culture times, NO production was shown to increase the amount of PG produced in the RWV and SF suggesting that simulated microgravity within the RWV may impact cell response during early culture times. One of the predominant factors contributing to this response is the induced fluid flow in both SF and RWV culture. However, during the 5 day culture period, decreased NO production and decreased PG synthesis was observed compared to static, free swelling cultures. Finally, ρ_x appears to have little affect on PG production under the three culture environments.

CONCLUSION

In summary, this study demonstrates that different culture environments can lead to differences in chondrocyte response. During early culture times, simulated microgravity has little affect on NO and PG production. Longer culture times may be needed to elucidate the chondrocyte pathways involved in microgravity.

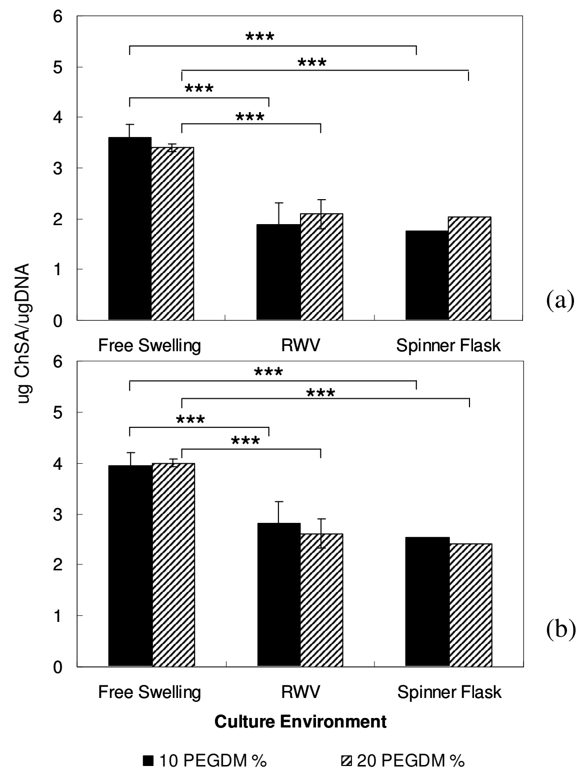

Figure 2. *PG production by chondrocytes encapsulated in 10 or 20% PEGDM gels and cultured under free swelling, simulated microgravity in a rotating wall vessel or within a spinner flask for 2 days (a) or 5 days (b). * p<0.05; ** p<0.01; ***p<0.001.*

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