Injectable Gellan Gum Hydrogels with Autologous Cells for the Treatment of Rabbit Articular Cartilage Defects

João T. Oliveira,[1,2] Leandro S. Gardel,[1,2,3] Tommaso Rada,[1,2] Luís Martins,[4] Manuela E. Gomes,[1,2] Rui L. Reis[1,2]

[1]3B's Research Group—Biomaterials, Biodegradables and Biomimetics, Department of Polymer Engineering, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, Zona Industrial da Gandra, S. Cláudio do Barco, 4806-909 Caldas das Taipas, Guimarães, Portugal, [2]IBB—Institute for Biotechnology and Bioengineering, PT Associated Laboratory, Guimarães, Portugal, [3]Department of Clinical Veterinary, Abel Salazar Biomedical Sciences Institute, University of Porto, Porto, Portugal
[4]Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, 4710-057 Braga, Portugal

Received 7 October 2009; accepted 10 January 2010
Published online 24 February 2010 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jor.21114

ABSTRACT: In this work, the ability of gellan gum hydrogels coupled with autologous cells to regenerate rabbit full-thickness articular cartilage defects was tested. Five study groups were defined: (a) gellan gum with encapsulated chondrogenic predifferentiated rabbit adipose stem cells (ASC + GF); (b) gellan gum with encapsulated nonchondrogenic predifferentiated rabbit adipose stem cells (ASC); (c) gellan gum with encapsulated rabbit articular chondrocytes (AC) (standard control); (d) gellan gum alone (control); (e) empty defect (control). Full-thickness articular cartilage defects were created and the gellan gum constructs were injected and left for 8 weeks. The macroscopic aspect of the explants showed a progressive increase of similarity with the lateral native cartilage, stable integration at the defect site, more pronouncedly in the cell-loaded constructs. Tissue scoring showed that ASC + GF exhibited the best results regarding tissue quality progression. Alcian blue retrieved similar results with a better outcome for the cell-loaded constructs. Regarding real-time PCR analyses, ASC + GF had the best progression with an upregulation of collagen type II and aggrecan, and a downregulation of collagen type I. Gellan gum hydrogels combined with autologous cells constitute a promising approach for the treatment of articular cartilage defects, and adipose derived cells may constitute a valid alternative to currently used articular chondrocytes. © 2010 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 28:1193–1199, 2010

Keywords: gellan gum; stem cells; cartilage; tissue engineering; in vivo

Articular cartilage is considered to be the most important type of cartilage due to its function in mobility and locomotion. Due to the tissues' intrinsic features (low metabolism, absence of vascularization), trauma and degenerative conditions (e.g., focal defects, osteoarthritis, rheumatoid arthritis) frequently create disability states leading to pain and decreased life quality. Although several therapies have been attempted, the outcomes are not fully satisfactory, and other lines of research have been put forward.[1–3] The use of biomaterials, cells and bioactive agents, either alone or combined, has been proposed[4–6] as an alternative to solve these problems, being this field defined as tissue engineering.[7] Natural and synthetic biomaterials have been studied as supports for cell development and formation of a functional cartilaginous tissue.[8,9] Hydrogels in the form of injectable systems that can be applied in a minimally invasive manner gelling in situ under physiological conditions have gathered considerable attention in cartilage regeneration approaches.[10,11] Gellan gum is a polysaccharide that forms thermoreversible gels, being noncytotoxic in different tested situations.[12–14] It is commonly applied in the food industry,[15] and has been previously used in the biomedical field for drug delivery.[16] Gellan gum has been originally proposed by our group[17] as a new biomaterial for cartilage tissue engineering applications, yet recent studies can be found on the same application[18] and on the general use of gellan gum for tissue engineering.[19] In our previous works, it has been used to encapsulate both human nasal and articular chondrocytes in vitro, and has been tested in vivo by subcutaneous implantation in nude mice with human articular chondrocytes.[20–22] In this work, autologous cells [adipose stem cells (ASC) articular chondrocytes (AC)] were combined with gellan gum and injected in rabbit knee full thickness size defects. The results show that gellan gum constructs are able to efficiently regenerate hyaline-like cartilage tissue in the created defects.

Correspondence to: J. T. Oliveira (T: +351-253510900; F: +351-253510909; E-mail: joao.oliveira@dep.uminho.pt)

METHODS

Five groups were defined for this work: (a) gellan gum with encapsulated chondrogenic predifferentiated [transforming growth factor beta1 (TGF-β1) and bone morphogenetic protein 2 (BMP-2)] rabbit adipose stem cells (ASC + GF); (b) gellan gum with encapsulated nonchondrogenic predifferentiated rabbit adipose stem cells (ASC); (c) gellan gum with encapsulated rabbit articular chondrocytes (AC) (standard control); (d) gellan gum alone (GELLAN) (control); (e) empty defect (EMPTY) (control). A total of 12 New Zealand White rabbits ($n = 12$) were used, 4 per time point (1, 4, and 8 weeks), and each rabbit had a total of 4 defects created in the same way in the right limb. The experiments with the model involved an autologous approach, meaning that the cells extracted from a specific rabbit were implanted in the same rabbit. The animal tests were approved by the Ethics Review Committee and their care was in accordance with institution guidelines.

Rabbit Adipose Stem Cells Isolation and Expansion

Rabbit adipose tissue was obtained from the intrascapular region of 10–11 weeks old/2.4–2.6 kg female New Zealand White rabbits. Charles Rives, Barcelona, Spain Briefly, the rabbits were preanaesthetized with ketamine (25 mg/kg i.m., Imalgene® 1000, Merial, Lyon, France) and medetomidine (0.15 ml/kg i.m., Domitor®, Orion Corporation, Espoo, Fin-

land). Adipose tissue was collected and digested in a 1 mg/ml collagenase type II (Sigma-Aldrich Co., St. Louis, MO) solution, and the released cells ressuspended in culture medium [Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich Co., St. Louis, MO), 10% (v/v) fetal bovine serum (FBS, Biochrom, Berlin, Germany; Heat Inactivated), 1% antibiotic], and seeded in tissue culture polystyrene flasks for expansion. The cells were divided in two groups: one group subjected to a chondrogenic predifferentiation period consisting of DMEM, sodium pyruvate 1.0×10^{-3} M, ascorbate-2-phosphate 0.17 mM, proline 0.35 mM, ITS 1X, and supplemented with 10 ng/ml TGF-β1 (Sigma-Aldrich Co., St. Louis, MO) for 2 days followed by 100 ng/ml BMP-2 (R&D Systems, Minneapolis, MN) for 3 days prior to in vivo implantation (ASC + GF); another group cultured with the same medium but without TGF-β1 and BMP-2 (ASC).

Rabbit Articular Chondrocytes Isolation and Expansion

The chondrocytes isolation and expansion protocol was based on previous reports from Crawford and Dickinson.[23] Rabbits were preanaesthetized as detailed before for adipose tissue collection. Fragments of articular cartilage were harvested from the femoral condyles and the cartilage pieces immersed in trypsin–EDTA solution, followed by a DMEM and sterile collagenase type II solution (2 mg/ml) and kept for 8–10 h at 37°C under constant agitation. The isolated cells were suspended in expansion medium consisting of DMEM, containing 10 mM HEPES buffer pH 7.4, 1% antibiotic, 20 mM L-alanyl glutamine, 1× MEM nonessential amino acids and 10% (v/v) FBS (Biochrom Berlin, Germany; Heat Inactivated), supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech, London, UK). Rabbit articular chondrocytes were plated into tissue culture flasks for expansion.

In Vivo Injection in Rabbit Articular Cartilage Defects of Gellan Gum-Cell Constructs

The different cells were used at passage 2 under the following procedure. Gellan gum powder (G1910, Sigma-Aldrich Co., St. Louis, MO) was mixed with sterile distilled water to obtain a final concentration of 1.25% (w/v). The solution was stabilized at 42°C under constant stirring. Articular cartilage full-thickness defects with a diameter of 4 mm were created in the rabbits medial femoral condyle and a 1 mm diameter hole was drilled to the subchondral bone (Fig. 1). Briefly, the rabbits were preanaesthetized with ketamine (25 mg/kg i.m., Imalgene® 1000, Merial, Lyon, France) and medetomidine (0.15 ml/kg i.m., Domitor®, Orion Corporation, Espoo, Finland). The cells were ressuspended in a small amount of PBS, the gellan gum solution was mixed at a temperature of 40–41°C with the cell suspension (final concentration of 10×10^6 cells/ml) and the mixture was then injected into the defect. A waiting time of 2–3 min was given for the gels to form in situ. Defects were also filled with gellan gum without cells and other defects were left empty. The experiments were conducted for periods of up to 8 weeks with data collection points at 1, 4, and 8 weeks. At the established time points, the animals were euthanized by injection of an overdose of pentobarbital sodium (Eutasil® Ceva Sante Animale, Libourne, France).

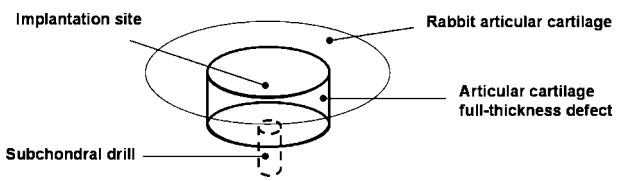

Figure 1. Schematic representation of the articular cartilage defect created in the rabbits femoral condyles.

Histology and Histological Scoring

Common histological analysis was performed on 4 μm thickness sections of the samples collected after 1, 4, and 8 weeks of implantation. The explants were dehydrated, embedded in paraffin and cut for posterior analysis using a microtome Leica RM2155 (Leica Microsystems, Nussloch, Germany). H&E staining was performed using an automatic processor according to in-house methodology (Leica TP1020-1, Leica Micro-Systems, Nussloch, Germany) and alcian blue staining followed standard histological methods. The Pineda scoring system[24] was used for histological evaluation of the explants. The degree and the quality of healing in all defects was assessed and scored blindly by five independent researchers. The maximum possible score in the Pineda scoring system is 0 points (increased regenerative potential) with a minimum of 14 points (decreased regenerative potential). Statistical analyses were conducted using SPSS Statistics 17.0 software, namely one-way ANOVA with Tukey correction for $n = 5$. Statistical significance level was $p < 0.05$ and the test of homogeneity of variances confirmed for $p < 0.05$.

Real-Time PCR (Sox9, col I, col II, Aggrecan)

Samples were collected after 1, 4, and 8 weeks of implantation upon exposure of the defect site area. They were immersed in TRIzol® (Invitrogen, Carlsbad, CA), and stored at −80°C for posterior analysis. RNA was extracted following the manufacturers' guidelines. The amounts of isolated RNA and A260/280 ratio were determined using Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Rockland, DE). A predetermined amount of RNA from each sample was reverse transcribed into cDNA using the IScriptTM cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) in a BioRad CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Cartilage related markers (Sox9, collagen type I, collagen type II, and aggrecan, using GAPDH as the housekeeping gene for normalization) were chosen to evaluate the chondrogenic phenotype of the cultured systems. The relative gene expression quantification was performed using the $2^{-\Delta\Delta C_t}$ (Livak) method. All the primer sequences were generated using Primer3 software[25] and acquired from MWG Biotech AG (Ebersberg, Germany). Real-time PCR was performed using a BioRad CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) and SYBR Green IQTM Supermix (Bio-Rad Laboratories, Hercules, CA) to detect amplification variations. The analyses of the results were performed with CFX Manager Software—version 1.0 (Bio-Rad Laboratories, Hercules, CA).

RESULTS

Macroscopic Observation and Histology

Macroscopic observation of the defects shows a smooth lateral integration and homogeneous appearance of the cell containing constructs, namely both groups of tested ASC, and also articular chondrocytes (Fig. 2). The results presented in Figure 3 show that the injected gellan gum hydrogels containing the different cell types studied were kept in the defect after injection and appear to be well integrated, both in the osteochondral junction and with the lateral native articular cartilage.

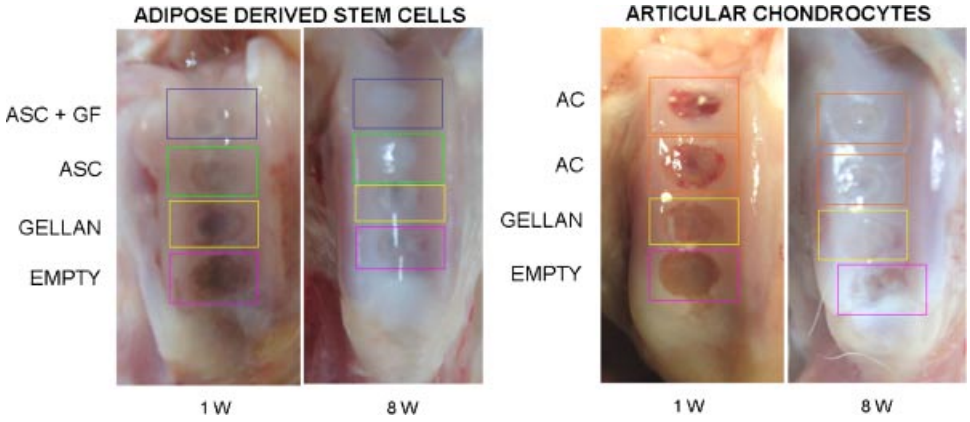

Figure 2. Macroscopical observation of the different studied groups after 1 and 8 weeks post-implantation.

This observation is evident in all the constructs with the exception of gellan gum alone where the inclusion and integration is apparently weaker. Regarding cell morphology, similar results were found for ASC + GF and ASC, with the constructs containing AC presenting a slightly different phenotype when compared to the native cartilage. Gellan gum alone and the empty defect presented the less similar morphologies, exhibiting only some focal spots of hyaline-like chondrocytes, which were detected more frequently in gellan gum hydrogels alone. Alcian blue methacromatic staining was detected in histological sections of all the explants, although with different extents (Fig. 4). Regarding the regularity of the staining profiles, gellan gum with ASC + GF and ASC appear to be the most uniform extending from the center of the defect site to the contact with the lateral cartilage present on each side. Some implantation sites (Fig. 3) presented evidences of tissue formation out of the articular plane, which may be an indirect result of the biopsy taken for real-time PCR analysis which morphologically altered the explants to some extent, or excess of hydrogel-cell constructs being injected in the defect.

Histological Scoring

The Pineda scoring system[26,27] was used to grade the tissue quality of the various groups in study. ASC + GF exhibited the best absolute values in terms of progression of a newly formed hyaline-like cartilage tissue (Fig. 5). When looking to the scores for 1, 4, and 8 weeks of implantation, ASC + GF constructs show a continuous increase in performance culminating at the final time point where they were ranked the best within the five groups. Gellan gum hydrogels with encapsulated ASC have demonstrated some variability throughout the implantation stages, but considerably improved after 8 weeks. The variations within AC constructs occurred in a smoother way presenting a better performance after 8 weeks. The gellan gum hydrogels alone showed slightly increasing quality of the newly formed tissue, maintaining nevertheless some stability in terms of the scoring. Finally, the empty defects exhibited an improvement tendency with time more noticeable at 4 weeks of implantation. From 4 to 8 weeks, no relevant variations are encountered and this group was ranked the poorest regarding the quality and performance, being statistically different from the cell-loaded constructs ($p < 0.05$).

Real-Time PCR (Sox9, Collagen Type I, Collagen Type II, Aggrecan)

The real-time PCR analyses results are graphically represented in Figure 6. In the gellan gum hydrogels with encapsulated ASC + GF, no Sox9 expression was

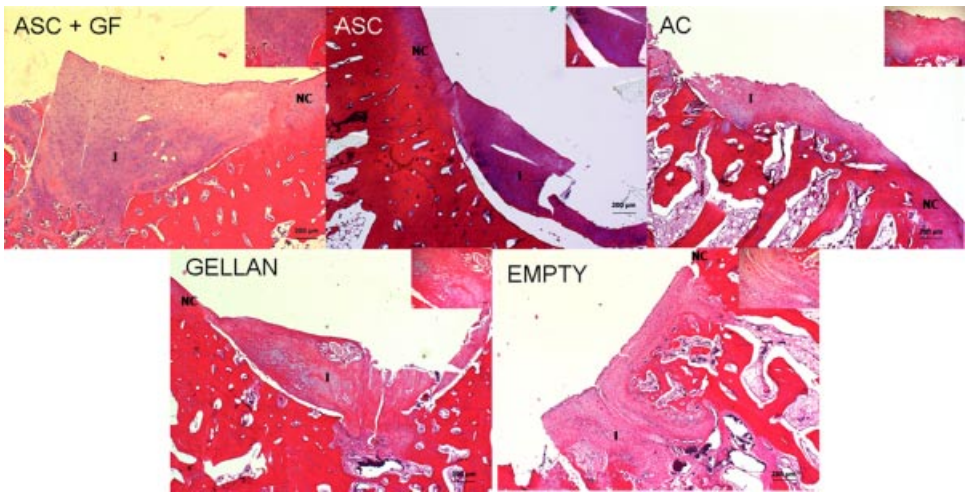

Figure 3. Optical microscopy pictures of histological sections of the explants after 8 weeks of culture and stained with hematoxylin–eosin (I, implant region; NC, native cartilage; scale bar = 200 μm).

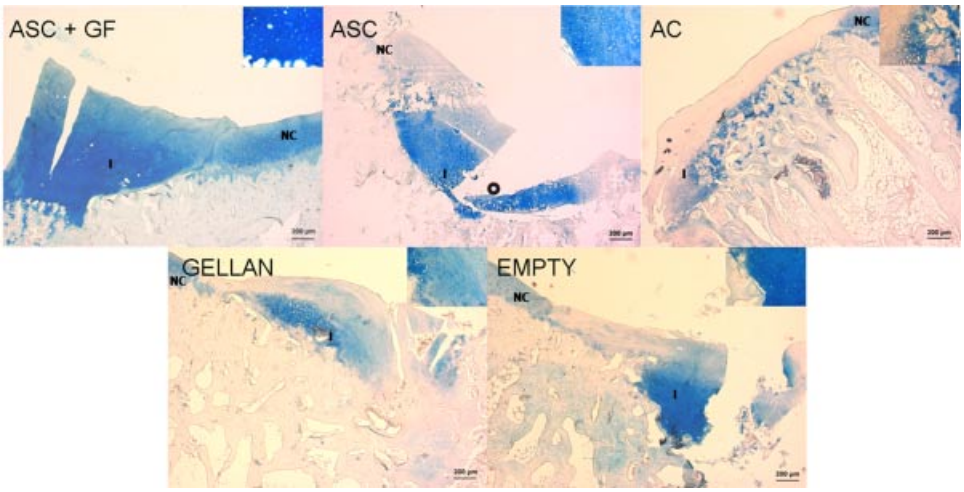

Figure 4. Optical microscopy pictures of histological sections of the explant after 8 weeks of culture stained with alcian blue (I, implant region; NC, native cartilage; scale bar = 200 μm).

detected. Collagen type I levels were residual at 4 weeks and were not observed after 8 weeks of implantation. Regarding collagen type II, an upregulation of approximately 30-fold was noticed from 4 to 8 weeks, and the same upregulation profile was identified for aggrecan, in this case with a 9-fold increase. ASC revealed no Sox9 expression at all time points, and collagen type I was also maintained at residual levels. Collagen type II experienced a 58-fold upregulation, and aggrecan evidenced only a slight increase from 4 to 8 weeks of implantation. Regarding the constructs with articular chondrocytes, no Sox9 was again detected, and collagen type I was noticed after 4 weeks but was downregulated approximately fourfold after 8 weeks. The same pattern of variation was observed with collagen type II and aggrecan, which exhibited a downregulation of two- and threefold from 4 to 8 weeks of implantation. Gellan gum alone did not present Sox9 transcripts and collagen type I levels were also residual. Collagen type II and aggrecan showed an upregulation of 13- and 2-fold, respectively. Finally, the empty defect showed no existence of Sox9, and evidenced a slight upregulation of collagen type I levels from 4 to 8 weeks. Collagen type II was upregulated 1.6-fold and aggrecan was also upregulated 1.8-fold. In terms of higher gene transcription levels after 8 weeks, the empty defect group showed the highest values for collagen type I; AC, followed by ASC + GF and ASC presented the highest collagen type II expression profiles, and ASC + GF followed by AC had the best results regarding aggrecan levels.

DISCUSSION

In this work, injectable gellan gum hydrogels combined with autologous cells were studied for the treatment of rabbit articular cartilage defects. After 1 week, the implants were similar in appearance, exhibiting a faint opaque white color, being this occurrence more pronounced and homogeneous in gellan gum with encapsulated cells (ASC + GF, ASC, AC). Even so, the gel transparency was still noticeable in all constructs. After 4 weeks, the white opaque nature of the defects was even more pronounced in the cell-loaded constructs when compared to gellan alone and empty defects, being this pattern also observed after 8 weeks.

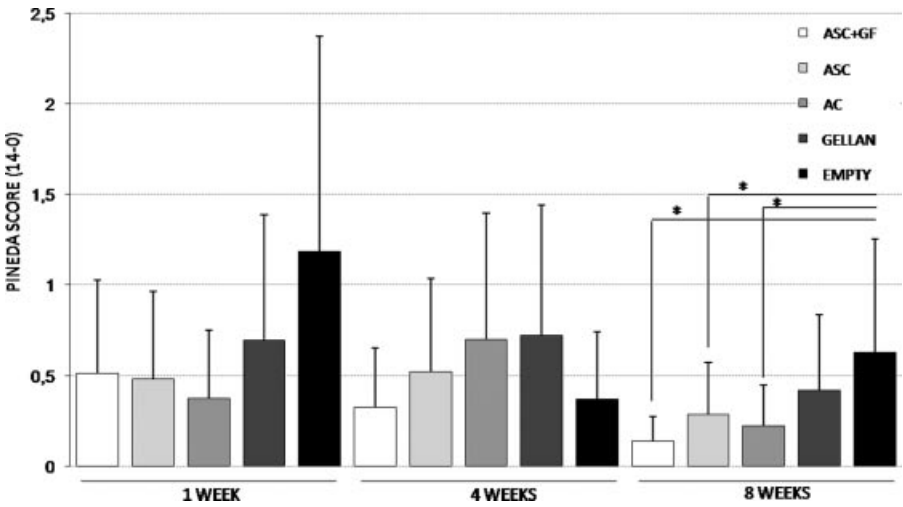

Figure 5. Graphical representation of the results obtained with the Pineda scoring on the explanted gellan gum-cell constructs after 1, 4, and 8 weeks of implantation (* statistical significant for a statistical level of 0.05 determined by one-way ANOVA with Tukey correction).

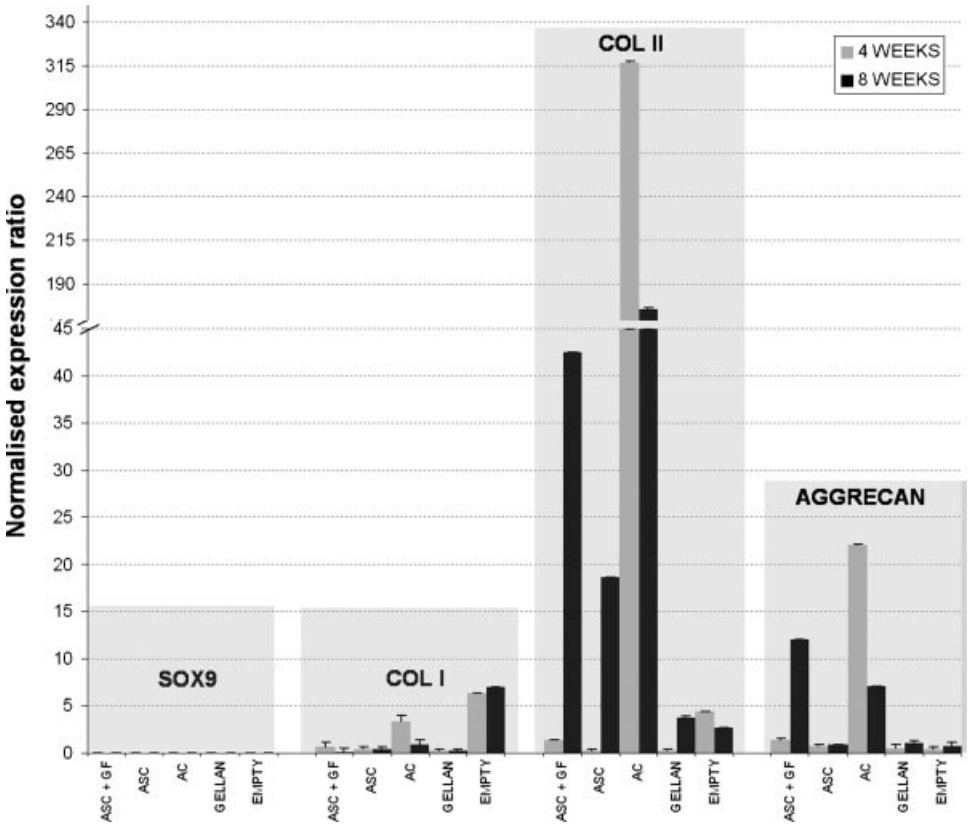

Figure 6. Graphical representation of the real-time semi-quantitative PCR analysis results for Sox9, collagen type I, collagen type II, and aggrecan performed on the explants collected after 1, 4, and 8 weeks of implantation.

The defects were homogeneously filled and well integrated with the surrounding cartilage presenting smooth transition zones between the implant and the native tissue in ASC + GF, ASC, AC. The results for gellan gum alone and the empty defects were more heterogeneous. The morphological appearance was much similar to the lateral native cartilage in those groups than in gellan gum alone and empty defects, particularly after 8 weeks of implantation, which demonstrates a difference between the constructs with cells and without cells. The staining of histological sections of the explants revealed the implants were all kept at the implantation site, even though unrestricted movement of the animals was permitted right after the surgeries. Therefore, gellan gum constructs are able to be injected, remain, and adapt to articular cartilage defects under normal load bearing conditions. A progressive integration was observed in all the constructs throughout the implantation periods. Both the osteochondral junction and the lateral native cartilage presented a continuous tissue bridging with the implants. The best results were observed for the cell containing gels mostly in prolonged stages of implantation. These results were somehow predictable since the cells incorporated are expected to confer an improved advantage in tissue formation and integration due to their autologous nature and the role they can develop in tissue rebuilding. Histological analyses suggest that in terms of cell morphology and overall cartilage tissue structure ASC + GF and ASC have performed as well or better than AC and the other constructs. The histological scoring system reinforced some of the conclusions obtained with macroscopic and histological analysis. The ASC + GF constructs had the best scores in terms of values calculated from the average of the individual scores of five independent researchers. However, the quality of the tissue formed at the end of the experiments was not statistically different between the different groups ($p > 0.05$), with the exception of the empty defects regarding the cell containing constructs, according to ANOVA analysis $p < 0.05$. After 8 weeks of implantation, ASC + GF appear to perform better than ASC, which suggests the importance that the chondrogenic predifferentiation environment may have in the final performance of the constructs. When compared to AC, both constructs with ASC appear to perform better in terms of absolute scoring values of the progression of the quality and integrity of the newly formed tissue. Real-time PCR analyses did not detect Sox9 transcripts in any of the samples during the course of the experiments. Previous studies have suggested its association with the chondrocyte phenotype maintenance and collagen type II expression, although this is not completely consensual.[29,30] Further studies should be conducted for a full gene transcription profiling of the implants, influence of in vitro expansion on markers expression, and tracking of other markers of the chondrocytes cell cycle. Collagen type I presence only in the initial periods in AC and residual for ASC + GF, ASC, and gellan is in

agreement with the formation of a cartilage tissue of hyaline nature. The detection and maintenance of collagen type I levels in the empty defects indicates otherwise. In fact, the creation of this defect resembles to some extent the Pridie drilling and microfracture surgical techniques applied to the treatment of cartilage pathologies.[31,32] The outcome of this and similar surgical procedures is frequently the formation of a fibrocartilage tissue with inferior mechanical properties, and therefore the upregulation of collagen type I levels typical of fibrocartilage was expected.[31,32] Collagen type II and aggrecan presented the highest levels in gellan gum-AC constructs although exhibiting a decrease from 4 to 8 weeks of implantation. This could be associated to an eventual hypertrophy of chondrocytes, which carries a decrease in collagen type II values.[33] Nonetheless, collagen type II levels are still significantly higher than for all the other constructs. The same is not true for aggrecan. ASC + GF presented the highest values for aggrecan expression with a steadily upregulation until the final implantation period. The same pattern of variation was observed for collagen type II which opens good prospects for the generation of a fully functional tissue engineered construct since these two molecules confer cartilage with its load bearing functions. ASC showed the same upregulation pattern in collagen type II but not in aggrecan which may be related with the prior chondrogenic stimulation given to ASC + GF. Altogether, these results suggest that gellan gum constructs combined with different cell types have potential application in the treatment of articular cartilage defects. Promising results were obtained with ASC preconditioned to chondrogenic differentiation in vitro, which may open the opportunity for clinically treating a defect without the disadvantages of a cartilage biopsy for chondrocyte isolation. Their performance should be further optimized by varying the culture cocktails and exploiting other cells parameters (number, stage, and subpopulation). The study through longer implantation periods and inclusion of other relevant controls, such as mosaicplasty, autologous chondrocyte implantation (ACI), and matrix-assisted autologous chondrocyte implantation (MACI), for example, should be conducted to compare and confirm the results obtained so far.

ACKNOWLEDGMENTS

J. T. Oliveira acknowledge the Portuguese Foundation for Science and Technology (FCT) for his grant (SFRH/BD17135/2004). The authors thank the medical and technical staff of the Institute for Biomedical Sciences Abel Salazar (ICBAS) of the University of Porto, Portugal and the Institute for Health and Life Sciences (ICVS) of the University of Minho, Portugal. The authors also thank Dr. Patrícia Malafaya, Cristina Correia, and Rui Pereira, for their help with the histological scoring. This work was carried out under the scope of the European NoE EXPERTISSUES, and partially supported by the European Project HIPPOCRATES.

REFERENCES

1. Hunt SA, Jazrawi LM, Sherman OH. 2002. Arthroscopic management of osteoarthritis of the knee. J Am Acad Orthop Surg 10:356–363.
2. Ge Z, Hu Y, Heng BC, et al. 2006. Osteoarthritis and therapy. Arthritis Rheum 55:493–500.
3. Brittberg M, Lindahl A, Nilsson A, et al. 1994. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 331:889–895.
4. Athanasiou KA, Agrawal CM, Barber FA, et al. 1998. Orthopaedic applications for PLA-PGA biodegradable polymers. Arthroscopy 14:726–737.
5. Santos MI, Tuzlakoglu K, Fuchs S, et al. 2008. Endothelial cell colonization and angiogenic potential of combined nano- and micro-fibrous scaffolds for bone tissue engineering. Biomaterials 29:4306–4313.
6. Li WJ, Chiang H, Kuo TF, et al. 2009. Evaluation of articular cartilage repair using biodegradable nanofibrous scaffolds in a swine model: a pilot study. J Tissue Eng Regen Med 3:1–10.
7. Langer R, Vacanti JP. 1993. Tissue engineering. Science 260:920–926.
8. Risbud MV, Sittinger M. 2002. Tissue engineering: advances in *in vitro* cartilage generation. Trends Biotechnol 20:351–356.
9. Hutmacher DW. 2000. Scaffolds in tissue engineering bone and cartilage. Biomaterials 21:2529–2543.
10. Hoemann CD, Sun J, Legare A, et al. 2005. Tissue engineering of cartilage using an injectable and adhesive chitosan-based cell-delivery vehicle. Osteoarthritis Cartilage 13:318–329.
11. Elisseeff J, Anseth K, Sims D, et al. 1999. Transdermal photopolymerization for minimally invasive implantation. Proc Natl Acad Sci USA 96:3104–3107.
12. Moorhouse R, Colegrove GT, Sanford PA, et al. 1981. PS-60: a new gel-forming polysaccharide. In: Brant DA, editor. Solution properties of polysaccharides. Washington, DC: American Chemical Society; pp 111–124.
13. Jansson PE, Lindberg B, Sandford PA. 1983. Structural studies of gellan gum, an extracellular polysaccharide elaborated by pseudomonas-elodea. Carbohydr Res 124:135–139.
14. Grasdalen H, Smidsrod O. 1987. Gelation of gellan gum. Carbohydr Polym 7:371–393.
15. Mao R, Tang J, Swanson BG. 2000. Texture properties of high and low acyl mixed gellan gels. Carbohydr Polym 41:331–338.
16. Sanzgiri YD, Maschi S, Crescenzi V, et al. 1993. Gellan-based systems for ophthalmic sustained delivery of methylprednisolone. J Control Release 26:195–201.
17. Oliveira JT, Picciochi R, Santos TC, et al. 2008. Injectable gellan gum hydrogels as supports for cartilage tissue engineering applications. Annual Tissue Engineering and Regenerative Medicine International Society—European Chapter Meeting. Porto, Portugal: Mary Ann Liebert, Inc.; p OP165.
18. Gong YH, Wang CM, Lai RC, et al. 2009. A novel injectable polysaccharide hydrogel: modified gellan gum for long-term cartilage regeneration *in vitro*. J Mater Chem 19:1968–1977.
19. Smith AM, Shelton RM, Perrie Y, et al. 2007. An initial evaluation of gellan gum as a material for tissue engineering applications. J Biomater Appl 22:241–254.
20. Oliveira JT, Martins L, Picciochi R, et al. 2009. Gellan gum: a new biomaterial for cartilage tissue engineering applications. J Biomed Mater Res A (Epub ahead of print).
21. Oliveira JT, Santos TC, Martins L, et al. 2010. Gellan gum injectable hydrogels for cartilage tissue engineering applications: *in vitro* studies and preliminary *in vivo* evaluation. Tissue Eng Part A 16:343–353.

22. Oliveira JT, Santos TC, Martins L, et al. 2009. Performance of new gellan gum hydrogels combined with human articular chondrocytes for cartilage regeneration when subcutaneously implanted in nude mice. J Tissue Eng Regen Med 3:493–500.
23. Crawford A, Dickinson SC. 2008. Chondrocyte isolation, expansion, and culture on polymer scaffolds. In: Hollander AP, Hatton PV, editors. Methods in molecular biology, biopolymer methods in tissue engineering. Totowa NJ: Humana Press, Inc.; p 147–157.
24. Pineda S, Pollack A, Stevenson S, et al. 1992. A semi-quantitative scale for histologic grading of articular cartilage repair. Acta Anat (Basel) 143:335–340.
25. Page DC, Lander ES. http://frodo.wi.mit.edu/.
26. Moojen DJ, Saris DB, Auw Yang KG, et al. 2002. The correlation and reproducibility of histological scoring systems in cartilage repair. Tissue Eng 8:627–634.
27. Dorotka R, Bindreiter U, Macfelda K, et al. 2005. Marrow stimulation and chondrocyte transplantation using a collagen matrix for cartilage repair. Osteoarthritis Cartilage 13:655–664.
28. Aigner T, Gebhard PM, Schmid E, et al. 2003. SOX9 expression does not correlate with type II collagen expression in adult articular chondrocytes. Matrix Biol 22:363–372.
29. Tew SR, Clegg PD, Brew CJ, et al. 2007. SOX9 transduction of a human chondrocytic cell line identifies novel genes regulated in primary human chondrocytes and in osteoarthritis. Arthritis Res Ther 9:R107.
30. Anderson AF, Richards DB, Pagnani MJ, et al. 1997. Antegrade drilling for osteochondritis dissecans of the knee. Arthroscopy 13:319–324.
31. Steadman JR, Rodkey WG, Rodrigo JJ. 2001. Microfracture: surgical technique and rehabilitation to treat chondral defects. Clin Orthop Relat Res 391:S362–S369.
32. Breinan HA, Martin SD, Hsu HP, et al. 2000. Healing of canine articular cartilage defects treated with microfracture, a type-II collagen matrix, or cultured autologous chondrocytes. J Orthop Res 18:781–789.
33. Pelttari K, Winter A, Steck E, et al. 2006. Premature induction of hypertrophy during *in vitro* chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. Arthritis Rheum 54:3254–3266.