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A computer-assisted microscopic analysis of bone tissue developed inside a polyactive polymer implanted into an equine articular surface

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Summary. One of the most promising applications for the restoration of small or moderately sized focal articular lesions is mosaicplasty (MP). Although recurrent hemarthrosis is a rare complication after MP, recently, various strategies have been designed to find an effective filling material to prevent postoperative bleeding from the donor site. The porous biodegradable polymer Polyactive (PA; a polyethylene glycol terephthalate - polybutylene terephthalate copolymer) represents a promising solution in this respect. A histological evaluation of the longterm PA-filled donor sites obtained from 10 experimental horses was performed. In this study, attention was primarily focused on the bone tissue developed in the plug. A computer-assisted image analysis and quantitative polarized light microscopic measurements of decalcified, longitudinally sectioned, dimethylmethylene blue (DMMB)- and picrosirius red (PS) stained sections revealed that the coverage area of the bone trabecules in the PA-filled donor tunnels was substantially (25%) enlarged compared to the neighboring cancellous bone. For this quantification, identical ROIs (regions of interest) were used and compared. The birefringence retardation values were also measured with a polarized light microscope using monochromatic light. Identical retardation values could be recorded from the bone trabeculae developed in the PA and in the neighboring bone, which indicates that the collagen orientation pattern does not differ significantly among these bone trabecules. Based on our new data, we speculate that PA promotes bone formation, and some of the currently identified degradation products of PA may enhance osteo-conduction and osteoinduction inside the donor canal.

Key words: Mosaicplasty, Donor tunnels, Bone, Polarized light microscopy, Computer-assisted image analysis, Horse

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Introduction

Articular cartilage has a poor capacity for repair and healing (Horas et al., 2003; Bedi et al., 2010). Articular cartilage performs the necessary functions of uniformly transferring and decreasing the load on the underlying bone (Shirazi and Shirazi-Adl, 2009); failing to do so can initiate further joint abnormalities (Ding et al., 2008). Therefore, an increasing number of theoretical and clinical studies have been designed to achieve the regeneration of organized articular cartilage.

One of the major innovative and biological approaches used to restore the function of articular cartilage for more than a decade has been mosaicplasty (MP). This approach focuses on reconstructing the joint articular surfaces via the transplantation of several small autologous osteochondral grafts (OCGs) in a rosette

pattern to form a stable plug that fills the lesion (Hangody et al., 1997, 2001a,b, 2008; Hangody and Fules, 2003; Hangody and Módis, 2006). The theoretical advantage of MP is based on a study published by Pap and Krompecher (1961), in which the authors demonstrated that an OCG functionally incorporates into the recipient's surrounding tissue, even in the absence of compatibility. However, the size and ratio of the graft have a significant influence on its survival. In the case of autologous osteochondral mosaicplasty, serology and tissue typifying are redundant. They also have significant restrictions, including the limited size of the useable donor area, donor site morbidity, differences in orientation and thickness, and the mechanical properties of the donor vs. recipient cartilage (Bedi et al., 2010).

A number of articles have addressed graft-host integration and post-operative function, but few have studied donor site events. Normally, the tunnels that remain after removing an OCG are filled with spongy bone within four weeks (similar to Pridie's drilling method) due to the invasion of mesenchymal stem cells and bleeding from the subchondral area (Hangody and Módis, 2006). The surfaces of the tunnels tend to be covered with a primer repair tissue, which becomes fibrocartilage when exposed to appropriate loading (Bodo et al., 2000). Unfortunately, in a few cases, excessive bleeding may spontaneously occur in the remaining empty donor tunnels, leading to hemarthrosis (Feczko et al., 2003). Ferric ions (Fe^{3+}) derived from the degraded normocytes in the blood may enter the joint cavity and irreversibly destroy the proteoglycan structure of the hyaline cartilage (Sokoloff, 1963).

Several clinical and preclinical experimental studies have been conducted on various materials, such as hydroxyapatite (Litvinov et al., 2000), carbon fiber (Meister et al., 1998), polyglyconate (Freed et al., 1994) and compressed collagen (Nixon et al., 1993), to exclude the possibility of post-operative hemarthrosis and to fill the donor tunnels. This results in cicatricial tissue or poor fibrocartilage formation on the surface. In our previous study, we investigated a copolymer material called Polyactive (PA), which consists of polyethylene glycol terephthalate - polybutylene terephthalate (PEGT/PBT; IsoTis OrtoBiologics, Bilthoven, the Netherlands). We have provided clear evidence that PA is one of the most appropriate filling materials in this context (Módis et al., unpublished data). Histological examinations of human samples have shown that PA helps fibrocartilage (and, in a few cases, hyaline-like cartilage) formation on the joint surface, in addition to bone and connective tissue generation inside the joint (Módis et al., 2005).

To the best of our knowledge, to date, there have been no publications concerning quantitative and qualitative analyses of the bone trabecules that have evolved in donor tunnels filled with any material. Therefore, in this study, we aimed to perform a quantitative and qualitative analysis of the trabecules in donor tunnels filled with PA.

Materials and methods

Mosaicplasty in horses

All of the procedures using animals were approved by the Ethics Committee for Animal Trials (Szent István University, Faculty of Veterinary Sciences), and the tissues were obtained in accordance with their guidelines. Osteochondral autograft transplantations were performed on 10 horses. The donor area was the femoral medial trochlea, and the recipient region was the medial or lateral trochlea of the distal third metacarpal bone in the animal's forelimb. This method has been described previously (Bodo et al., 2000). Briefly, the horses were positioned in lateral recumbency under general anesthesia. Four OCG pieces (each approximately 6.5 mm in diameter and 20 mm in length) containing healthy joint cartilage and spongiosa were collected from the same site. The grafts were kept in a sterile isotonic solution (0.9% NaCl) until implantation. The OCGs were placed into 22-mm-deep tunnels at the recipient site. Half of the donor areas were filled with biodegradable polymers (Polyactive [PA]; IsoTis OrtoBiologics, Bilthoven, the Netherlands; Figure 1); the other donor areas were left empty.

Tissue processing for histological analysis

After a long-term (2-year) follow-up, the horses were sacrificed in accordance with the study guidelines and the donor tunnels that had been filled with PA were obtained with their surrounding tissues intact. The samples were immediately transferred into Sainte-Marie's fixative modified according to Tuckett and Morriss-Kay (1988). After fixation, the tissues were placed into 10 % ethylenediamine-tetraacetate (EDTA; Solon, Ohio, USA) for approximately 3 weeks. The decalcified and dehydrated tissue samples were embedded in paraffin, and 5-7-μm-thick longitudinal sections were cut (Microm HM335E; Microm International GmbH., Walldorf, Germany) perpendicular to the surface of the articular cartilage. The paraffin sections were mounted onto gelatin-coated glass slides and dried overnight at 37°C.

After 24 hours at 37°C, dewaxing and rehydration, the sections were stained with dimethylmethylene blue (DMMB, Aldrich, Steinheim, Germany), picrosirius red (PS, Polysciences, Warrington, USA), and hematoxylin-eosin (H&E), according to the protocols provided by the manufacturers (Constantine and Mowry, 1968; Módis, 1974, 1991; Kiraly et al., 1996). After staining, the sections were covered with DPX (Fluka Chemie, Buchs /Switzerland).

Computer-assisted image analysis of the PA-filled and unfilled donor tunnels

During our preliminary microscopic observation, we found numerous thick trabecular bones with abnormal

structures in the donor tunnels filled with PA. However, thin osteon units with normal structures were visible in the control (surrounding) areas of the bone (Figs. 2, 3). To quantitatively analyze our observations, computer-assisted image analysis was performed, as described briefly below.

Images of various samples from 7 different horses were captured using a Nikon Eclipse 800 microscope (Nikon Corporation Instruments Company, Japan) equipped with a Spot RT slider (Diagnostic Instruments, Sterling Heights, MI, USA) CCD camera. The acquired and presented images were representative of all of the samples examined using a 60x Plan Fluor Nikon objective (Nikon Corporation Instruments Company, Japan). After system calibration, 500x720-μm areas from each of the samples were digitalized. Together, 31 control and 31 PA-filled donor tunnels were compared to one another. The quantitative analysis was performed using Image Pro 5.1 (Media Cybernetics, Inc., Silver Spring, MD, USA).

The Mann-Whitney test was used to statistically analyze the results.

Polarized light microscopy measurements

A special polarized light microscopy technique was used to study the spatial orientation of the collagen at the submicroscopic level. The optical anisotropy of the collagen was amplified by a picrosirius red staining (Constantite and Mowry, 1968; Módis, 1991). After staining, the Sirius red molecules with a planar configuration are bound parallel to the long axis of the collagen fibrils. Therefore, collagen appears red when using normal (non-polarized) transilluminating white light and exhibits optical anisotropy between a crossed polarizer and an analyzer if the collagen molecules and fibrils are spatially ordered within a collagen fiber bundle. After staining, the sign of birefringence is positively related to the long axis of the fibers. The retardation value (gamma value) of the birefringence, which is proportional to the extent of the submicroscopic structure, can be measured using a compensator plate. Generally, $\lambda/4$ and proper monochromatic light are used for such measurements (Módis, 1991). Our specimens were analyzed using a polarization microscope (Zetopan Pol; Reichert, Wien, Austria) equipped with a $\lambda/4$ compensator plate, a 10x eyepiece, a 40x objective lens, a 100-W halogen lamp, and an interference filter transmitting monochromatic light with a wavelength of 591.4 nm. (For more technical details, see Módis, 1991.) The birefringence retardation values were determined. One hundred independent measurements per individual sample were taken in the bone trabeculae from both the PA-filled tunnels and the surrounding normal (control) areas. The data collected from the PA-filled and control areas were compared using statistical probes.

Results

A macroscopic inspection showed that the tissue accumulated in the PA-filled donor tunnels was distinctly softer than the tissue in the neighboring regions.

The residual PA helped provide orientation during the microscopic examinations of all three staining procedures. The surfaces of the PA-filled donor tunnels were recovered by fibrocartilage; the inner trabecules were thicker and exhibited abnormal organization compared to the trabecular meshwork of the surrounding tissue (Figs. 2, 3). In some cases, giant polynuclear cells were found next to the residual PA. Compared to the orthochromatic trabecules of the receiving area, the osteon units of the PA-filled donor tunnels exhibited purple-red metachromasia on the DMMB-stained sections (Fig. 2).

Computer-assisted image analysis

Here, we provide clear evidence that the area occupied by the bony trabecules developed in the PA-filled donor tunnels was significantly wider ($p<0.05$) than the area occupied by the control trabecules (Figs. 4, 5).

The data were analyzed using the Mann-Whitney test. The analysis resulted in a significant difference between the averages of the areas covered by the trabecules in each ROI ($p=5.34x10-6$; Fig. 5). The area occupied by the bony trabecules in the PA-filled donor tunnels was 25 % greater than the area in the intact spongiosa next to the control tunnels (Fig. 5).

Polarized light microscopy measurements

We analyzed the structure and the pattern of the osteon unit orientation on the PS-stained sections where the PA residuals showed a homogenous dual-fraction

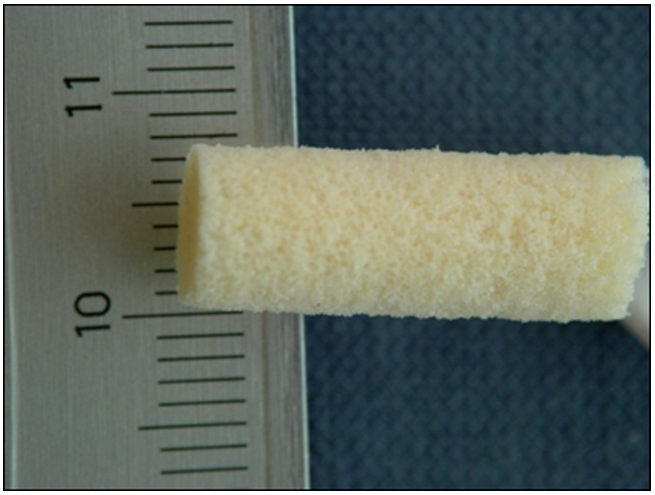

Fig. 1. An image of the biodegradable porous polymer used to fill the tunnels. Scale bar: 1 mm

(Fig. 3). One hundred parallel measurements per sample were performed to explore the collagen fiber orientation, and the statistical analysis did not reveal a significant difference between the PA-filled donor tunnels and the control areas (p=0.5229; Fig. 6). This similarity may be due to the similar spatial orientations of the collagen molecules in the trabecules in the donor tunnels filled with PA vs. those in the control areas.

Discussion

The most important result of our study was that several new osteon units developed in the PA-filled donor tunnels in the two-year period following mosaicplasty. These new osteon units showed microscopically irregular but submicroscopically intact and regular collagen structures.

It has previously been shown that PA has an osteoconductive effect (Radder et al., 1996; Du et al., 2002). To the best of our knowledge, this the first study to compare bone tissue growth under the influence of porous PA to that of the original tissue.

First, using the literature, we aimed to compare the structure of the growing bone tissue under the influence of porous PA to that of the original tissue.

It has been previously reported that PA promotes the reconstruction of the auricular surface in humans, which suggests the clinical usefulness of this biopolymer (Módis et al., 2005a,b). We aimed to answer the following questions: (1) How does bone evolve in the PA-filled material placed into the osteochondral tunnel? (2) Why do the osteon units developed here have a more

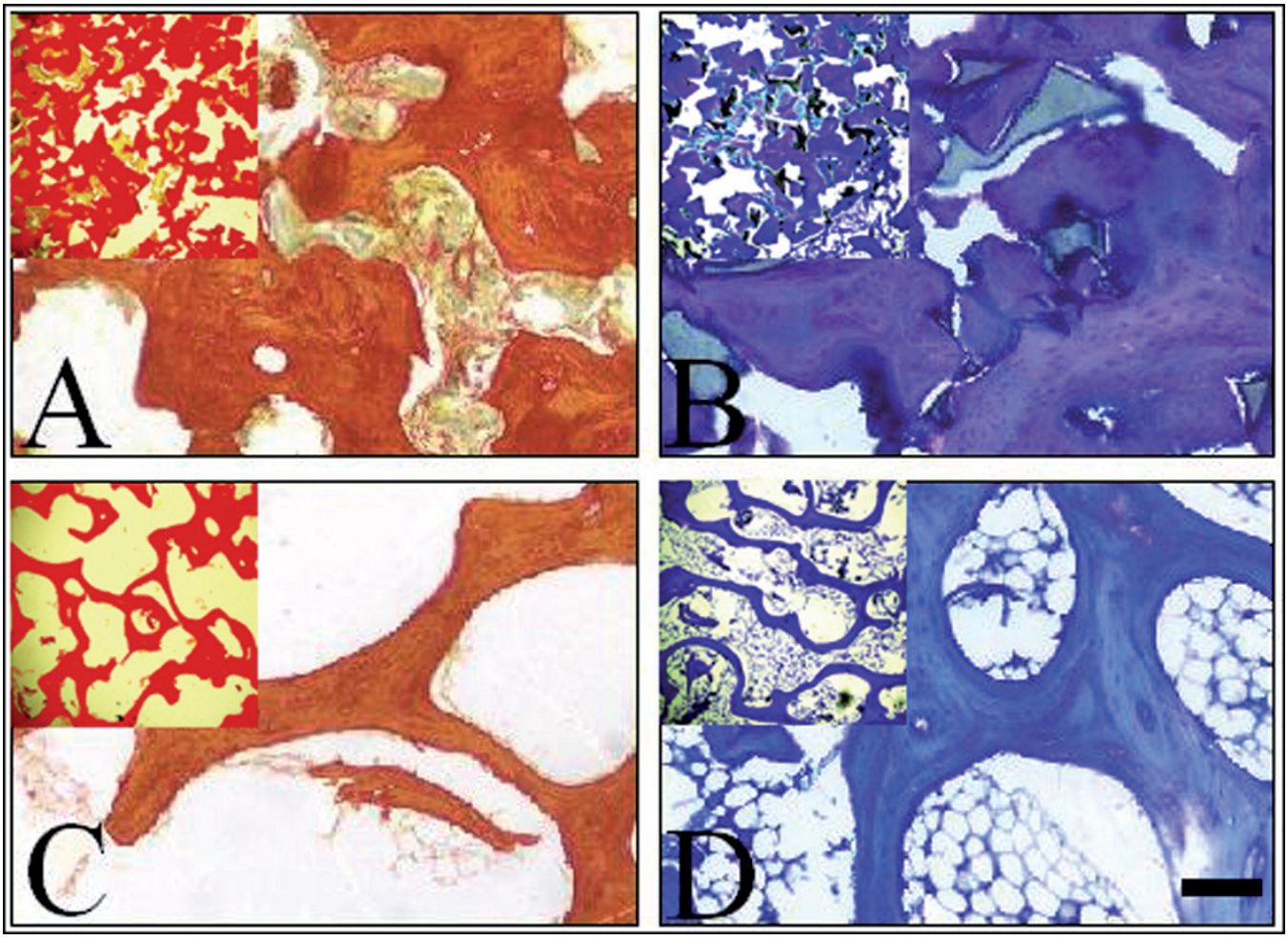

Fig. 2. Representative low (inserts) and high power light microscopy images of picrosirius red- (PS; **A and C**) and dimethylmethylene blue (DMMB; **B and D**)-stained sections of the PA-filled donor tunnels (**A and B)** and the surrounding area (**C and D**; 100x magnification). Meta- vs. orthochromasia (**B vs. D**), the different thicknesses (**A and B vs. C and D**), and the differential regularities of the osteon units of the PA-filled tunnels vs. those in the surrounding area are notable. Scale bar: 100 μm

massive and irregular microscopic structure compared to the intact spongiosa surrounding it? (3) How does one explain the fact that the most abundant macromolecular component of the organic bone matrix, collagen, exhibits an irregular structure under a microscope but still has a regular submicroscopic orientation pattern?

The first question can be answered relatively easily. The porous PA contacts the bone marrow and surrounding intact bone tissue. Osteoprogenitor cells can migrate from the bone marrow (possibly from the surface of the surrounding bone trabecules) to the surface of the PA particles, where they differentiate into osteoblasts that produce the bone matrix. We were unable to determine the mineralization level of the newly evolved bone because we analyzed decalcified tissue sections. We also do not know which PA degradation product can initiate chemical signaling in osteoblasts.

We can suggest a speculative answer to the second question. The surface of the PA is never covered by hyaline cartilage. Rather, it is covered by fibrocartilage, which has a much lower load-bearing capacity. We observed such coverage in some of our samples, but it has also been reported in human biopsy samples (Módis, et al., 2005a,b). It is well known that the proteoglycan (PG) content of hyaline cartilage is significantly higher than that of fibrocartilage (Röhlich, 2006). These molecules, which form massive aggregates and have significant hydration shells, are responsible for the weight-bearing capacity of intact articular cartilage (Helminem et al., 1987). It is possible that the increased amount of trabecular bone compensated for the decreased weight-bearing capacity of the fibrocartilage.

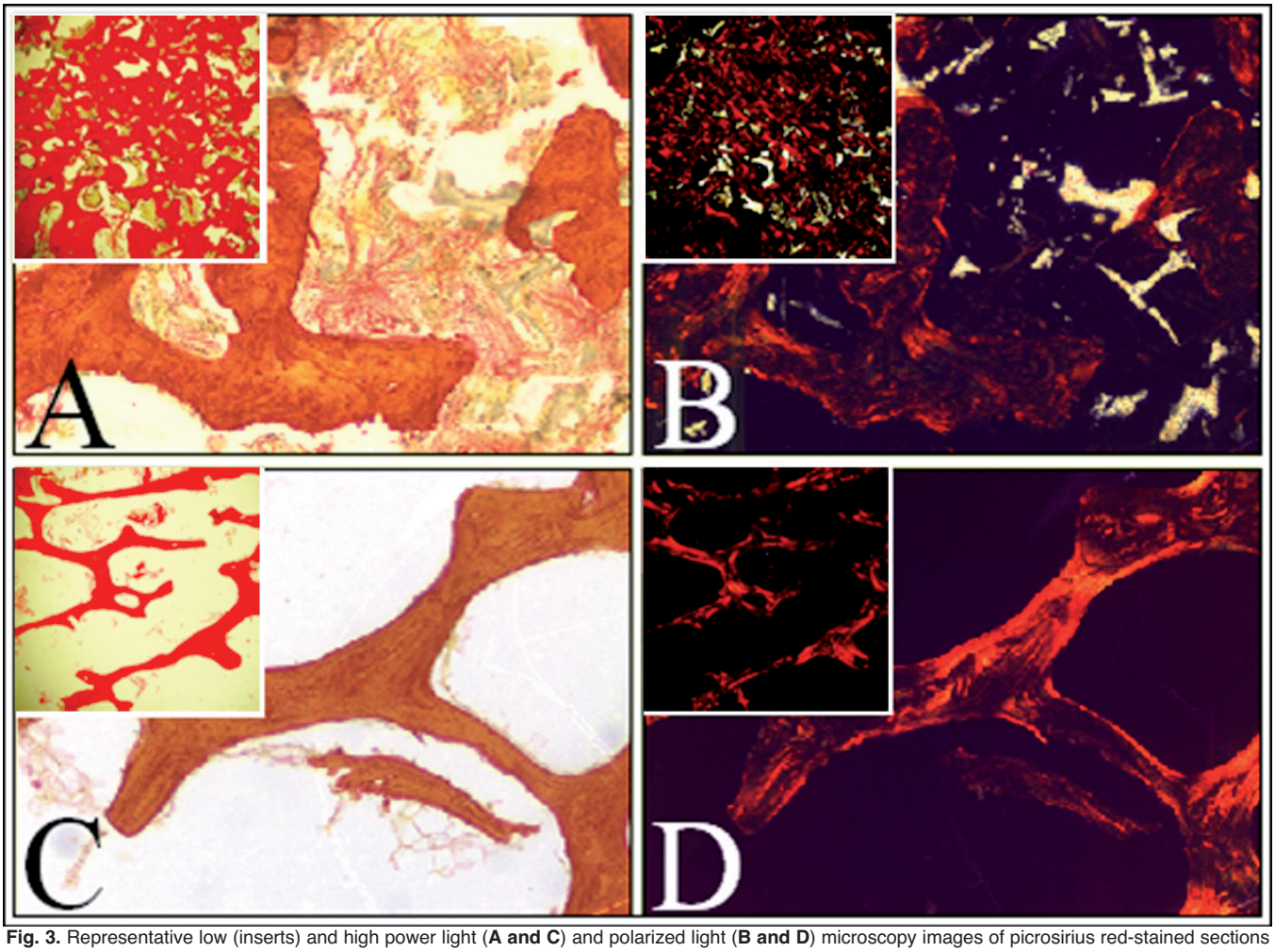

Fig. 3. Representative low (inserts) and high power light (**A and C**) and polarized light (**B and D**) microscopy images of picrosirius red-stained sections of PA-filled donor tunnels (**A and B**) and the surrounding area (**C and D**). The PA particles exhibited a homogenous dual-fraction under polarized light (**B**). Furthermore, the lamellae in the thick new trabecules that evolved in the PA-filled donor tunnels exhibit an irregular ordination compared to their neighbors (**B vs. D**). Scale bar: 100 μm

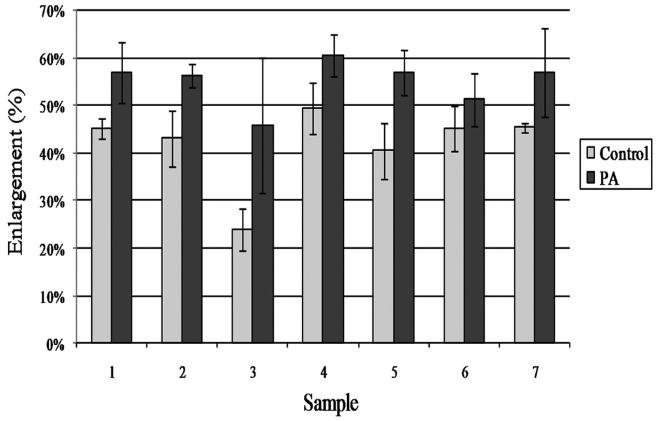

Fig. 4. A column diagram showing the mean area occupied by the bony trabecules ROI (%) in each sample. Five ROI per sample were digitalized from both the PA-filled and control areas. The size of the occupied areas of the trabecules developed in the PA-filled donor tunnels are represented by the black columns, and the control areas are represented by the gray columns.

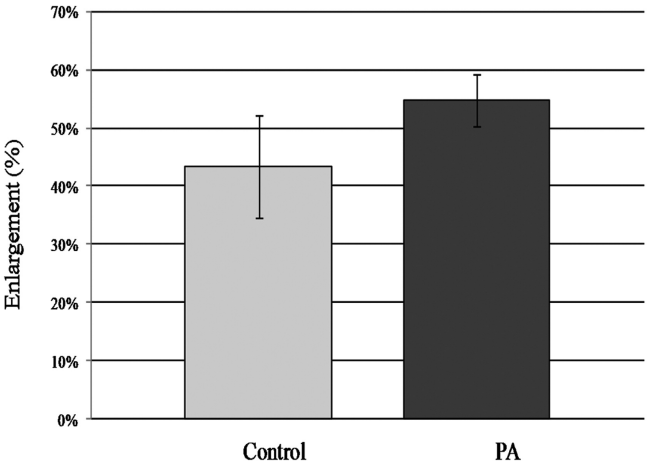

Fig. 5. A diagram showing the average area occupied by the trabecules in each ROI (%). The area enlargement (%) of the control tunnels is in grey, and the area enlargement of the PA-filled donor tunnels is in black. The size of area occupied by the trabecules in the PA-filled donor tunnels was greater than that of the control tunnels.

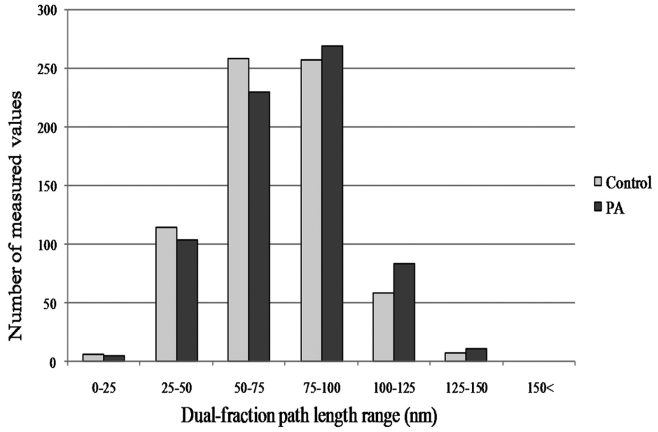

Fig. 6. The results of quantitative polarized light microscopy experiments for the determination of the collagen fiber orientation in the trabecules. The diagram shows the number of measured values belonging to various dual-fraction path length ranges (nm). The grey columns represent the control samples, and the black columns represent the values for the PA-filled donor tunnels. The distribution of the values (nm) of the PA-filled donor tunnels closely resembled the values of the control tunnels. A Mann-Whitney statistical analysis did not reveal a significant difference between the PA-filled and control tunnels (p=0.5229). There was no observed difference between the collagen fiber orientations of the control trabecules vs. the PA-filled trabecules.

The most difficult question to answer is why the orientation pattern of the collagen molecules does not change, although the bone trabecules exhibit an irregular structure compared to the surrounding tissue. The (predominantly type-I) collagen molecules, which comprise the collagen fibers, connect to one another via covalent cross-linking in the extracellular space (Hay, 1991, Módis, 1991, Röhlich, 2006). It is possible that self-assembly creates the regular structure of the collagen fiber until fibrillogenesis is induced by the enzymes that are responsible for the covalent bonds among the collagen molecules, the various glycoproteins that interact with the collagen fibrils, and the PG molecules in the microenvironment. We did not examine these components; we only registered the final result of the process.

Experiences based on additional models in the fields of cell biology and biochemistry could establish the use of this biodegradable and biocompatible material.

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