Fabrication of Precise Cylindrical Three-Dimensional Tissue Engineering Scaffolds for In Vitro and In Vivo Bone Engineering Applications

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It is sometimes necessary to form highly porous polymeric tissue engineering scaffolds into various shapes and sizes. Ideally, in these cases, the three-dimensional morphology should be maintained to the outer margins of the scaffold so as to provide optimum function. Many biodegradable polymeric scaffolds are soft and delicate, however, and their poor physical strength presents a challenge when cutting these materials into the required shapes. We describe a simple device that can be used quickly and accurately to cut cylindrical shapes from such delicate polymeric scaffold materials, which maintain their morphological features to the margins of the shapes produced. We demonstrate that the device can be used to create scaffolds with reproducible dimensions having an SD in mass of less then 6%. The in vitro utility of scaffolds cut with the device was established through demonstrating bone marrow–derived cell invasion into fibrin-filled scaffolds that fit precisely into the wells of 24-well plates. We also demonstrate the in vivo utility of precise cylindrically shaped scaffolds by observing rapid bone invasion into 2.4-mm diameter scaffolds that have been placed into drill hole defects in the distal femur of young rats. When scaffolds are filled with fibrin before implantation as part of a bone tissue engineering strategy, less blood fills the defect site and the fibrin is gradually remodeled and replaced by bone. The ability to cut precise cylindrical scaffolds in the millimeter size range has allowed for the creation of a new small animal model that may prove useful for screening tissue engineering scaffolds for further study.

Key Words: Foam, poly(lactide-*co*-glycolide), tissue engineering scaffold, trabecular bone, fibrin-filled scaffolds

Tissue engineering has evolved as an alternative strategy to autografts and allografts, which have inherent limitations such as donor site morbidity and risk of disease transmission and immune rejection. Tissue engineering strategies generally involve the use of highly porous scaffolds, which may serve as a delivery vehicle for cells and/or growth factors, and the ultimate goal is to replace injured or diseased organs and tissues. During the past decade, many highly porous biodegradable scaffolds have been created and used for a variety of these strategies.[1–3] Methods for reproducibly shaping these scaffolds in a manner that retains the original scaffold porosity and geometry to the margins of the material have not yet been explicitly described. The need for cutting these scaffolds to desired shapes and sizes is of great importance for in vivo applications to fit specific defects and for in vitro applications, where consistent and reproducible samples must be used to perform controlled experiments.

One method to manufacture scaffolds of a desired shape involves the use of individual molds.[4,5] These molds can be used to create a variety of shapes and sizes of scaffolds, but there are many limitations

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with the use of such molds. One potential disadvantage is that the porosity at the outer margins of the created scaffolds that contact the mold is often compromised by the creation of an area with significantly reduced porosity or "skin."[1,2,6] This outer skin occludes the pores and therefore must be removed to ensure cell and tissue ingrowth. Molds are particularly disadvantageous when creating small scaffolds in the millimeter scale range, which may be required for some applications. Furthermore, the cost of making many individual molds for smaller shapes and cleaning them afterward may present considerable inconvenience to commercial scale-up production.

Compression molding can also be used to create scaffolds of various shapes and dimensions.[7,8] The scaffold is formed under pressure and may be compressed at elevated temperatures[5] between matching mold halves. A disadvantage in using compression molding, where elevated temperatures are needed, is that biologically active species, which can be incorporated into the starting materials, may be compromised or the polymer may thermally degrade.

Other methods used for shaping scaffolds involving free-hand cutting methods with cork borers[9,10] or other sharp objects are time-consuming and do not allow production of small-sized scaffolds having reproducible dimensions.

This work describes a device that can be used to cut soft materials such as biodegradable tissue engineering scaffolds efficiently and accurately while preserving interconnected macroporosity to the margins of the scaffold. Cylindrical scaffolds with reproducible dimensions are useful for exploring a variety of bone tissue engineering strategies. In vivo results are discussed when scaffolds prepared with the described cutting device were implanted into the distal femur of rats and demonstrated to support rapid invasion of host bone tissue. Furthermore, both in vitro and in vivo results are described for an emerging bone tissue engineering strategy,[11] which involves filling scaffolds with a cell-invasive matrix.

Materials and Methods

Scaffold Fabrication

Pure poly(lactide-*co*-glycolide) (PLGA) 75:25 scaffolds were produced by modifying a previously described technology.[12] Briefly, PLGA with an inherent viscosity of 1.13 dl/g was purchased from Birmingham Polymers (Birmingham, AL). Analytical grade dimethylsulfoxide was purchased from BDH (Toronto, Ontario, Canada). Sugar crystals were purchased form Redpath Sugar (Toronto, Ontario, Canada). US standard sieves (0.85-mm and 1.18-mm pore size) and Teflon fluorinated ethylene propylene (FEP)-coated aluminum foil were purchased from VWR Canlab (Mississauga, Ontario, Canada). Deionized double-distilled water was obtained from Millipore Milli-RO 10 Plus and Milli-Q UF Plus systems (Bedford, MA) operated at 18 MΩ resistance. The starting PLGA 75:25 three-dimensional scaffold blocks were prepared by dispersing glucose crystals having 0.85- to 1.18-mm dimensions in a solution of PLGA 75:25 in dimethylsulfoxide (5%, 6%, 7%, 8%, 9%, and 10% PLGA [wt/vol]). The sugar/polymer mixture was then placed in a Teflon FEP-coated aluminum mold 10 cm × 10 cm × 3 cm and allowed to set. When the polymer precipitated, the glucose crystals were extracted from the polymer, which resulted in a three-dimensional scaffold block having macroporous interconnected porosity. The dimensions of the resultant scaffold were 10.0 cm × 10.0 cm × 1.2 cm.

For the CaP-PLGA scaffolds, an equimolar mixture of dicalcium and tetracalcium phosphate was reacted to form hydroxyapatite, and this was broken into small particles. Particles less than 45 μm were dispersed with glucose crystals having 0.85- to 1.18-mm dimensions in a solution of 11.5% (wt/vol) PLGA in dimethylsulfoxide. The sugar/polymer/calcium phosphate mixture was placed in the Teflon FEP mold, allowed to set, and processed as described previously, resulting in macroporous scaffolds.

Cutting Device

The cutting device was turned on a lathe from a stainless steel (316 grade) cylinder. Three different cylinder sizes (10.0 mm, 4.4 mm, and 2.4 mm in diameter) were manufactured. One end of the cylinder was used to create the cutting edge, whereas the other end was reduced in diameter to 0.508 or 0.3175 cm to fit either a standard drill or Dremel (Model 398), respectively. The use of a high-speed Dremel with a digital control and a drill press (Multipro Deluxe drill press stand, Model 212, type II) allowed for precisely controlled travel of the cutting device through the foam. The cylindrical scaffolds were cut to the desired length using a custom-made Teflon guiding device and a standard double-edged razor blade. The cutting devices were ultrasonically cleaned with acetone and then with Decon and were then rinsed with double-distilled water, 70% ethanol, and 100% ethanol before use.

Cell Isolation

Rat bone marrow cells were obtained according to previously published methods.[13] Briefly, femora

were excised from young adult (115–125 g) Wistar rats (Charles River, Quebec, Canada) and transferred into an antibiotic/Fungizone solution consisting of penicillin G (1,670 U/ml), gentamicin (500 μg/ml), and amphotericin B (3 μg/ml). After 10 minutes, femora were transferred into a fresh aliquot of the same solution. This washing procedure was repeated three times. Subsequently, the femora were transferred into α-minimal essential medium. Epiphyses were removed, and the marrow from each diaphysis was flushed out with 15 ml of α-minimal essential medium supplemented with 15% (vol/vol) fetal calf serum, 100 mg L-ascorbic acid, 5 mM β-glycerophosphate, and 10^{-8} M dexamethasone, together with antibiotics and Fungizone at one tenth of the concentration used during excision. Marrow cells of both diaphyses were collected and cultured in the supplemented α-minimal essential medium.

Cell Culture

Subcultured rat bone marrow cells were placed into individual wells of 24-well plates at a seeding density of 10^4 cells/cm^2. After 5 to 6 days, depending on cell confluency, fibrin was polymerized into a scaffold on top of the cells. Medium was changed three times a week.

Surgical Implantation

For testing bone ingrowth in vivo, ethanol prewetted PLGA-CaP cylindrical scaffolds (2.4 mm in diameter × 2.5 mm in height) were washed with culture medium and filled with fibrin before implantation into bone defects (approximately 2.5 mm in diameter) in the middiaphysis of both the right and left femurs of six young (125–150 g) male Wistar rats. The maintenance and use of animals were in accordance with the Canadian Council of Animal Care Guidelines. Controls consisted of implanting empty prewetted scaffolds. After 2, 4, and 7 days in vivo, the femurs were removed, fixed, decalcified, and embedded in low-melting-point paraffin. Serial sections perpendicular to the long axis of the implant were obtained using a Spencer 820 microtome. The sections were stained with Masson trichrome stain.

Filling Scaffolds With Fibrin

For in vitro work, 1.5-cm diameter, 3-mm long scaffolds cut with our custom-built cutting device were prewetted with 70% ethanol and rinsed three times with double distilled H_2O before being placed on top of day 5 to 6 subcultured cells located within individual wells of 24-well plates. Autoclaved Silastic tubing was placed on top of the scaffolds to secure them in place. Two hundred fifty microliters bovine fibrinogen–containing solution was pipetted into the scaffold, followed by 250 μl thrombin-containing solution, and this was quickly mixed. The fibrin was prepared to a have final concentration of 3 mg/ml fibrinogen, 1.25 NIH U/ml thrombin, and 20 μmol/ml $CaCl_2$. Medium was changed three times a week with the addition of 225 KIU/ml aprotinin, and after 9 days in culture, samples were fixed in a 2% formaldehyde and 2% glutaraldehyde mixture and prepared for cryosectioning. Bovine fibrinogen, thrombin, and aprotinin were obtained from Sigma Chemical Company (St. Louis, MO).

For in vivo work, the same concentration of fibrin used for the in vitro experiments and a similar process for filling scaffolds with the fibrin were used. Briefly, Silastic tubing was firmly secured at the bottom of individual wells of 96-well culture plates, and scaffolds were placed into the wells and pushed to the bottom. Fifteen microliters reconstituted bovine fibrinogen (6 mg/ml) was pipetted into the scaffold, followed by 15 μl reconstituted thrombin (2.5 NIH U/ml) containing 40 μmol/ml $CaCl_2$. The two solutions were quickly mixed by pipetting in the scaffold and allowed to gel in an incubator for 30 to 60 minutes at 37°C, 5% CO_2, and 100% relative humidity. Scaffolds were removed from the multiwell plates and closely examined under a dissecting microscope to ensure that the scaffolds were completely filled with fibrin. These scaffolds as well as empty control scaffolds were placed into an α-minimal essential medium 225-KIU/ml bovine aprotinin solution, and this was placed in an incubator at 37°C overnight.

Cryosectioning Fibrin-Filled Scaffolds

After 9 days in in vitro culture, fibrin-filled scaffolds were removed from 24-well plates, rinsed in double distilled H_2O, and placed into wells of a 12-well culture plate. Tissue freezing medium (Fisher Scientific) was poured onto these scaffolds and subsequently placed under a vacuum at 635 mm Hg for 4 hours and cryosectioned at –30°C (Ames Lab-Tek cryostat, Elkhart, IN). Glass slides used to collect the sections were acid-cleaned before use and coated with gelatin. Sections on glass slides were maintained at 40°C overnight. Before staining, slides were immersed in water for 10 minutes to remove the tissue freezing medium. Sections were stained with either Gomori trichrome or Toluidine blue. Histological sections were examined by light microscopy (Leitz, Heerbrugg, Switzerland), and pictures were taken with 200 ASA film.

Results and Discussion

Scaffold blocks (10 cm × 10 cm × 1.2 cm) (Fig 1A) were created using large molds from which individual scaffolds were cut. Thus, many small-sized highly porous scaffolds were cut using the cutting device described below while maintaining the individual scaffold geometry. The cutting device comprised an elongated shank and a hollow cutting cylinder with a smooth continuous circular cutting edge (see Fig 1B). The device was made from medical grade (316) stainless steel, which was resistant to corrosion and could be readily cleaned and sterilized. The shank can be made to different dimensions to fit various drill chucks. After the device was mounted in a Dremel (see Fig 1C), it was used to cut very small to very large cylindrical foams as well as cylindrical channels within the scaffolds (Fig 2). The cylindrical features can be varied according to diameter and length.

To test the reproducibility with which the device can cut cylindrical scaffolds of various dimensions, we first created large starting blocks (10.0 cm × 10.0 cm × 1.2 cm) of PLGA scaffolds having a pore size of approximately 0.85 to 1.18 mm in diameter. Next, these starting scaffolds, which resemble the trabecular bone in morphology,[14] were used as sample materials for the cutting device.

We assessed the cutting precision by first obtaining the mass deviation of 100 10.0-mm diameter scaffolds, which were randomly selected. Next, we used a selection criterion that only included scaffolds with a mass of 23.0 mg ± 3.0 mg. Implementation of the selection criterion was necessary, because some of the scaffolds had a nonhomogeneous pore nature as a result of the processing. Of 100 samples, 9 were eliminated from the sample pool because they were considered outliers. The SD in mass calculated for the 10.0-mm diameter scaffolds was 5.3% and can be considered an acceptable value of error.

To determine the limitations of the device with respect to the physical strength of the scaffold, PLGA scaffold blocks having 5%, 6%, 7%, 8%, 9%, and 10% (wt/vol) PLGA concentrations in dimethylsulfoxide were cut. The physical strength of the tested scaffolds was found to increase with increasing PLGA content.

The limiting factor during manufacturing of the final cylindrical scaffold was found to be the physical strength of the initial scaffold block. The 5% (wt/vol) PLGA scaffolds were found to be fragile to handle and collapsed during the cutting process. All the other scaffolds that were made from 6% to 10% (wt/vol) PLGA were cut while maintaining the interconnected macroporosity throughout the scaffold (Fig 3). It was also found that cutting the block scaffolds while they were wet improved the scaffold durability. This may have resulted from the fact that poly(DL-lactic-co-glycolic acid) has a relatively low

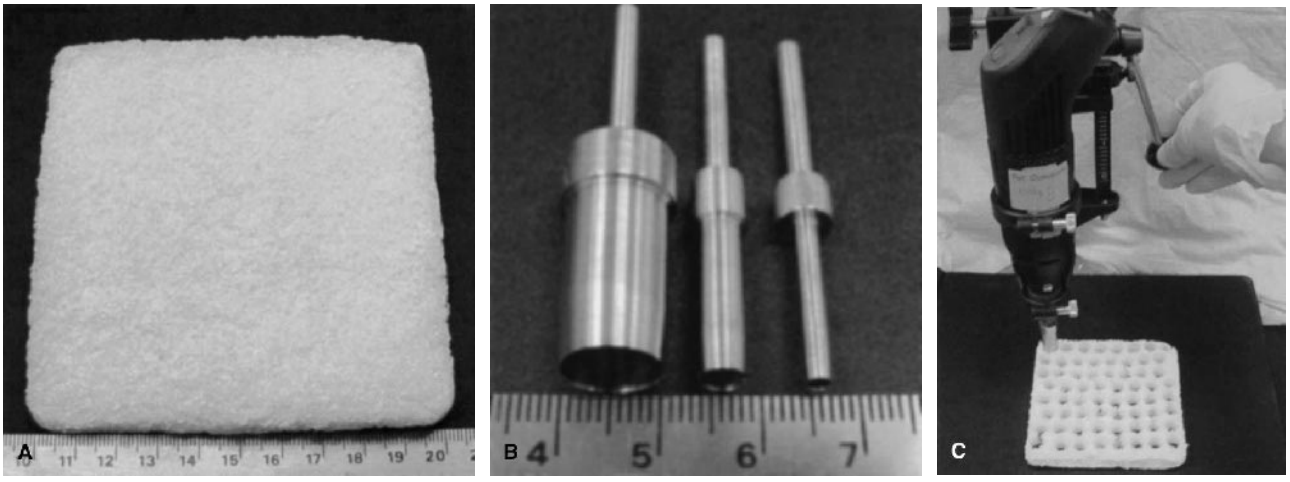

Fig 1 (A) Macroporous scaffold block. Large poly(lactide-*co*-glycolide) (PLGA) scaffold blocks (10.0 cm × 10.0 cm × 1.2 cm) were created using the Teflon fluorinated ethylene propylene (FEP)-coated aluminum mold. (B) Stainless steel cutting tools. Cutting devices having various diameters were fabricated. From left to right, the diameters of the devices are 10.0 mm, 4.4 mm, and 2.4 mm. Smaller diameter devices can be used to cut scaffolds with a reduced pore size. (C) Cutting the PLGA scaffolds. Many cylindrical scaffolds were cut from one PLGA scaffold block by placing the cutting tools into a digitally controlled high-speed Dremel housed in a Dremel press. This allowed for precisely controlled rotational speed and travel of the cutting device through the scaffold blocks.

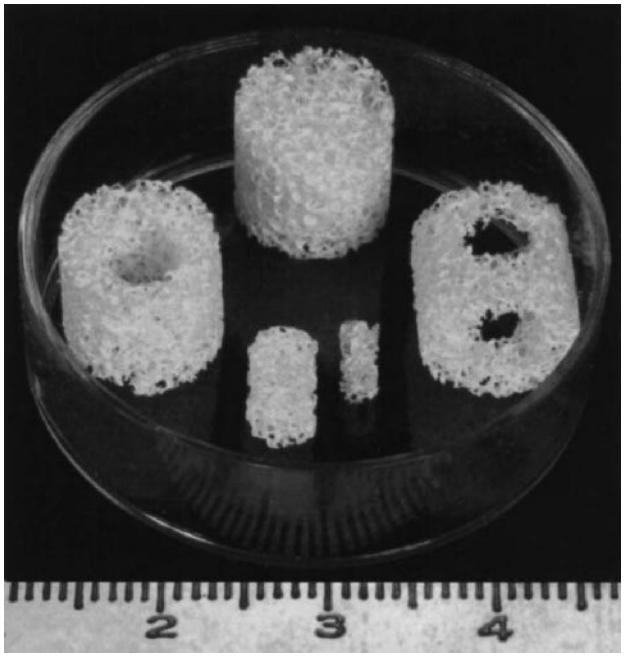

Fig 2 Samples of cut scaffolds. The cutting device can cut a wide variety of sizes of cylindrical scaffolds from larger scaffold blocks. In addition, the device can be used to create channels, both radial and axial, within scaffolds for promoting cell migration and vascular ingrowth. These channels can also be used for incorporation of drug delivery devices and other organic and inorganic materials as well as for seeding such scaffolds with cells. The scaffolds have a high degree of interconnected macroporosity, which mimics the structure of trabecular bone (space between each number represents 1 cm).

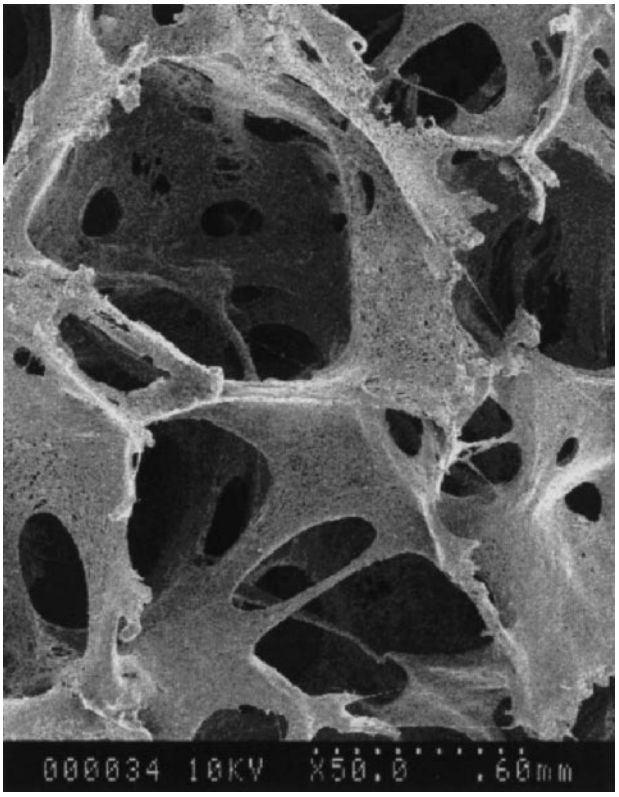

Fig 3 Representative image of cut scaffold. Once cut, the resultant scaffolds maintain their morphological features to the outer margins of the shapes produced.

glass transition temperature (approximately 45°C–50°C),[15] and the presence of water may have produced less heat.

In Vitro Experimentation

The utility of the described cutting tool was demonstrated through an in vitro cell invasion assay (Fig 4) used to determine if bone marrow–derived cells could attach to a distant surface after migrating through a fibrin-filled scaffold. The cutting tool allowed the creation of scaffolds that fit exactly into the wells of a 24-well plate. After 9 days, cells were observed at positions above the underlying cell layer (Fig 5), and many cells had a spindle-like morphology, which is representative of migrating cells. Cells migrated from the underlying substrate, through the fibrin matrix, to the scaffold surface. Once the cells

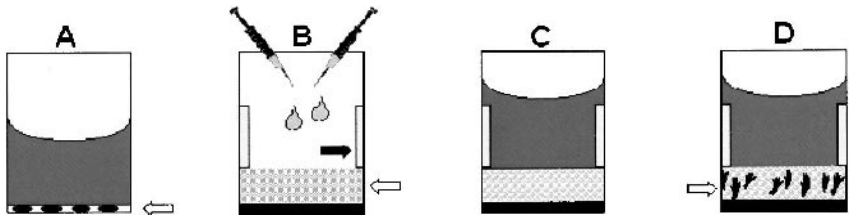

Fig 4 Procedure for filling scaffolds with fibrin (in vitro). (A) Subcultured rat bone marrow–derived cells were seeded into individual wells of a 24-well plate. (B) Once the cell layer became confluent, fibrin was polymerized in a scaffold that was secured over the underlying cell layer with Silastic tubing. (C) Medium was changed three times a week. (D) After 9 days, cultures were terminated and fixed.

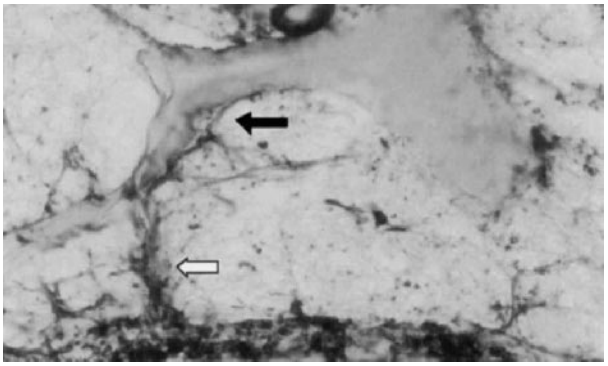

Fig 5 In vitro cell invasion of fibrin-filled scaffolds. Rat bone marrow cells can been seen migrating from an underlying cell layer through fibrin and attaching to the scaffold surface after 9 days in vitro. A collagenous matrix containing cells (white arrow) is observed, most probably having been deposited by the migrating cells. Once cells reach the scaffold surface, they seem to attach and migrate along the scaffold surface (black arrow).

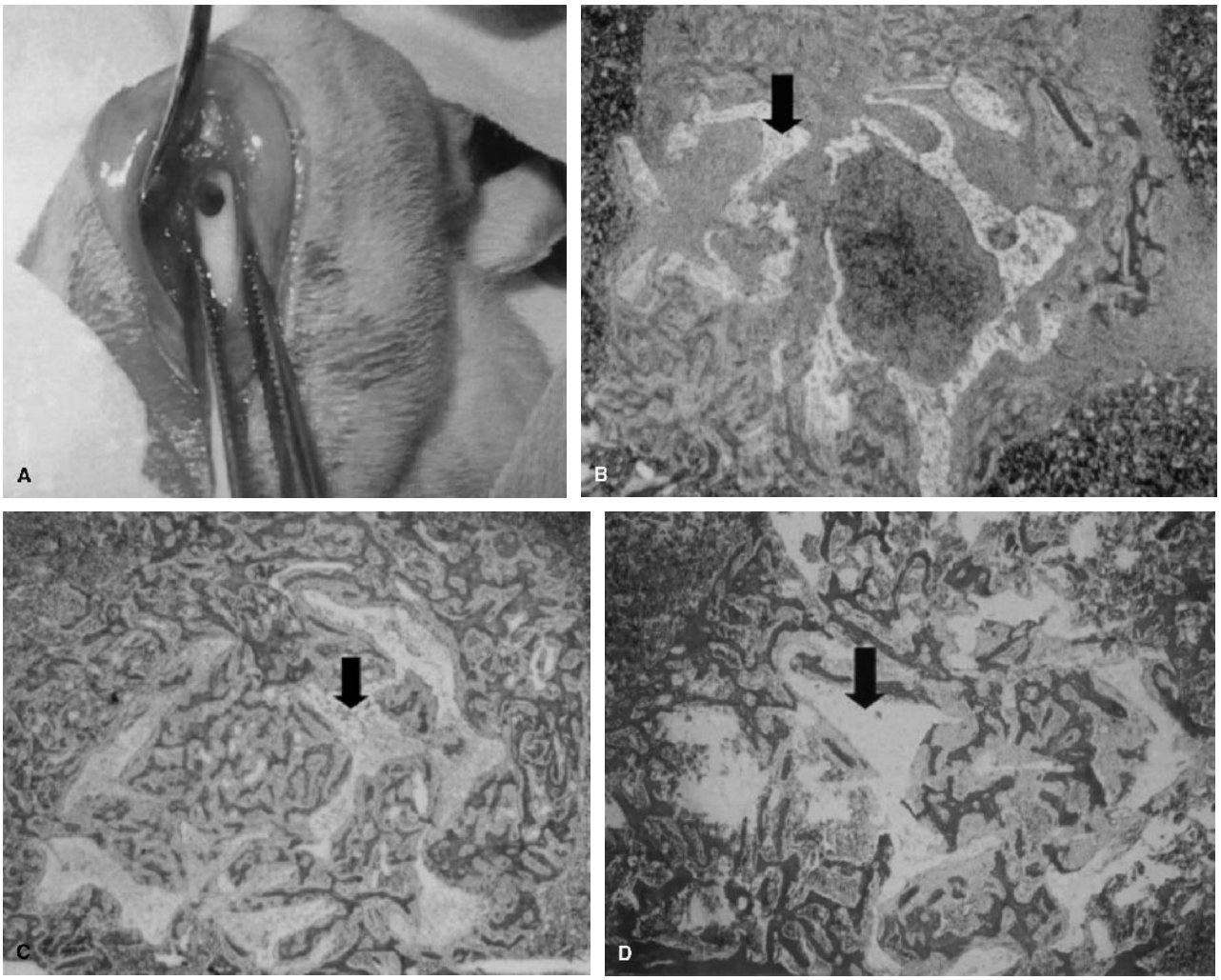

Fig 6 Surgical procedure and in vivo histology. (A) Rats were anesthetized, and a drill hole defect was created in the right and left distal femurs. (B) A significant amount of bone tissue had already invaded poly(lactide-*co*-glycolide)–CaP scaffolds after 4 days in vivo. (C) The pores of these scaffolds were filled with bone tissue after 7 days in vivo. (D) Connective tissue also rapidly invaded scaffolds prefilled with fibrin as evidenced after 7 days in vivo. Arrows point to regions once occupied by the scaffold, which have been dissolved during histological processing. All field widths are 3.2 mm.

reached the scaffold surface, they seemed to attach and migrate along the scaffold. It also seemed that struts in the scaffold touching the underlying cell layer on the tissue culture dish supported the direct migration of bone marrow–derived cells (not shown). Therefore, some cells seemed to have migrated solely along the scaffold surface, which may provide a conduit to the middle of the scaffold.

In Vivo Experimentation

The utility of the cutting tool was further demonstrated by creating 2.4-mm diameter scaffolds that fit precisely into the approximately 2.5-mm defect sites (Fig 6A). This allowed for the creation of a new small animal model for testing small-diameter scaffolds that would not have otherwise been possible. After 4 days in vivo (see Fig 6B), bone had already begun to form within the pores of the scaffold. This was evidenced from the morphology and location of color in the histological sections stained with Masson trichrome, which stains collagen blue. After 7 days in vivo (see Fig 6C), bone had completely invaded the pores of the empty CaP-PLGA scaffold.

Fibrin-filled scaffolds reduced the amount of blood that filled the defect site, as evidenced after 2 days in vivo (not shown). After 7 days, the fibrin had been completely remodeled and replaced by new connective tissue, and bone was observed throughout the pores of the scaffold (see Fig 6D). Such experiments help to validate the use of filling scaffolds with a cell-invasive matrix for tissue engineering applications.

Taken together, these data demonstrate that the cutting tool we have created provides great utility in investigating tissue engineering strategies both in vitro and in vivo. Precise cylindrical scaffolds with millimeter-sized diameters have allowed the creation of a new small animal model that may prove useful for histomorphometric screening of tissue engineering scaffolds.

Conclusions

The cutting device we have created can be used quickly and accurately to fabricate cylindrical shapes from delicate polymeric scaffold materials having interconnected macroporosity. The resultant scaffolds maintain their morphological features in the bulk and at the margins of the shapes produced. The device can be machined to cut different size scaffolds, especially those with small diameters, which would otherwise be difficult to fabricate. Furthermore, scaffolds cut with this device can be used for a variety of in vitro and in vivo tissue engineering applications.

The authors thank Keith Porter for custom making our stainless steel cutting tools.

This work was supported by grants from BoneTec Corporation and the Ontario Research and Development Challenge Fund as well as a Natural Sciences and Research Council of Canada postdoctoral fellowship to Dr Rzeszutek and an Ontario Graduate Scholarship postgraduate fellowship awarded to Jeffrey M. Karp.

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