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Effects of Initial Cell Seeding Density for the Tissue Engineering of the Temporomandibular Joint Disc

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Abstract—Tissue engineering may provide a better treatment modality for postoperative discectomy patients. The TMJ disc is an ideal candidate for tissue engineering approaches because of its lack of an intrinsic regenerative ability. Unfortunately, basic knowledge related to TMJ disc tissue engineering is still at an infancy level and not on par to that related to articular cartilage tissue engineering. The objective of this study was to examine the effects of initial cell density of TMJ disc cells seeded in nonwoven poly-glycolic acid (PGA) scaffolds on the biochemical and biomechanical properties of constructs examined at 0, 3, and 6 weeks after seeding. Low, medium, and high seeding densities were chosen to be 15, 30, and 120 million cells per ml of scaffold, which were seeded using a spinner flask. Significant differences were found temporally and as a function of seeding density in morphology, total collagen, GAG content, and permeability of the constructs, but not in aggregate modulus. The high seeding density group outperformed the low and medium groups in collagen and GAG content at all time points measured. The high-density group produced a total of $55.37 \pm 3.56\ \mu$g of collagen per construct, maintained $15.77 \pm 1.86\ \mu$g of GAG per construct, and only shrunk to 50% of the original scaffold size. Permeability of the constructs at 6 weeks was decreased by 70% compared to 0 weeks.

Keywords—TMJ disc, Cell seeding density, Biochemistry.

INTRODUCTION

The temporomandibular joint (TMJ) disc is a specialized fibrocartilaginous tissue located between the mandibular condyle and the glenoid fossa-articular eminence of the temporal bone.[21,39] The TMJ disc probably functions to increase the contact area between the two joint surfaces, thus reducing stress, and to absorb shock. The TMJ disc also divides the joint into separate compartments for different movements in the joint.[28] Removal of the TMJ disc is sometimes a preferred treatment modality for severe degenerative TMJ disorders. When the disc becomes an obstacle in TMJ rotational and translatory movements, often resulting in internal derangement with concomitant degeneration, surgeons have no choice but to perform a discectomy.[15,22,25,26] However, disc removal is a drastic operation exhibiting a debatable success rate.[20,27]

The lack of an intrinsic regenerative ability in the TMJ disc renders it a suitable candidate for tissue engineering approaches. A strategic tissue engineering approach is needed for the *in vitro* regeneration of the TMJ disc utilizing appropriate animal models.[15] The porcine animal model has been identified as a suitable model for the human TMJ disc.[8,11,43] Tissue engineering approaches for the porcine TMJ disc should consist of detailed characterization studies of the native tissue and thorough cell culture studies.

A hurdle in the design of strategic tissue engineering attempts for the TMJ disc is the lack of a comprehensive understanding of its native biochemical and biomechanical properties. However, many recent characterization studies have shed some insight into the TMJ disc. The two major biochemical components of the TMJ disc are collagen and glycosaminoglycans (GAG).[1] GAGs have been the most characterized biochemical component of the TMJ disc, yet there is still controversy on total content and distribution. The total GAG content of the TMJ disc has been observed to vary between 0.6 and 10% by dry weight.[3,5,37,38,41] In contrast, collagen content, which is the major constituent of the TMJ disc, has not been as thoroughly investigated as GAG content.[1] Reports in the literature are scarce and not always consistent. Collagen content has been reported to be 58% by volume,[10] or about 37% by wet weight,[23] or 68%[3] to 83%[38] by dry weight. It has been well established in the literature that type I collagen is the most abundant collagen type found in the TMJ disc by far.[12,31,33–35]

The cells in the TMJ disc responsible for this matrix production have been referred to as fibrochondrocytes[9,31] or as a mixture of chondrocyte-like cells and fibroblasts cells.[1,15] Chondrocyte-like cells are distributed heterogeneously throughout the TMJ disc.[33,34] In the porcine TMJ disc, the majority of cells appear fibroblastic in phenotype with the ratio of fibroblasts to chondrocyte-like cells approximately four to one.[19]

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In terms of mechanical properties, there have been few studies examining the compressive or tensile behavior of the TMJ disc.[6,7,16,40,44,45] On the particular animal pig model examined in the current study, the TMJ disc aggregate modulus ranges from 16 to 29 kPa,[29] while its tensile moduli are approximately 20 MPa.[16] The disparity between compressive and tensile moduli suggests that the TMJ disc is better suited to resist tensile loads than compressive loads.

Successful tissue engineered constructs should possess similar biochemical and biomechanical properties to the native tissue. There have been few tissue engineering studies focused on the TMJ disc, and only four studies thus far have used TMJ disc cells.[2,17,42,46] However, recent attempts have identified a potential scaffold, nonwoven PGA, for TMJ disc tissue engineering using a porcine animal model.[2] A spinner flask method of seeding was identified as an appropriate method of seeding TMJ disc cells into nonwoven PGA meshes.

The objective of the current study was to examine the effects of seeding density of TMJ disc cells in nonwoven PGA scaffolds on the biochemical and biomechanical properties of constructs as a function of culture time. Cells at three densities, 15, 30, and 120 million cells per ml of scaffold, were allowed to seed on the scaffolds for 1 week using a spinner flask. Biosynthesis was assessed by measuring total collagen and GAG content at Weeks 0, 3, and 6 post-seeding. Mechanical properties were measured by a creep indentation test at Weeks 0 and 6.

MATERIALS AND METHODS

Cell Isolation

TMJ disc cells were obtained from discs harvested from P.I.C. Genetic Breed female hogs weighing 70–80 kg, procured from a local abattoir. TMJ discs were obtained by removing each TMJ *en bloc* unopened and placing it in 100% ethanol. The block was then allowed to air dry under sterile conditions in a laminar flow hood, and subsequently scrubbed with a sterile iodine pad. Then the capsule was breached and the disc was removed and minced with a scalpel. Each disc was digested in 40 ml of 2.5 mg/ml of collagenase (Type 2, Worthington Biochemical, Lakewood, NJ). Cells were passaged twice to obtain the necessary cell numbers required for seeding the scaffolds. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX™ (Biowhittaker, East Rutherford, NJ). Each 500 ml bottle of DMEM was supplemented with 50 ml of fetal bovine serum (Biowhittaker, East Rutherford, NJ), 5 ml Penicillin–Streptomycin–Fungizone (Biowhittaker, East Rutherford, NJ), 5 mL nonessential amino acids (Life Technologies, Carlsbad, CA), and 25 μg/mL L-ascorbic acid (Sigma, St. Louis, MO) in 5 ml of PBS, all added to 500 ml of the culture medium.

Cell Seeding

TMJ disc cells were seeded into scaffolds that were 2-mm thick ellipses with 7- and 5-mm major and minor axes, respectively, were obtained by cutting a nonwoven PGA mesh sheet (Albany International, Albany, NY). According to the manufacturer the PGA mesh was 45–55% crystalline and 95% porous. The scaffolds were seeded at 15, 30, and 120 million cells per ml of scaffold using a spinner flask corresponding to a low, medium, and high cellular concentration, as previously described.[2] Briefly, following spinner flasks autoclaving, the PGA scaffolds were placed onto a thin wire, and the spinner flasks and scaffolds were sterilized with ethylene oxide and aired for 1 day. One day prior to seeding, the scaffolds were wetted with sterile-filtered ethanol, followed by two washes with PBS, and then left overnight in 200 ml of culture medium at 37°C. Cells were then added to the spinner flasks in a total volume of 250 ml of culture medium, and stirred at 90 RPMs. Stirring was stopped after 5 days, and cells were given an additional 2 days in the spinner flasks to allow them to adhere to the scaffolds.

Assays

Constructs were assessed by gross morphology, cell, proliferation, and ECM synthesis at 0, 3, 6 weeks. Biomechanically, they were evaluated at 0 and 6 weeks. As explained earlier, we refer to $t = 0$ as the time when the constructs were removed from spinner flasks and placed in the well plates. Analysis of proliferation and biosynthesis was performed as previously described.[2] Briefly, constructs were lyophilized for 2 days and then digested overnight in 1.5 ml of papain at 60°C. Proliferation was then assessed by picogreen assay (Molecular Probes; Eugene, OR) for measuring DNA. A conversion factor of 7.7 pg DNA/cell for TMJ disc cells was reported previously.[3] A dimethylmethylene blue (DMMB) dye-binding kit was used to detect glycosaminoglycan (GAG) content with the use of a chondroitin sulfate standard (Biocolor; Newtonabbey, Northern Ireland). Collagen content was measured with a modified hydroxyproline assay[2] using a collagen standard (Accurate Chemical and Scientific Corporation; Westbury, NY).

Mechanical integrity was evaluated under compression, as previously described for the native TMJ disc.[29] Briefly, a constant feedback-controlled compressive force of 0.5 g was applied to hydrated constructs until equilibrium was reached, with maximum time of 45 min. A porous 1.66 mm diameter indenter tip was used to apply the creep indentation. The linear biphasic theory with a numerical solver was used to calculate aggregate modulus, shear modulus, Poisson's ratio, and permeability of the engineered constructs.[4,29,30,36]

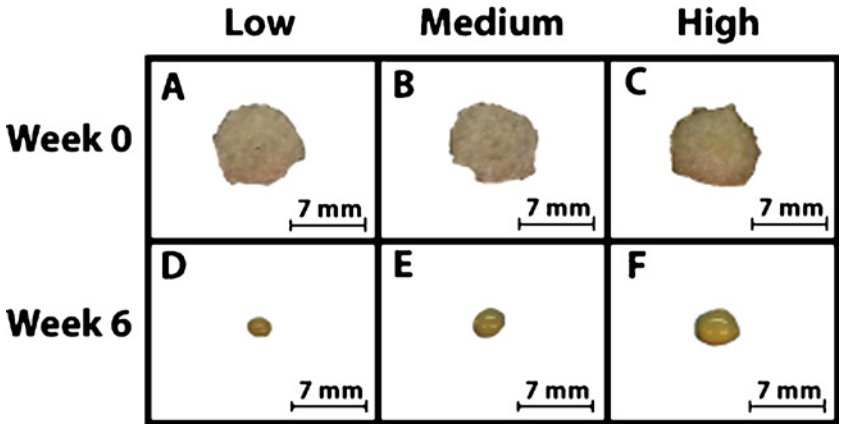

FIGURE 1. Macroscopic view of constructs from the low, medium, and high seeding groups at Weeks 0 and 6. (A) Low seeding density construct, Week 0. (B) Medium seeding density construct, Week 0. (C) High seeding density construct, Week 0. (D) Low seeding density construct, Week 6. (E) Medium seeding density construct, Week 6. (F) High seeding density construct, Week 6.

Statistical Analysis

All data were compiled and presented as mean ± standard deviation. Statistical analysis was performed with an analysis of variance (ANOVA), and when significance was detected with ANOVA ($p < 0.05$), comparisons among groups were made with a Fisher's Protected Least Significance Difference post hoc test. A single-factor ANOVA with $n = 6$ (biochemical tests) or $n = 4$ (biomechanical test) was performed. A two-factor ANOVA was also performed for group and time to observe temporal differences.

RESULTS

Gross Morphology

At the Week 0 time point all three groups appeared grossly the same [Fig. 1(A)–(C)], although the high seeding density group appeared to be a bit more opaque. These Week 0 groups were different at the microscopic level (Fig. 2). All groups were uniformly seeded but the number of cellular nodules was different. The low group had a fair number of nodules distributed throughout the construct [Fig. 2(A)], while the medium group possessed a higher number of nodules [Fig. 2(B)]. The high-density constructs, which were the most densely packed, with cells, had the appearance of large continuous cellular nodules [Fig. 2(C)].

There was a marked difference in macroscopic appearance of the three groups at Week 6 [Fig. 1(D)–(F)]. The low group contracted to about 20% of the original scaffold size in terms of length of the long axis [Fig. 1(D)], the medium to about 30% [Fig. 1(E)], and the high group to about 50% [Fig. 1(F)]. Constructs from all groups were much more opaque than at Week 0, also exhibiting a pronounced glistening at the surface of the construct. At the microscopic level there were no observable differences between groups.

Cellularity

There was a significant decrease in the number of cells contained in each construct over the culture period (Fig. 3), as previously observed.[17,18] The constructs had a higher cellularity at Week 0 than the rest of the time points ($p < 0.0001$), and there were more cells in the constructs at Week 3 than Week 6 ($p < 0.0001$). The high seeding density group possessed more cells than the medium and low groups at every time point measured ($p < 0.0001$), and the medium seeding density group had a higher cellularity

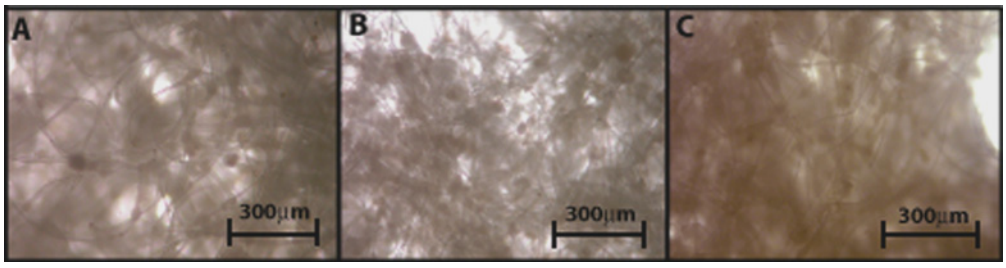

FIGURE 2. Microscopic view of constructs from the low, medium, and high seeding groups at Week 0. (A) Low seeding density construct, Week 0. (B) Medium seeding density construct, Week 0. (C) High seeding density construct, Week 0.

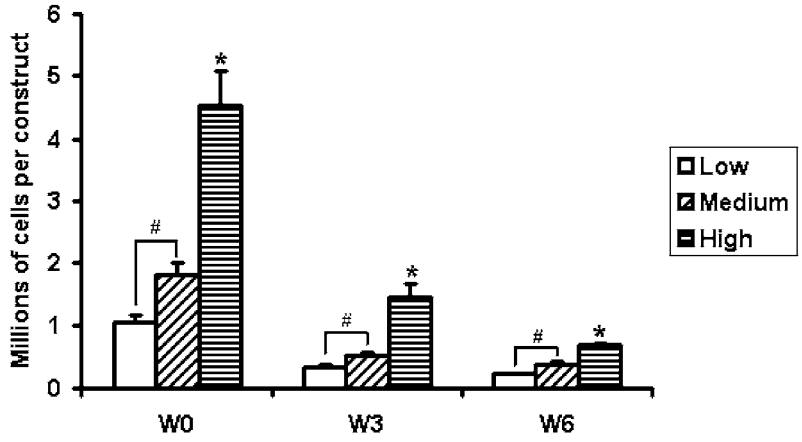

FIGURE 3. Cellular content of constructs from the low, medium, and high seeding groups at Weeks 0, 3, and 6. Symbols* and # indicate significance ($p < 0.05$) between the seeding density groups at each time point. Error bars show standard deviation ($n = 6$).

than the low group at every time point ($p < 0.05$). The seeding efficiencies for the low and medium groups were about 100%, while the high-density group had an efficiency of only about 60%. The high-density constructs started at 4.55 ± 0.53 million cells per construct at Week 0, the medium at 1.83 ± 0.18 million cells per construct, and the low-density constructs at 1.03 ± 0.13 million cells per construct. At the Week 6 time point the high density constructs ended at 0.70 ± 0.03 million cells per construct, the medium at 0.39 ± 0.03 million cells per construct, and the low density constructs at 0.24 ± 0.01 million cells per construct.

Biochemical Content

The biochemical content correlated well with the initial cell seeding numbers. The GAG content of the high-seeding density group was significantly higher ($p < 0.0001$) than the low and medium groups for every time point taken (Fig. 4), and the medium seeding group possessed a significantly higher GAG content than the low group at the Weeks 3 and 6 time points ($p < 0.005$). The total GAG content seems to increase slightly in each group from Week 3 to Week 6, but the change was not statistically significant. At the Week 6 time point the high-seeding density group had $15.77 \pm 1.86\,\mu$g of GAG per construct, the medium had $8.81 \pm 2.38\,\mu$g of GAG per construct, and the low-seeding density group had $4.70 \pm 1.14\,\mu$g of GAG per construct.

The collagen content per construct increased over the 6-week culture period (Fig. 5). The high-seeding density group outperformed the low and medium seeding density groups in total collagen production at every time point ($p < 0.01$). At Week 6 the collagen content of the medium seeding density group was statistically higher than the low seeding group ($p < 0.005$). The highest increase in collagen production occurred between Week 3 and Week 6 for the high seeding density group. The high seeding density group at Week 6 contained $55.37 \pm 3.56\,\mu$g of collagen per construct, the medium contained $23.76 \pm 3.56\,\mu$g of collagen per construct, and the low-density group possessed $18.26 \pm 0.69\,\mu$g of collagen per construct.

Creep Indentation

There was no correlation between the extracellular matrix content measured and the mechanical properties observed. There were no significant differences in aggregate modulus between groups at Week 0 and Week 6, and there was no difference in modulus among groups between Week 0 and Week 6 (Fig. 6). The average aggregate modulus observed was 8.0 ± 0.9 kPa for all groups at the Week 0 and Week 6 time points. There was a significantly lower permeability ($p < 0.05$) in the high seeding density constructs than the other two groups at Week 0 (Fig. 7). There was no statistical difference among groups in terms of permeability at Week 6. There was a significant decrease in permeability from Week 0 to Week 6 ($p < 0.001$), from $32.8 \pm 17.6 \times 10^{-14}$ m^4/N s to $7.0 \pm 0.9 \times 10^{-14}$ m^4/N s.

DISCUSSION

The field of tissue engineering of the TMJ disc is a fairly new field when compared to hyaline articular cartilage tissue engineering.[1] This study, for the first time, examined the effect of cell seeding density of TMJ disc cells on the culture of PGA nonwoven scaffolds. Low, medium, and high seeding densities were attempted at 15, 30, and 120 million cells per ml of scaffold, respectively. The study demonstrated that in terms of initial cell seeding density a saturation effect was achieved between 30 and 120 million cells per ml of scaffold. This was evidenced by the seeding efficiency which decreased from 100% at low and medium

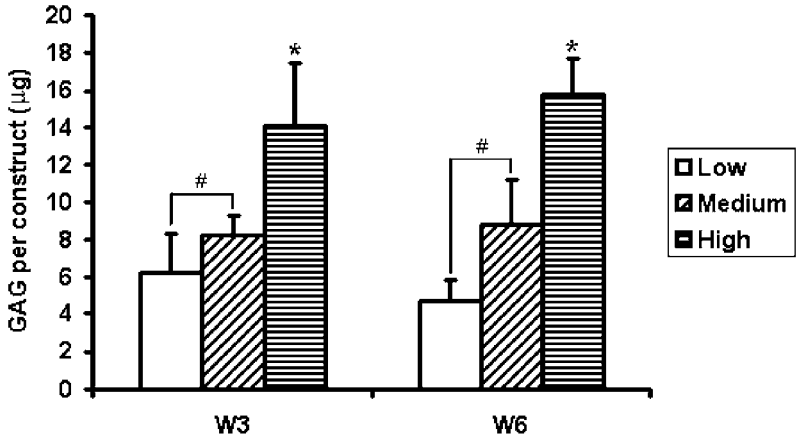

FIGURE 4. GAG content of constructs from the low, medium, and high seeding groups at Weeks 3 and 6. Symbols* and # indicate significance ($p < 0.05$) between the seeding density groups at each time point. Error bars show standard deviation ($n = 6$).

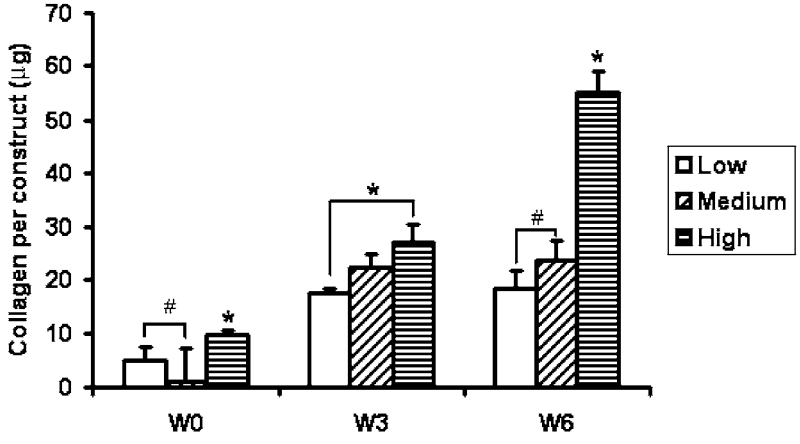

FIGURE 5. Collagen content of constructs from the low, medium, and high seeding groups at Weeks 0, 3, and 6. Symbols* and # indicate significance ($p < 0.05$) between the seeding density groups at each time point. Error bars show standard deviation ($n = 6$).

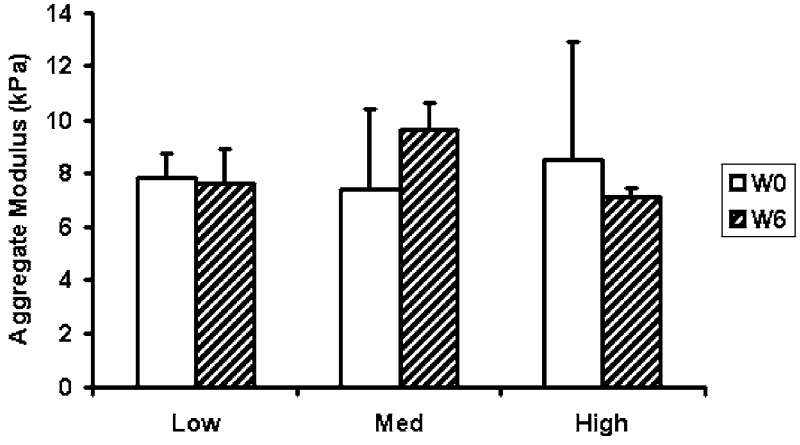

FIGURE 6. Aggregate modulus of constructs from the low, medium, and high seeding groups at Weeks 0 and 6. Error bars show standard deviation ($n = 4$).

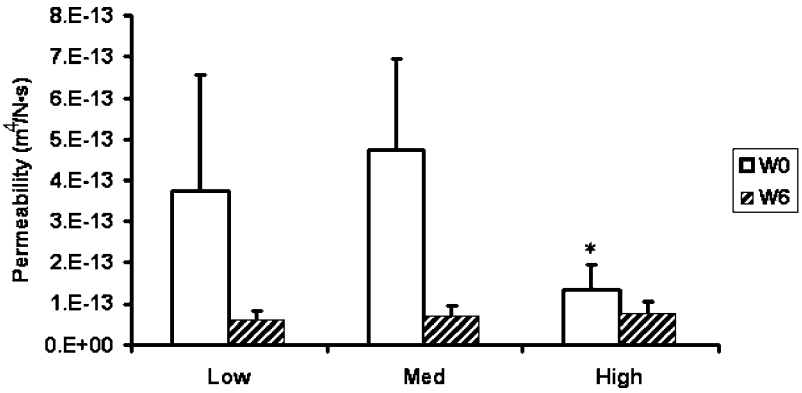

FIGURE 7. Permeability of constructs from the low, medium, and high seeding groups at Weeks 0 and 6. An asterisk (*) indicates significance ($p < 0.05$) between the seeding density groups at each time point. Error bars show standard deviation ($n = 4$).

densities to 60% at the high seeding density, and resulting in 75 million cells per ml of scaffold. However, with culture time, cellularity decreased significantly in all density groups demonstrating that there maybe a nutrient transport problem at all the cell densities used. The high seeding density group was the best in terms of construct morphology and biochemical content. No changes in aggregate modulus were observed between the groups at the beginning and end of the culture period.

All previous studies of porcine TMJ disc cells on PGA nonwoven meshes have yielded constructs with dimensions at a fifth of the original size of the scaffold.[2,17,18] This study is the first to show a final decrease in dimensions by only one half at the end of a 6-week culture for TMJ disc cells. These findings correlate well with a study performed on passaged chondrocytes that appeared fibroblastic in nature and were then seeded onto the same PGA nonwoven mesh.[32] The investigators also found a 50% decrease in construct dimensions after a similar culture period.

This study also produced constructs with higher collagen content[2,17,18] and total GAG content[2,17] than previous TMJ disc cells studies on PGA nonwoven meshes. In terms of matrix content and cells remaining, there was a linear increase of GAG production with increasing cellular content at Week 6. However, there was a quadratic increase in collagen production with increasing cellular content at Week 6. These results are encouraging because in trying to mimic the high collagen content and the low GAG content of the native TMJ disc,[1,15] it is beneficial that more collagen is produced per cell than GAG.

In terms of mechanical properties, the high seeding density constructs were less permeable than the low and the medium density groups immediately postseeding, as expected from a larger number of cells and concomitant ECM present. However, the aggregate modulus was about the same for all three groups at both Week 0 and Week 6, and there was no difference in permeability at Week 6. The lack of difference in aggregate modulus seems to indicate that a certain threshold value must be reached in biochemical content before differences can be manifested in a measurable fashion in the creep indentation test. The lack of difference in permeability at Week 6 may indicate that a plateau permeability was reached by these constructs; this value is close to the permeability observed in the native TMJ disc.[29] Specifically, the native TMJ disc exhibits a permeability value of $2.4 \times 10^{-14}\,\mathrm{m}^4/\mathrm{N\,s}$ compared to $7.1 \times 10^{-14}\,\mathrm{m}^4/\mathrm{N\,s}$ for the Week 6 constructs. It is important to note that the lack in difference of modulus between time points indicates that the original mechanical integrity provided by the PGA was replaced by the matrix produced over time since the polymer degrades fully be Week 6.

This investigation shows promise for the field of tissue engineering of the TMJ disc in terms of production of matrix by TMJ disc cells in PGA scaffolds. In comparison to two articular chondrocyte studies on PGA,[24,47] TMJ disc cells behaved in a similarly positive manner. Collagen production increased significantly over time and GAG content was maintained. The total collagen produced in this current study was only $55\,\mu\mathrm{g}$, smaller than the amount typically produced by articular chondrocytes in the other studies. However, TMJ disc cells are a different type of cell than chondrocytes,[1] and thus TMJ disc cells may need other factors to produce more matrix. It is important to note that the chondrocytes used in the previous studies were obtained from a young animal (2–3 weeks old) and were utilized at zero passage (P0).[24,47] The TMJ disc cells originated from an older animal (10-month-old), and were used after second passage (P2). Clearly the age of the animal may have an impact on the capabilities of the cells to produce matrix. Additionally, passaged chondrocytes do not behave the same as P0 chondrocytes.[13,14,32]

In conclusion, the highest seeding density of TMJ disc cells produces the highest amount of matrix when seeded on PGA nonwoven meshes, despite the fact that a measurable saturation effect was noted between medium and high initial cell densities. Thus, it is recommended that studies focused

on an implantable tissue-engineered TMJ disc construct use high initial seeding densities, not exceeding 75 million cells per ml of PGA scaffold. If the objective of a study is to simply observe the behavior of cells to different conditions, then a lower seeding density may be used. However, if too few cells are seeded onto the PGA nonwoven meshes, these constructs do not develop the mechanical integrity to last for longer than 2–4 weeks in culture.[17,48] Interestingly, not matter what initial seeding density was used all constructs decreased in cellularity over the culture period. Thus, although the cells have a maximum volume they can occupy, it seems that they do not like being packed in. Diffusional problems from too many cells may account for such a behavior, for if cells cannot obtain the necessary nutrients to survive they will die or leave the scaffold. Additionally, the decrease in cellularity may also be a developmental function of fibrocartilaginous cells after secreting matrix, since fibrocartilaginous tissues are fairly acellular.

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