

## Enhanced Oxygen Supply Improves Islet Viability in a New Bioartificial Pancreas

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The current epidemic of diabetes with its overwhelming burden on our healthcare system requires better therapeutic strategies. Here we present a promising novel approach for a curative strategy that may be accessible for all insulin-dependent diabetes patients. We designed a subcutaneous implantable bioartificial pancreas (BAP)—the “ $\beta$ -Air”—that is able to overcome critical challenges in current clinical islet transplantation protocols: adequate oxygen supply to the graft and protection of donor islets against the host immune system. The system consists of islets of Langerhans immobilized in an alginate hydrogel, a gas chamber, a gas permeable membrane, an external membrane, and a mechanical support. The minimally invasive implantable device, refueled with oxygen via subdermally implanted access ports, completely normalized diabetic indicators of glycemic control (blood glucose intravenous glucose tolerance test and HbA<sub>1c</sub>) in streptozotocin-induced diabetic rats for periods up to 6 months. The functionality of the device was dependent on oxygen supply to the device as the grafts failed when oxygen supply was ceased. In addition, we showed that the device is immunoprotective as it allowed for survival of not only isografts but also of allografts. Histological examination of the explanted devices demonstrated morphologically and functionally intact islets; the surrounding tissue was without signs of inflammation and showed visual evidence of vasculature at the site of implantation. Further increase in islets loading density will justify the translation of the system to clinical trials, opening up the potential for a novel approach in diabetes therapy.

Key words: Diabetes; Bioartificial pancreas (BAP); Islets; Implantation; Immune barrier; Oxygen

### INTRODUCTION

A major goal in the treatment of insulin-dependent diabetes is to improve the patient's quality of life by providing the patient with an insulin source that regulates the glucose levels on a mandatory minute-to-minute basis. Presently, this can be achieved only following transplantation of donor pancreatic islets (32). Many efforts have been made to make islet transplantation a widely applicable procedure to cure diabetes (10). Unfortunately, in recent years, many obstacles that prevent islet transplantation from

being an acceptable alternative for insulin therapy have been identified. The first is the requirement for more than one donor per recipient (34). This is a major drawback in a world of severe shortage of donor organs. Second, immunological and morbidity issues have been described (10). Furthermore, the procedure may not allow retransplantation as recipients may develop allotypic immunization (9), which may obstruct future transplantations in the same patient, and, last but certainly not least, islet allotransplantation requires lifelong use of immunosuppressive therapy.

Thus, islet transplantation is currently not considered an acceptable alternative for insulin therapy.

All the aforementioned issues could be solved with immunoisolation of pancreatic islets. Immunoisolation does not require the use of immunosuppressive agents and, theoretically, allows for transplantation of islets from non-human sources, thereby solving the donor shortage problem. The principle applicability of immunoisolation was previously shown in experimental systems (1,12,14,24) and in pilot clinical trials (17). However, encapsulated islets have a limited graft survival time that has been attributed to lack of sufficient supply of oxygen (13,21).

A mathematical model developed by Dulong and Legallaise (15) clearly predicts the physiological limitations of encapsulated islets when transplanted into a site with venous-type oxygen tension. Essentially, inadequate oxygen supply caused loss of islet viability and reduced their capacity to secrete insulin (13,21). These effects are exacerbated in encapsulated islets (2,3,11,16,27,28). Several solutions have been proposed to generate oxygen close to the capsules or to reduce transport limitations imposed by the encapsulation system. Some experimentally tested systems include direct supply of oxygen to cultured cells by means of electrochemical generator (39) or to isolated islets by local photosynthesis (6). Unfortunately, these systems cannot generate the amount of oxygen required to maintain the viability of islets in clinical doses. Other approaches to increase oxygen supply and graft survival include inducing angiogenesis around the devices (26), the application of islet cell clusters of small diameter to reduce diffusional distance to the islet's core cells (20,25,30), use of hypoxia-resistant islet cells (38), and the use of islet cells engineered to contain intracellular oxygen carrier such as myoglobin (35). Notably, none of these approaches attempted to increase oxygen supply by active means. An approach utilizing active supply of oxygen has been the focus of our research efforts in recent years; such an approach not only enhances the *in vivo* survival rates of islets in the current devices implanted in the subcutis but may also facilitate increasing the islet dose in the devices.

In this study, we investigated a comprehensive approach that allows for active oxygen supply to the islets in an immunoisolating device. It involves an immune barrier, an adequate oxygen supply, and a minimally invasive surgical procedure.

## MATERIALS AND METHODS

### *Animals, Diabetes Induction, and Pretreatment*

All experiments were approved by the Israeli National Ethical Committee. Lewis and Sprague–Dawley rats were purchased from Harlan (Rehovot, Israel). Diabetes was

induced in Lewis rats (260–280 g) by a single intravenous infusion of 85 mg/kg body weight of streptozotocin (STZ; Sigma, Rehovot, Israel). This treatment induced irreversible diabetes (nonfasting blood glucose >450 mg/dl) in >90% of rats in our colony. In addition, levels of c-peptide, measured from serum samples by ELISA (Alpco Diagnostics, Salem, NH, USA), in induced animals ( $n=6$ ) were under detection limits 30 min after receiving a glucose (Sigma) bolus of 0.5 g/kg body weight (data not shown). Animals were considered diabetic when nonfasting blood glucose exceeded 450 mg/dl for  $\geq 4$  consecutive days. To prepare the diabetic animals for device implantation in a nonstressing, close-to-normal blood glucose environment, 1.5 capsules of slow released insulin (Linplant, LinShin, Toronto, ON, Canada) were inserted under the skin of the diabetic animals and removed 48 h after implantation (so the device would become the only source for insulin). Animals were considered ready for implantation when their nonfasted blood glucose was <250 mg/dl for  $\geq 3$  consecutive days. Efficacy of glycemic control was followed for 30–180 days postimplantation. The functionality of the device was assessed by three different diagnostic tests: daily measurements of nonfasting blood glucose, intravenous glucose tolerance tests (IVGTT) performed at 3, 9, and 12 weeks postimplantation, and the level of glycosylated hemoglobin (HbA1c) determined at day 90 postimplantation. To avoid false-positive results associated with regeneration of the native pancreas, blood glucose was followed for a period of time after the removal of the device.

### *Islet Isolation, Cultivation, and Enumeration*

Pancreata were obtained from 9- to 10-week-old male Lewis or Sprague–Dawley rats weighing 260–280 and 300–320 g, respectively. Standard collagenase digestion of the donor pancreata was used with some modifications. Briefly, each pancreas was infused with 10 ml enzymatic digestive blend containing 15 PZ units collagenase NB8 (Serva, Heidelberg, Germany) and 1mg/ml bovine DNase (Sigma, cat. no. 159001) in Hank's balanced salt solution (HBSS; Biological Industries, Bet HaEmek, Israel) for 14 min. Islets were purified on discontinuous Histopaque gradient [1.119/ 1.100/1.077/ RPMI (Sigma)] run for 20 min at 1750 g/max in the cold (6°C). They were then washed twice and cultured in complete CR medium [Connaught Medical Research Laboratories (CMRL): Roswell Park Memorial Institute (RPMI) medium (1:1) supplemented with 10% fetal bovine serum (all from Biological Industries)] for a period of 5–8 days before being integrated into the implantable devices.

For determining number of cells in a rat islet equivalent (IEQ), we selected 21 different lots containing 50–60 standard islets each. Islets were defined as “standard” when diameters of both  $x$ - and  $y$ -axes were estimated

to be 150  $\mu\text{m}$ . For enumeration of cells, the method of Pisania et al. (33) was used and a value of  $1,556 \pm 145$  cells/IEQ was obtained. Immediately after isolation, rat islets were subjected to the same enumeration protocol and found to contain  $1,430 \pm 185$  cells/islet ( $n=10$ ). At the time of device assembly (6–8 days after isolation), we enumerated  $1,270 \pm 280$  cells/IEQ ( $n=107$ ). During the cultivation period, cell count in an average islet declined by  $<20\%$ , and therefore, at time of implantation, an islet particle was estimated to correspond to 0.8 IEQ.

Islet particles were either isogenic (i.e., derived from Lewis rats and implanted into diabetic Lewis rats) or allogeneic (i.e., derived from Sprague–Dawley rats and implanted into diabetic Lewis rats).

#### *Oxygen Consumption Rate*

Oxygen consumption rate (OCR) of naked islets was determined with samples of 250 IEQ suspended in RPMI:CMRL (1:1) and supplemented with 1% fetal bovine serum. Measurements were carried inside a 620- $\mu\text{l}$  chamber equipped with stir bar and placed in a temperature control unit (Eurotherm 808; Eurotherm, Worthing, UK) kept at constant temperature of 37°C. OCR of preimplanted, encapsulated islets was similarly determined with samples of 250 IEQ immobilized in 30  $\mu\text{l}$  of ultrapure, high guluronic acid (68%) alginate (UP-MVG, Novamatrix). OCR of postimplanted islets was determined following explantation of the device, release of the alginate slab, and manual counting of islets using doses of  $\geq 200$  islets. Oxygen concentration in the gas chamber of the device was determined by injecting a sample taken from the interior of the gas chamber into the oxygen measurement cell in a gas phase, into which a Clark-type oxygen electrode (Unisense, Aarhus, Denmark) was inserted. Oxygen partial pressure in liquids and gases was measured using a Unisense oxygen measurement system including a micromanipulator, Clark-type microelectrode, and PA2000 picoammeter controller (Unisense). Data were analyzed using Labview 7.2 software (National Instruments, Austin, TX, USA). OCR of naked islets was calculated as previously described (31). OCR of the device was calculated from the change in oxygen concentration in a gas chamber of known volume 24 h after refueling with a known oxygen concentration. The decrease in oxygen concentration was compared to the total OCR of the islets during the 24 h (see below).

#### *Subkidney Capsule Transplantation*

Doses of 900 IEQ of either isogenic (Lewis) or allogeneic (Sprague–Dawley) rat islets were used for these experiments. Islets were separated from the tissue culture medium by low force ( $140 \times g$ ) centrifugation and concentrated to a volume of 5  $\mu\text{l}$ . For naked islet transplantation, a 3-mm incision was made in the kidney capsule

and the entire dose of islets was infused into the formed pouch. For implantation of encapsulated islets, 40  $\mu\text{l}$  of 2.2% (w/v) high mannuronic acid (M=54%) alginate (UP-MVM, Novamatrix, Sandvika, Norway) was mixed with the islet pellet and poured into a stainless steel mold (diameter, 9 mm; width, 0.5 mm; generated in house from 316 stainless steel) at density of 1,400 IEQ/ $\text{cm}^2$  in face surface area. In order to obtain homogeneous cross linking of the hydrogel polymer, the mold containing the islet-alginate suspension was covered with a sintered glass disc (Robu Glas 1530, Robu Galsfilter Gerate, Hattert, Germany) presoaked with the cross-linking solution [70 mM  $\text{SrCl}_2$  (Sigma); 20 mM NaCl (Sigma), 20 mM HEPES pH 7.4 (Biological Industries); 270 mOsm] for 12 min. A 9-mm incision was made in the kidney capsule, separated from the kidney cortex, and the slab was inserted into the formed pouch using watchmaker forceps (Bochem Instrumente GmbH, Weilburg, Germany). Two identical slabs were implanted, one underneath each kidney capsule.

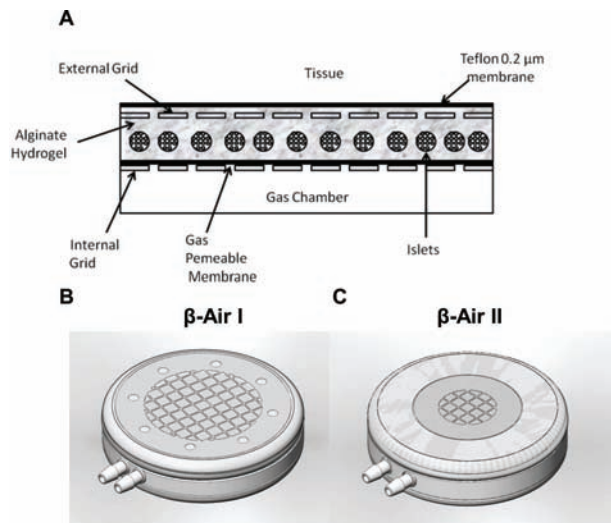
#### *Implantable Devices*

The bioartificial pancreas (BAP) prototypes (Fig. 1A) are subcutaneously implanted disc-shaped devices consisting of two major components—an islet module containing islets encapsulated in an alginate hydrogel slab, which is separated from the implantation pocket, and a gas chamber, which is separated from the islet module.

The housing of the device is made of clinical grade polyether ether ketone (PEEK Optima LT1R40; Invibio, Thornton Cleveleys, Lancashire, UK). Two BAP prototypes were investigated, designated “ $\beta$ -Air” I and II. The two prototypes have identical external diameters and materials of construction and were loaded with the same islet dose. They differ in the surface area of the islet-containing slab, the method of gas chamber ventilation, and the composition of the infused gas mixture. Surface density of the islets in “ $\beta$ -Air II” is more than twice that of “ $\beta$ -Air I.” Also, “ $\beta$ -Air I” uses a continuous supply of atmospheric air whereas oxygen-enriched gas mixture (40% oxygen and 5%  $\text{CO}_2$ ) is injected once a day into the gas chamber of the “ $\beta$ -Air II” prototype (see below for details).

The exterior diameter and height of “ $\beta$ -Air I” (Fig. 1B) is 31.3 and 7 mm, respectively. The gas chamber (volume, 3 ml) is connected to an external air pump and an outlet port by two transcatheter silicone tubes (1 mm OD; Chen Shmuel Chemicals, Haifa, Israel). The internal islet module (diameter, 18 mm; surface area, 2.54  $\text{cm}^2$ ; height, 0.5 mm) contains 2,500 donor IEQ embedded in ultrapure, high guluronic acid (68%) alginate.

A custom-fabricated 25- $\mu\text{m}$ -thick Silon IPN (interpenetrating network of polydimethylsiloxane and polyethylenefluoroethylene) oxygen-permeable membrane (Bio Med



**Figure 1.** The implantable bioartificial pancreas device. (A) Cross section. (B) The “ $\beta$ -Air I” prototype-external view. (C) The “ $\beta$ -Air II” prototype-external view.

Sciences, Allentown, PA, USA) separates the gas chamber from the islet module and a 25- $\mu$ m hydrophyzized polytetrafluoroethylene (PTFE) membrane (“Biopore”; Millipore, Schwalbach, Germany) with a pore size of 0.4  $\mu$ m creates a barrier between the islet module and the recipient’s tissue. A metal grid on each side of the islet module provides mechanical strength. The oxygen permeability of the silon IPN membrane, measured in a diffusion apparatus at 37°C, is  $4.36 \times 10^{-14}$  mol/(s $\times$ cm $^2$  $\times$ mmHg).

The outer dimensions of “ $\beta$ -Air II” are identical to those of “ $\beta$ -Air I”; however, the diameter of the islet module in “ $\beta$ -Air II” is reduced to 11.5 mm (net surface area, 1.05 cm $^2$ ) to enable testing of a larger islet density (Fig. 1C). As such, the surface density of the encapsulated islets is increased from about 1,000 IEQ/cm $^2$  in the “ $\beta$ -Air I” device to >2,000 IEQ/cm $^2$  in the “ $\beta$ -Air II.” The device is connected to two

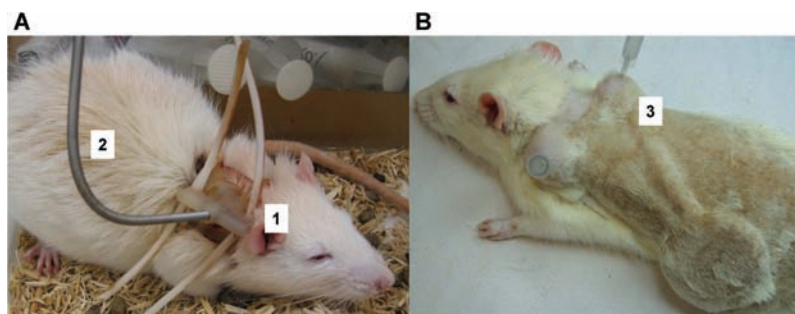
implanted subcutaneous access ports (Soloport; Instech Solomon, San Antonio, TX, USA) placed on the dorsal side of the animal between the scapula by short polyurethane tubes (part of the port kit) (Fig. 2A). The footprint of the oxygen delivery system is seen on a shaved animal shown in Figure 2B.

#### Device Assembly

Doses of 2,200–2,600 IEQ were collected by brief centrifugation (140 $\times$ g). Each pellet was gently mixed with 1.8% (w/v) of high guluronic acid alginate (G=0.68) and transferred to a sterile mold (diameter, 10.6 or 18 mm; thickness, 0.5 mm). Alginate was cross-linked by the slightly hypotonic strontium cross-linking solution mentioned above. Thereafter, the islet-containing slab (Fig. 3A, B) was released from the mold and washed twice at 37°C in complete CR medium for 5 min each. The slab was then installed into an islet module and a Biopore membrane was fixed onto the device using a Viton75/27 mm O-ring (McMaster Carr, Los Angeles, CA, USA). The membrane was sealed to the plastic housing with Med 1000 silicone glue (Nusil, Carpinteria, CA, USA). Fully fabricated devices (Fig. 3C, D) were washed in complete CR medium in 37°C for 2 h before implantation.

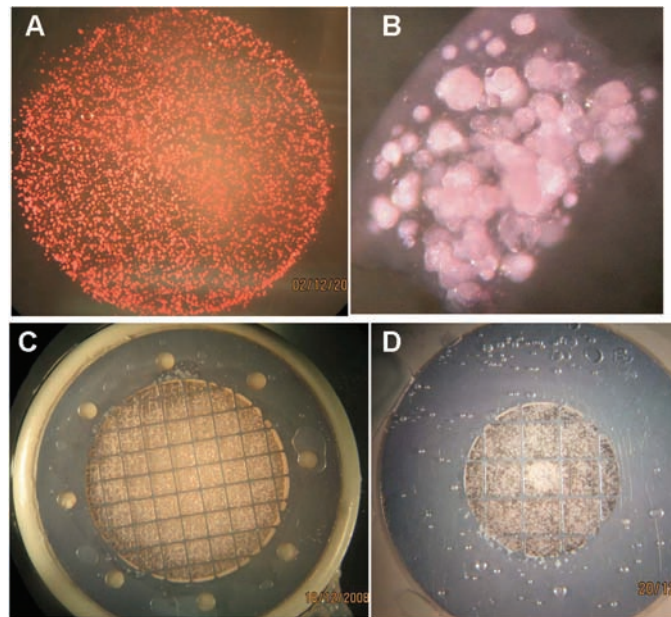
#### Device Implantation

Rats were anesthetized by ketamin/xylazine (Vetoquinol, Lure Cedex, France) followed by isofluran (Abbott Laboratories Ltd., Maidenhead, Berkshire, UK) inhalation. A 3-cm incision was made on the dorsal skin, and muscles were separated from the hypodermis. A second incision was made in the skin between the shoulder blades, and two channels, connecting this site with the device implantation site, were created by traversing 3-mm-wide stainless steel needles (made in house) under the skin. Two silicon tubes, part of the gas infusion system of the “ $\beta$ -Air I” device, were threaded via these channels to connect the device with an external gas supply. The body of the device was inserted



**Figure 2.** Rats carrying implanted devices. (A) “ $\beta$ -Air I”. 1, restraining system; 2, metal wire pipe holding silicone tubes. Atmospheric air was periodically injected through one of these tubes. (B) “ $\beta$ -Air II”. 3, implanted access ports; oxygen-enriched gas was injected once a day through one of the implanted septa and cleared via the collateral septum. The animal was shaved before photographing to illustrate the relative positions of the device and the subcutaneous access ports.





**Figure 3.** The islet module before implantation. (A) Surface view of the islet module. (B) Cross section in an islet module. (C) “ $\beta$ -Air I” ready for implantation. (D) “ $\beta$ -Air II” ready for implantation. The external diameters of the two prototypes are identical.

under the dorsal skin incision with the islet module facing the fascia and skin was sutured and fixed with a tissue adhesive (Histoacryl B Braun, Tuffingen, Germany). The outlet of the silicone tubes were inserted into a restraining system (CIH95 Covance Infusion Harness; Instech Laboratories, Plymouth, PA, USA) and connected to an external air pump (Fig. 2A). In “ $\beta$ -Air II,” the tubes (made of polyurethane) and the dedicated subcutaneous access ports were both implanted under the skin at a place remote from the device. An oxygen-enriched gas mixture was infused daily into the gas chamber through this relay mechanism (Fig. 2B).

#### Gas Ventilation

For the “ $\beta$ -Air I” device, filtered atmospheric air from a compressed tank with pressure-reducing valve, was infused for a period of 15 min at a rate of 5 ml/min every 2 h through one of the silicone rubber tubes. The tubes remained open to the external air for the rest of the time. Gas chambers of “ $\beta$ -Air II” devices were replenished daily by flushing a gas mixture through the integral subcutaneous access ports (Fig. 2B). In initial tests using gas mixture containing either 20% or 30% oxygen, the islet grafts failed to function within a short period of time or displayed improper glycemic control. Subsequently, further refueling of the gas chamber was carried out with gas mixtures containing 40% oxygen, 55% nitrogen, and 5% CO<sub>2</sub>. The CO<sub>2</sub> helped to maintain physiological pH in the islets module.

#### Functional End Points

Nonfasting blood glucose concentration was measured twice daily in whole blood with a portable glucometer (Acu-Check; Hoffmann La Roche, Basel, Switzerland). For IVGTT, dextrose solution (Cure Medical, Emek Hefer Industrial Park, Israel) at a dose of 0.5 g/kg body weight was infused during a 15-s period via the penile vein following an overnight fast. Blood glucose was measured before infusion and at 10, 30, 60, 120, and 180 min postinfusion. HbA1c was determined by a service provider using dried blood samples on filter paper (Flexsite Inc. Services, Palm City, FL, USA).

#### Histology and Immunohistochemistry

At the end of the planned implantation period, grafts were retrieved for histological examination. Tissues were dehydrated with an automated device (Tissue-Tek VIP6 Sakura; Finetek, CA, USA), embedded in paraffin, and sectioned at approximately 4–5  $\mu$ m. For toluidine blue staining, sections were deparaffinized in xylene (Sigma), rehydrated in distilled water, and incubated in 0.1% toluidine blue (Sigma) in 1% NaCl for 1 min. Stained slides were washed, dehydrated, cleared in xylene, mounted, and covered. For glucagon and insulin immunostaining, slides were incubated overnight at 60°C, deparaffinized, and rehydrated in tap water. Slides were then boiled in acidic citrate solution (Invitrogen, Camarillo, CA, USA), blocked for 15 min with CAS block (Invitrogen), and incubated with mouse anti-human insulin antibody (Biogenex,

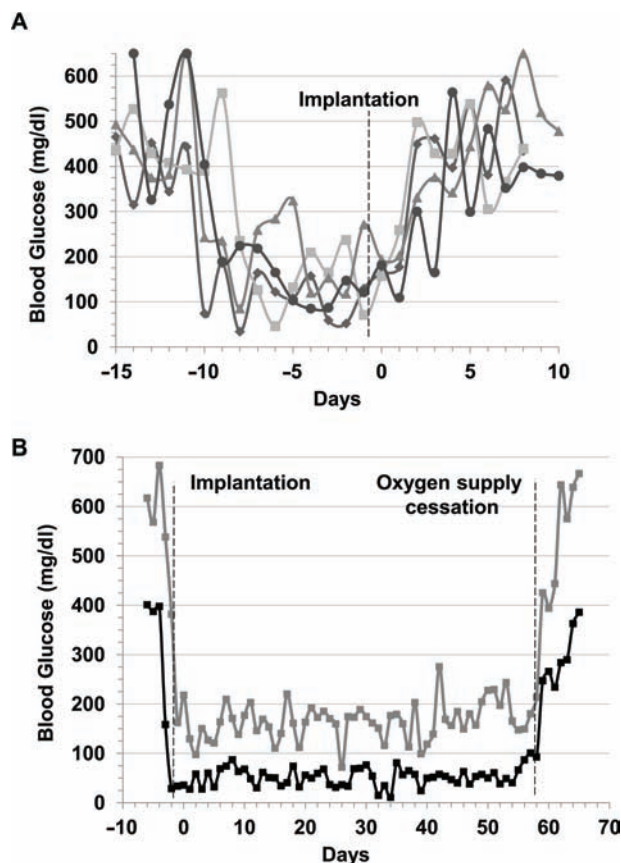
Fremont, CA, USA) diluted 1:300 for 1 h at room temperature or with rabbit anti-human glucagon antibody (ab18461; Abcam, Cambridge, UK) diluted 1:100. Slides were rewashed, incubated with Super Picture Ploy HRP conjugate reagent (Invitrogen) for 15 min, color developed with DAB substrate solution (Invitrogen) followed by a 8-min wash in running tap water. All slides were counterstained in Mayer's hematoxylin (Sigma), dehydrated, cleared in xylene, mounted, and covered. For Ki67 immunostaining, slides were incubated overnight at 64°C, deparaffinized, and rehydrated in tap water. Slides were then boiled in basic nuclear decloaker solution (Biocare Medical, Concord, CA, USA), blocked for 10 min with CAS block, and incubated with rabbit monoclonal anti-human Ki67 antibody (Thermo Fisher Scientific, Loughborough, Leicestershire, UK) diluted 1:200 for 1 h at room temperature. Slides were rewashed, incubated with Super Picture Poly HRP conjugate reagent for 15 min, color developed with DAB substrate solution followed by a 8-min wash in running tap water. Slides were counterstained in Mayer's hematoxyline, dehydrated, cleared in xylene, mounted, and covered. For SMA staining, monoclonal anti- $\alpha$ -smooth muscle actin (SMA; clone 1A4; Sigma product A2547) was used according to the manufacturer's instructions.

## RESULTS

### *Enhanced Oxygen Supply Facilitates Islet Survival in a Subcutaneous Implant*

Our initial objective was to determine whether adequate oxygen supply facilitates islet survival in our BAP devices by first establishing that, without oxygen, the islets in our devices fail after a short period of time. To this end, we normalized blood glucose levels of four diabetic rats using slow-release insulin capsules, implanted one "β-Air II" device, (each containing ~2,300 isogenic IEQ at a density of 2,250 IEQ/cm<sup>2</sup>) in each rat, and then removed the insulin capsules without pumping oxygen into the gas chamber of the device at any time. Indeed, our analysis of nonfasting blood glucose levels over time (Fig. 4A) demonstrated that, following implantation, normal nonfasting blood glucose levels were sustained for a period of only 1–2 days, after which the graft failed, as indicated by the elevated levels of blood glucose. These findings are consistent with comparative analyses of the islet module. Following 9 days of implantation, nearly 50% of the original IEQ loaded into the device were recovered; however, <7% of the original total OCR and total insulin content were retained at the end of the implantation period (Table 1).

We then evaluated the ability of an oxygen-refueled "β-Air II" device to normalize blood glucose levels. To this end, we implanted "β-Air II" devices (each device



**Figure 4.** "β-Air II" function is dependent on oxygen supply. (A) Four diabetic rats were implanted with standard "β-Air II" devices for a period of 9 days, but oxygen was not refueled at any time. Level of nonfasting blood glucose was monitored daily. (B) Six rats were made diabetic and blood glucose normalized by a slow release insulin capsule. On day 0, the insulin capsules were removed and "β-Air II" devices were implanted for a period of 58 days. The gas chamber was refueled once a day. At the end of this period, oxygen supply to the devices was disabled. Blood glucose was followed for 5 additional days. Gray and black lines represent 99% upper and lower confidence limits of the averages, respectively.

contained ~2,400 isogenic IEQ at a surface density of 2,300 IEQ/cm<sup>2</sup> and was refueled daily with gas blend containing 40% oxygen) into six diabetic rats (one device per recipient) and removed the slow release insulin capsules. Our analysis of nonfasting blood glucose levels over time demonstrated that the devices restored normal nonfasting blood glucose levels for a period of 58 days (Fig. 4B). Furthermore, upon stopping the oxygen supply to the device (after 58 days), we observed an abrupt rise in nonfasting blood glucose, further confirming that the islets in our device could not survive in the subcutaneous environment without an exogenous supply of oxygen.

**Table 1.** Comparison of IEQ Number, Total OCR, and Total Insulin Content Before Implantation and After a 9-Day Implantation Period

	Preimplantation	Postexplantation	Fraction Remaining (%)
IEQ	2,257 ± 170	1,092 ± 332	48.4
Total OCR (pmol/min)	9,819 ± 370	674 ± 192	6.9
Insulin (µg/graft)	240 ± 63	15 ± 3	6.4

Data presented are means ± SD ( $n=4$ ). IEQ, islet equivalent; OCR, oxygen consumption rate.

### *Functional Islets Are Maintained by Exogenous Oxygen Supply*

Our subsequent objective was to characterize the functionality of our BAP devices. For this purpose, we implanted 17 diabetic Lewis rats with devices containing isogeneic donor islets (10 rats received “β-Air I” and 7 rats received “β-Air II” devices) for a period of 90 days. Mean values (±SD) of islet dose, total OCR at implantation time, and packing densities are summarized in Table 2.

Our analysis of nonfasting blood glucose levels over time (from the removal of the slow-release insulin capsule and device implantation) showed that normoglycemia was attained and maintained in both prototype devices throughout the entire implantation period and that device explantation was followed by a rapid blood glucose increase (Fig. 5), suggesting that effective grafts could be maintained at surface densities of 1,000 and 2,000 IEQ/cm<sup>2</sup> once adequate oxygen is being supplied. Several animals were implanted for extended periods of time (up to 250 days) with similar results (data not shown).

The performance of the device was further characterized using a retrospective analysis of 107 implantation experiments, which demonstrated that total OCR of the implanted graft was a better correlate of device performance (nonfasting blood glucose levels) at 1 month than the implanted IEQ or insulin dose (data not shown).

### *Most of the Oxygen Supplied During Each Refueling Event Is Consumed by the Islets*

Next, we wanted to explore the consumption of supplied oxygen by the islets in our BAP device by studying the oxygen concentrations at the device gas chamber over time. To this end, we used seven animals implanted with “β-Air II” devices. Gas mixture containing 40% oxygen was flushed daily into the corresponding gas chambers

and oxygen levels were recorded twice weekly before replenishment event was performed (i.e., when the oxygen concentration was at its nadir). During the 90-day period of the experiment, the mean (±SD) of nonfasting blood glucose was 109 ± 49.6 mg/dl.

At the end of a 24-h period between two refueling events, the average oxygen concentration in the gas chambers decreased from a fixed level of 40% to 25.1 ± 2.0% ( $n=145$ ). For a 3-ml chamber, the corresponding oxygen disappearance rate was 19.9 ± 0.14 µmol/day. The islet grafts had a total OCR of 8,900 ± 1,600 pmol/min (measured at time of implantation), corresponding to consumption rates of 12.8 ± 2.3 µmol/day. Thus, assuming a stable OCR over time, about 65% of the supplied oxygen was consumed by the islets. The remainder probably diffused through the alginate matrix and the adjoining tubes into the tissue surrounding the implantation pocket.

### *Intravenous Glucose Tolerance Test Is Normalized by Implanted Devices*

A subsequent objective was to further characterize the functionality of the “β-Air” device using an acute phase test. The question arisen was whether the semipermeable diffusion barrier between the implanted islets and the local vascular bed may create a delay in insulin response to a glucose challenge. To this end, we performed IVGTT assays in diabetic rats carrying implanted devices containing isogeneic islets at 3, 9, and 12 weeks postimplantation (Fig. 6).

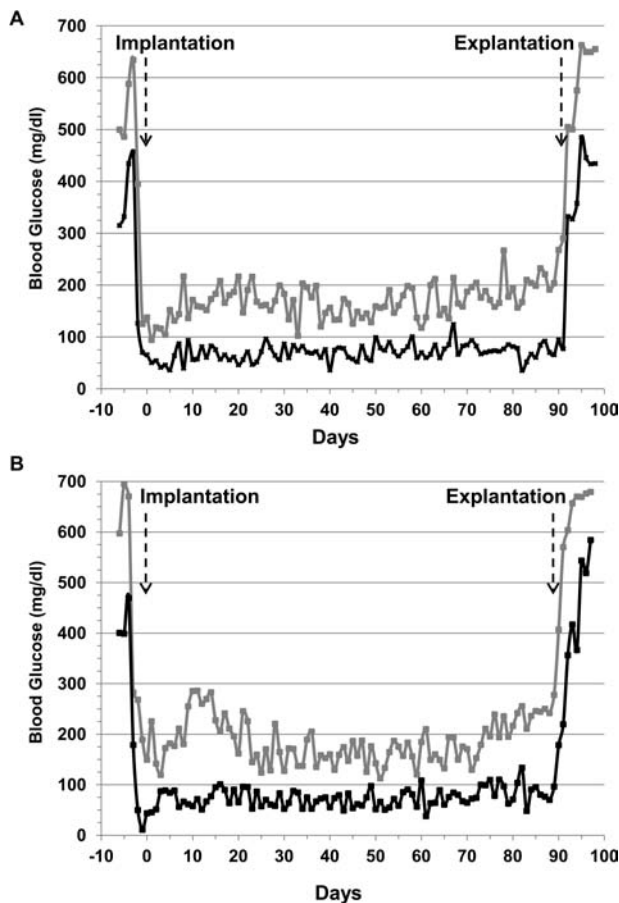
The response profiles of the implanted animals, containing either device type, were similar to those of healthy rats and markedly different from those of diabetic rats (Fig. 6A, B). Although the areas under the curves (AUC, 120-min period) of the implanted rats were increased compared with healthy rats (by 5–33%), this increase was

**Table 2.** Islets Functional Parameters at Time of Device Manufacturing

	IEQ per Device	Total OCR (pmol/min)	Packing Density (IEQ/cm <sup>2</sup> )
“β-Air I” ( $n=10$ )	2,530 ± 40	9,200 ± 1,300	1,000 ± 16
“β-Air II” ( $n=7$ )	2,240 ± 100	8,900 ± 1,600	2,120 ± 95

Data presented are means ± SD. IEQ, islet equivalent; OCR, oxygen consumption rate.





**Figure 5.** “ $\beta$ -Air” devices normalize blood glucose levels in diabetic rats throughout the implantation period. Diabetic rats were implanted with either “ $\beta$ -Air I” (A;  $n=10$ ) or “ $\beta$ -Air II” (B;  $n=7$ ) devices containing isogeneic islets. Nonfasting blood glucose concentrations were monitored twice daily. Devices were explanted after the 90-day implantation period and nonfasting blood glucose was measured for 5 additional days. Gray and black lines represent 99% upper and lower 99% confidence limits of the averages, respectively.

much smaller compared with the corresponding increase observed in diabetic rats (by  $>400\%$ ). Moreover, the AUC values of both device types at 3, 9, and 12 weeks postimplantation were statistically comparable (Fig. 6C). Combined, our findings suggest that the implanted devices normalized IVGTT findings in implanted animals.

#### *Implanted Devices Immunoprotect Allogeneic Islet Grafts*

Next, we wanted to explore the ability of our device to immunoprotect allogeneic islet grafts. To this end, we first compared the ability of isogeneic and allogeneic islets to cure diabetes in a simple animal model. We normalized blood glucose of diabetic Lewis rats using slow release insulin capsule, transplanted either Lewis

or Sprague–Dawley islets under the kidney capsule of four experimental groups ( $n=9$ ), and removed the slow release insulin capsule. The transplanted islets included either naked islets (900 IEQ) or a similar dose of islets encapsulated in flat geometry high M alginate slabs. Our analysis of nonfasting blood glucose levels over time (Fig. 7) demonstrated that naked or encapsulated isogeneic islets were active throughout the entire experimental period, whereas allotype islets—either naked or encapsulated—were rejected within 10 days of implantation, as evidenced by the increase in blood glucose levels and microscopic examinations (data not shown).

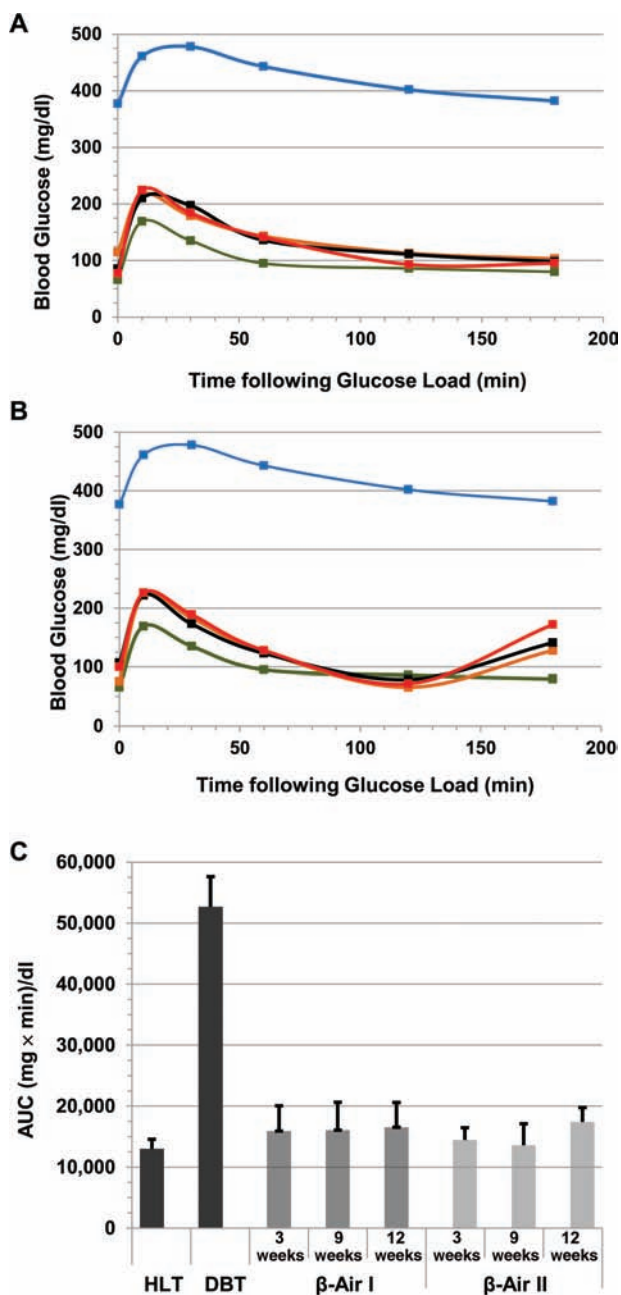
As a simple alginate hydrogel immune barrier failed to provide adequate protection to allogeneic transplanted islets, we explored whether the immunobarrier of our devices could do so by implanting seven diabetic Lewis rats with “ $\beta$ -Air II” devices containing Sprague–Dawley islets (IEQ,  $2,130 \pm 60$ ;  $2,000$  IEQ/cm<sup>2</sup>). Our analysis of nonfasting blood glucose over time (Fig. 8A) demonstrated that all the implanted animals achieved normoglycemia during the 90-day evaluation period and quickly reverted to the diabetic state upon device explantation (notably, in several animals an initial lag period that spanned the first 2 weeks was observed).

To confirm and expand upon our findings, we performed IVGTT assays on diabetic animals implanted with devices containing allotype islets (3, 9, and 12 weeks after implantation) (Fig. 8B, C). Our analysis demonstrated that the implanted devices were functional not only in preserving nonfasting blood glucose levels but also in controlling acute glucose load. Similar to our findings with devices containing isogeneic islets, AUCs (120-min period) of the implanted rats were increased compared with healthy rats (by 20–45%); however, this increase was much smaller than the corresponding increase observed with diabetic animals ( $\sim 350\%$ ), and the integrated AUC levels at 3, 9, and 12 weeks postimplantation were statistically comparable (Fig. 8C).

#### *Low Levels of HbA1c Are Observed in Experimental Rats With Implanted Devices*

To further characterize the functionality of our devices (carrying either isogeneic or allogeneic islets), we evaluated HbA1c levels in diabetic rats postimplantation [HbA1c is a surrogate measure of the time-averaged blood glucose concentration over the mean lifetime of red blood cells, which in rats is approximately 60 days (5)]. We determined the levels of serum HbA1c 90–120 days postimplantation of devices carrying either isogeneic ( $n=17$ ) or allogeneic ( $n=13$ ) islets (Fig. 9). Collectively, the implanted rats had slightly (albeit statistically significant) elevated levels of HbA1c compared with healthy rats (increase of 21% and 33% relative to healthy rats for





**Figure 6.** Implanted “ $\beta$ -Air” devices normalized glucose tolerance test profiles in diabetic rats. Lewis healthy ( $n=26$ ), diabetic ( $n=22$ ), and diabetic rats carrying “ $\beta$ -Air I” (A;  $n=10$ ) or “ $\beta$ -Air II” (B;  $n=7$ ) devices equipped with isogeneic islets were fasted overnight. They were then intravenously infused with glucose (0.5 g/kg), and blood glucose was periodically sampled for 3 h. Dark green line, healthy rats; blue line, diabetic rats. Orange, black, and red lines represent animals with implanted devices at 3, 9, and 12 weeks postimplantation, respectively. Area under the curves (C; AUC, mean  $\pm$  SD) was calculated from the individual data for the first 120 min after glucose infusion. HLT, healthy rats; DBT, diabetic rats.

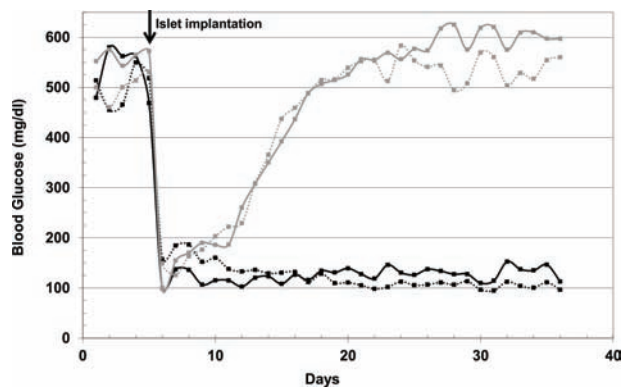
the isogeneic and allogeneic implanted animals, respectively). However, this increase was only one seventh of the increase observed with diabetic rats (275% increase).

#### *Biocompatibility, the Islet Module, and the Fibrotic Pocket of the BAP Devices Upon Explantation*

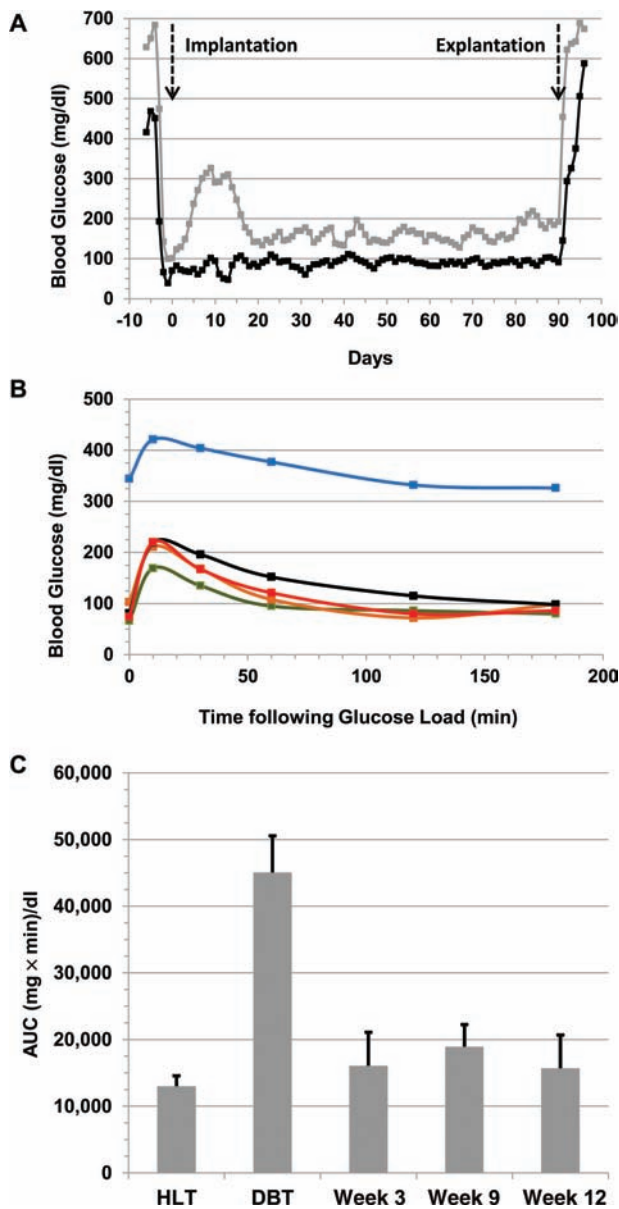
Our last objective was to evaluate the biocompatibility of our BAP device. To this end, we explanted the devices after a period of 90–180 days, inspected them for tissue response and integrity of the islet module, and examined the fibrotic pocket enveloping the device for growth of vascular bed. The implantation pocket of animals implanted with devices carrying either isogeneic or allogeneic islets appeared visually healthy and nonirritated with no signs of local inflammatory reaction (data not shown). The housing of the explanted devices was intact and the “Biopore” membrane was complete and undamaged.

We inspected the islet modules visually before separating them from the housing. They appeared intact and undamaged (Fig. 10A). Immunohistological examination of the islets within the module demonstrated that they contained functional islets as demonstrated by insulin and glucagon staining (Fig. 10B, C) and that they retained some proliferation capacity (demonstrated by Ki67 staining) (Fig. 10D).

Four fibrotic pockets surrounding explanted “ $\beta$ -Air II” devices were processed for histological and immunohistochemical examinations. The pockets were comprised of band-like mature films measuring between 350 and 550  $\mu$ m in thickness. On both faces of the device, networks of



**Figure 7.** Islet grafts [naked or encapsulated in high mannuronic acid (M) alginate] are rejected in allogeneic recipients. Following diabetes induction of Lewis rats, slow release insulin capsules were used to reduce blood glucose and animals were then transplanted with 900 IEQ naked isogeneic islets ( $n=9$ ; dotted black line) or naked allogeneic islets ( $n=9$ ; dotted gray line). Two additional groups were implanted with two doses of 900 IEQ each of either isogeneic islets ( $n=9$ ; continuous black line) or allogeneic islets ( $n=8$ ; continuous gray line) encapsulated in high M alginate hydrogel slabs. Data are reported as means of nonfasting blood glucose concentrations.



**Figure 8.** Implanted “ $\beta$ -Air” devices protect allogeneic islet grafts against rejection. (A) Nonfasting blood glucose profiles. Seven diabetic Lewis rats were made normoglycemic using slow insulin-releasing capsule. They were then implanted with “ $\beta$ -Air II” devices equipped with islets isolated from Sprague–Dawley rats. Devices were explanted after the 90-day implantation period, and nonfasting blood glucose was measured for 5 additional days. Gray and black lines represent 99% upper and lower 99% confidence limits of the averages. (B) Intravenous glucose tolerance test (IVGTT) profiles. Lewis healthy ( $n=26$ ), diabetic ( $n=11$ ), and diabetic rats carrying “ $\beta$ -Air” devices ( $n=7$ ) equipped with Sprague–Dawley islets were fasted overnight and assayed for IVGTT. Data are reported as means. Dark green line, healthy rats; blue line, diabetic rats. Orange, black, and red lines represent animals with implanted devices at 3, 9, and 12 weeks postimplantation, respectively. (C) IVGTT – AUC. Means are calculated from data represented in (B) for the first 120 min (data reported are means  $\pm$  SD; HLT, healthy rats; DBT, diabetic rats).

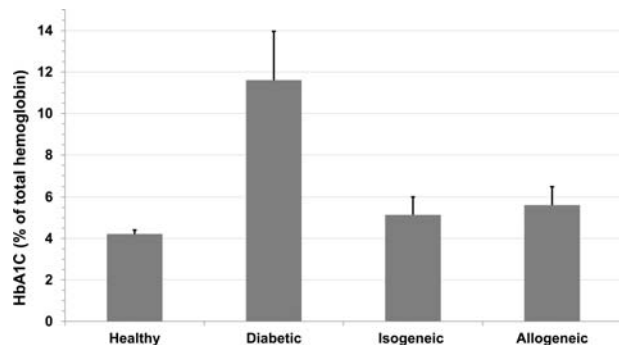
capillaries were observed (0; Fig. 11). Capillary densities averaged  $18 \pm 7.5$  blood vessels per high power optical field (area of  $0.2 \text{ mm}^2$ ) on the side facing the active side of the device and  $12 \pm 3.8$  on the contralateral side ( $n=4$  animals; not a statistically significant difference).

## DISCUSSION

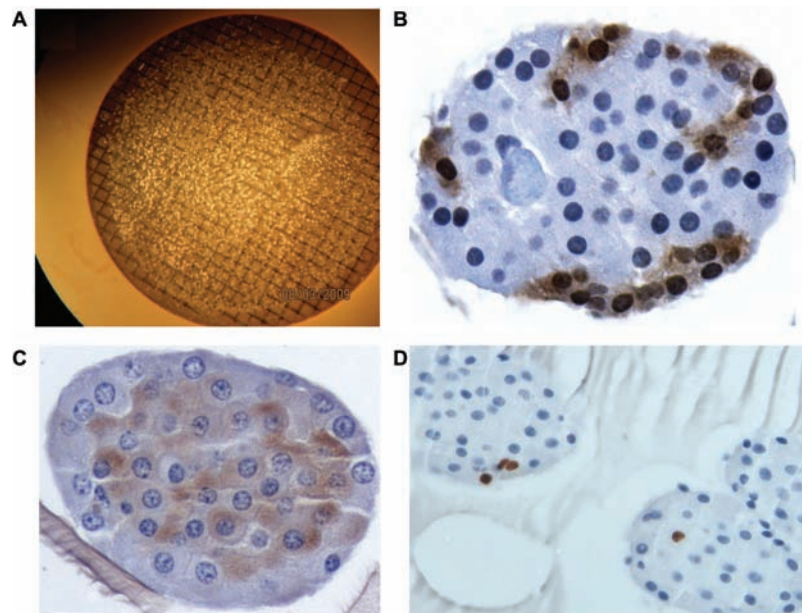
Widespread use of pancreatic islet transplantation is mainly limited by general shortage of donor organs, the need for more than one donor per recipient, and the necessity for lifelong use of immunosuppressants. In this study, we examined a mechanically stable, retrievable, implantable device design that facilitates a more efficient use of tissue by enhancing oxygen supply to the islets and achieving immunoisolation from the allogeneic immune response through the use of a physical immunobarrier. Additional characteristics of our device that may facilitate its clinical development include its biocompatibility and subcutaneous location.

Visual and histological examination of the implantation pocket at time of device retrieval demonstrated physiological responses involving the formation of an ordered fibrotic capsule around the device without an inflammatory reaction. In addition, the fibrotic tissue surrounding the device exhibited vascular structures with densities comparable to those recently reported by Veriter et al. (36). Notably, close proximity of blood vessels and the encapsulated islets create short diffusion distances for glucose and insulin and consequently a nearly normal IVGTT.

The advantages of the subcutaneous location of our BAP device (in contrast to current procedures in which islets are either infused into the portal system or transplanted into the peritoneal cavity) include implantation that requires a minimally invasive surgery, visualization during the implantation period, and easy device retrieval. However, since the subcutaneous space is characterized



**Figure 9.** Implanted “ $\beta$ -Air” devices containing isogenic or allogeneic islets nearly normalize HbA1c levels in diabetic rats. Glycated hemoglobin (HbA1c) was analyzed in Lewis diabetic rats carrying either isogenic ( $n=17$ ) or allogeneic ( $n=13$ ) islets in devices following a 90- to 120-day implantation period. Controls included healthy ( $n=10$ ) and diabetic ( $n=11$ ) Lewis rats.



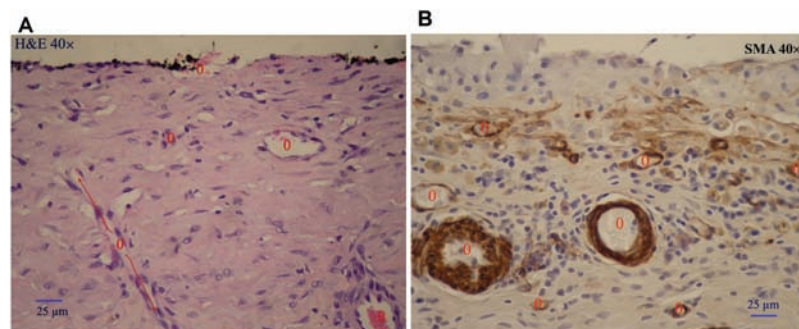
**Figure 10.** The islet module of the “ $\beta$ -Air” implantable devices after explantation. (A) “ $\beta$ -Air I” – surface view. (B–D) Representative islets recovered from an islet module of “ $\beta$ -Air II” devices and stained for glucagon (B), insulin (C), and Ki67 (D).

by low oxygen partial pressure and low vascularity, it may not be an appropriate transplantation site for high oxygen-consuming islets, although some success has been reported using low oxygen consuming porcine islets (14).

Oxygen supply is essential for islet functionality as during isolation, islets are separated from their blood supply, the intraislet vascular network is lost, and islets become dependent on diffusion for oxygen supply. Islets exposed to hypoxia undergo hypoxia-mediated inflammatory reactions, oxidative stress, and apoptosis (4,29,40). Encapsulation can exacerbate the oxygen supply problem, as has been demonstrated in detailed studies with mathematical models (15,20). Our data show that encapsulated islets at high surface density (1,400 IEQ/cm<sup>2</sup>) survive for 1 month at a well-perfused environment like

the subcapsular space of the kidney even if not actively supplied with oxygen. However, when implanted in the poorly oxygenated subcutis, encapsulated islets in the device become completely nonfunctional within a very short period of time if not supplied with exogenous oxygen.

Our BAP device provides a unique solution for adequate islet oxygenation. Gas-phase oxygen is contained in a chamber that is an integral part of the device, and the oxygen diffuses across a thin gas-permeable membrane and through an adjacent alginate hydrogel to supply the islets. We observed that the functionality of donor islets integrated in devices at a density of  $\sim$ 1,000 IEQ/cm<sup>2</sup> (“ $\beta$ -Air I”) could be maintained with atmospheric air for periods as long as 180 days until experiments were electively terminated. All glycemic indicators were corrected to normal values by the



**Figure 11.** Histological analysis of the fibrotic pocket surrounding an explanted “ $\beta$ -Air II” device carrying isogenic islets. Hematoxylin–eosin (A) and smooth muscle actin (SMA) (B) stained fibrotic capsule. 0, blood vessels. The upper side of the image was facing the explanted device.



" $\beta$ -Air I" implanted devices and quickly returned to the diabetic state upon device explantation. At a higher islet density ( $>2,000$  IEQ/cm<sup>2</sup>), the islets could not be sustained by atmospheric air in the gas chamber, and therefore, we developed a different ventilation approach wherein a gas mixture containing 40% oxygen was infused daily through subcutaneous access ports into the gas chamber of the " $\beta$ -Air II" device. Our data show that 24 h after refueling (i.e., immediately prior to the subsequent oxygen replenishment), the oxygen in the chamber decreased considerably suggesting that 60–75% of the originally contained oxygen was consumed by the graft. The remainder probably diffused into the surrounding host tissue without any observed toxicity. The oxygen partial pressures in the gas chamber ranged from 304 to 198 mmHg. For an average OCR (8,000 pmol/min/device), an area of 1.05 cm<sup>2</sup> (for the " $\beta$ -Air II" device), and a 25- $\mu$ m-thick Silon IPN membrane with permeability of  $4.36 \times 10^{-14}$  mol/(s  $\times$  cm<sup>2</sup>  $\times$  mmHg), the partial pressure drop across the membrane is calculated to be  $\sim 7$  mmHg. Consequently, the islet cells close to the membrane are exposed to an oxygen level which is similar to that in the gas chamber. Although the partial pressure drops as oxygen diffuses and is consumed within the 500- $\mu$ m thick islet module, a significant fraction of islet tissue is expected to be exposed to an oxygen partial pressure, which is substantially greater than the 100 mmHg level typical of arterial blood. Nonetheless, oxygen toxicity was not observed, and the performance of the graft, as measured by all glycemic indicators, was maintained for prolonged periods of time of up to 180 days. The efficacy of the encapsulated islet tissue in the device was further confirmed using immunohistochemical analyses, which demonstrated the presence of insulin-containing  $\beta$ -cells and cell proliferation in explanted grafts. Our observation is consistent with the findings of Lacy et al. (22) and Hughes et al. (19), both of whom demonstrated beneficial effects of high oxygen concentrations before and during implantation period on the performances of the transplanted grafts.

After demonstrating the feasibility of transplanting high oxygen-consuming islet grafts in an otherwise oxygen-poor environment, we explored the immune protection of donor islets against the host immune system in an allogeneic transplantation setting. We clearly showed that our BAP device immunoprotects the graft (at least for the timeframe of the experiments) against the host immune system as all glycemic indicators of the implanted animals were normalized for long periods of time (up to 180 days) and no differences were observed between the functional capacities of isogenic and allogeneic islets in these devices. In our system, such immunoprotection could not be provided when islets were encapsulated only in a simple slab of alginate hydrogel. The immune barrier established by our BAP device is based on a mechanical solution. Donor islets are contained in a housing fabricated mainly of PEEK impermeable

plastic and are separated from the host immune system by a two-layer barrier (overlying membrane and the alginate gel). This barrier is hypothesized to prevent physical contact between the islets and any of the host immune cells, thereby inactivating the T-cell-mediated immune rejection and delaying inward diffusion of immunoglobulins.

Control of blood glucose involves many players, of which insulin and glucagon play a key role. Insulin monotherapy for diabetes uses the most important player; still, glucagon is important to avoid hypoglycemia. Therefore, glucose homeostasis controlled by islets should provide a better solution than insulin therapy. However, intrahepatic transplanted islets contain fewer  $\alpha$ -cells than normal islets (23) and glucagon expression in these islets decreases over time (18). Bohman's observation that  $\alpha$ -cells are retained in encapsulated islets for a period of 100 days (7) is confirmed in our experiments showing glucagon staining in islet-containing devices explanted after a period of 180 days. As such, an added benefit of our solution is that patients implanted with the " $\beta$ -Air" device might experience a better glycemic control compared with those transplanted with the currently available state-of-the-art therapy.

The physiologically efficient glucose metabolism is facilitated by the anatomy of the pancreas draining into the portal system, the cephalic and intestinal signals that enhance insulin release, and the highly permeable islet vascularity that facilitates rapid insulin and glucagon release. In contrast, the BAP device delivers insulin away from the liver and might present less efficient kinetics of insulin release. Consequently, the pharmacokinetic profile of blood glucose after meals might be expected to lead to longer periods with higher glucose levels and less efficient shutoff of insulin release resulting in later hypoglycemia. However, this hypothesis is not supported by our IVGTT findings in the implanted animals. The integrated AUC (120 min) of diabetic animals is increased by more than 300% over the AUC of normal, healthy rats (52,700 vs. 13,000). At the same time, AUC of diabetic rats implanted with either isogenic or allogeneic islet grafts differed from that of healthy animals by a modest factor of 25% only. Two additional findings refute the "delayed device response" hypothesis. First, the 3-h time points of the IVGTT assays did not present any significant level of hypoglycaemia; and second, results of IVGTT assays performed 3 weeks post-implantation did not differ from those of assays performed 12 weeks postimplantation. Our observations support the notion that the kinetics of glucose intake and insulin release by the implanted devices is sufficiently rapid so as to nearly normalize IVGTT assays. This kinetics, in turn, might be explained by the observed vascularization of the fibrotic tissue encapsulating the device (a vascularization that may be induced by proangiogenic factors released from the islets) (8,36,37).



Assuming an islet surface density of  $\sim 2,000$  IEQ/cm<sup>2</sup> (the density of the “ $\beta$ -Air II” devices), a surface area of 50 cm<sup>2</sup> would be required for each 100,000 IEQ implanted. For humans (in whom a total of 300–500K IEQ is required), this density translates to a required surface area of 150–250 cm<sup>2</sup>, which is probably clinically impractical, even if divided into multiple devices. Therefore, additional research focused on increasing islet density is needed.

In summary, in this report, we present a comprehensive approach that addresses the critical issues of current islet transplantation in a single device and demonstrate the efficacy of this approach in a small animal model of diabetes. A physical immune barrier, adequate oxygen supply, and minimally invasive surgical procedure are the hallmarks of this new approach.

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