Improvement of Islet Engrafts by Enhanced Angiogenesis and Microparticle-Mediated Oxygenation

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Abstract

A major hindrance in islet transplantation as a feasible therapeutic approach for patients with type 1 diabetes is the insufficient oxygenation of the grafts, which results in cell death in portions of the implant. Here we address this limitation through the application of oxygen-generating microparticles (MP) and a fibrin-conjugated heparin/VEGF collagen scaffold to support cell survival by using a β cell line and pancreatic rat islets. MP are composed of a polyvinylpyrrolidone/hydrogen peroxide (PVP/H₂O₂) core and poly(D,L-lactide-co-glycolide) (PLGA) shell, along with immobilized catalase on the shell. The presence of MP is sufficient to reduce hypoxia-induced cell dysfunction and death for both cell types, resulting in localization of hypoxia-inducible factor (HIF-1 α) into the cytoplasm and enhanced metabolic function. After co-transplantation of MP and a reduced islet mass (250 islet equivalents) within an angiogenic scaffold in the omental pouch of streptozotocin-induced diabetic nude mice, we have observed significantly enhanced graft function as evidenced by improved blood glucose levels, body weight, glucose tolerance, serum C-peptide, and graft revascularization. These results suggest that the developed platform has great potential to enhance the efficacy for implants in cases where the cell dosage is critical for efficacy, such as islet transplantation and ischemic tissues.

Keywords: Diabetes, Islet transplantation, Oxygen-generating microparticle, Angiogenesis

Introduction

Transplantation of isolated islets of Langerhans is an emerging, promising treatment for type 1 diabetes mellitus (T1D) [1]. Recent advances have increased the yield of insulin independency by up to 44%, following three years of islet transplantation [2]. However, several shortcomings still restrict the mode of progression (reviewed in [3]), such as the lack of oxygen which can lead to impairment of islet function and death during the early post-transplantation period [4].

Under normal conditions, glucose-stimulated insulin secretion (GSIS) depends on oxidative phosphorylation. Pyruvate is produced from glucose uptake through glycolysis and utilized in the production of adenosine triphosphate (ATP) via mitochondrial oxidative pathways [5]. In contrast, hypoxia alters aerobic glucose metabolism to anaerobic glycolysis and attenuates GSIS [6], finally leading to activation of caspase-3 and apoptosis of islet cells [7].

Although under hypoxic conditions, activation of HIF in islet cells induces the secretion of angiogenic factors such as vascular endothelial growth factor (VEGF) [6]. According to research, the endogenous angiogenic factors produced by transplanted islets are inadequate to induce angiogenesis during the early post-transplant period [8]. Therefore, it is necessary to protect islets from the irreversible damaging effects of hypoxia prior to blood vessel formation [9].

Mechanisms utilized to supply oxygen during the early post-transplant period include the use of a prevascularized device-less site [10], induction of angiogenic factors over-expressed by islets or helper cells [11, 12], and proangiogenic factors concomitant with transplanted islets [13, 14].

Other approaches directly oxygenate the transplant site such as *in situ* generation of oxygen with hydration of solid peroxide [9], the use of a device that consists of a minimally invasive implantable chamber to supply oxygen [15], application of oxygen carrier agents such as hemoglobin [16], and perfluorocarbons accompanied by islets [16, 17].

In this study, we generated oxygen-generating microparticles (MP) with a core-shell structure composed of a polyvinylpyrrolidone (PVP)/H₂O₂ core and a poly(lactide-co-glycolide) PLGA shell that was coated with immobilized catalase enzyme on the surface of the MP in order to provide sustained release of oxygen. Carboxyl-end groups of PLGA were activated to bind with the tetrameric structure of the catalase, which allowed the enzyme to remain in its active form for at least two weeks [18, 19] in order to decompose H₂O₂ upon its release from the MP. For induction of angiogenesis, we generated a composite scaffold composed of a collagen sponge as the frame and fibrin-conjugated heparin/VEGF as the sustained delivery system for VEGF. This scaffold composition was reported to be appropriate for angiogenesis [20]. Short-term delivery of VEGF was more effective via this approach [21]. We investigated the impact of combined oxygenation and angiogenesis induction on the outcome of the islet grafts.

2. Materials and methods

2.1. Materials and chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise noted.

2.2. Oxygen-generating microparticles (MP)

2.2.1. Fabrication of core-shell structural MP

H₂O₂ was mixed with PVP at a molar ratio of 3:1 [repeating unit N-vinylpyrrolidone (VP)] and stirred overnight at 4°C in order to obtain a uniform mixture. The core-shell microspheres were fabricated by homogenizing PVP/H₂O₂ in a PLGA (RG 504 H, Boehringer Ingelheim, Germany) solution (5% weight in dichloromethane) with a 1:25 (V/V) ratio at 26000 rpm for 3 min. Phase separation was induced upon addition of olive oil that contained Span 80 (4% V/V) at a 6:1

(V/V) ratio and homogenized at 20000 rpm for 1 min. The coacervate polymer droplets that surrounded PVP/H₂O₂ were hardened in n-heptane for 3 h. The particles were washed three times in n-heptane, centrifuged at 1000 rpm, and vacuum dried.

2.2.2. Immobilization of catalase on the MP surface

MP (5 mg) were suspended in 1 ml of a 10 mM N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride/N-Hydroxysuccinimide (EDC/NHS) solution for 2 h under continuous stirring, and then washed three times with distilled water. Afterwards, the activated particles were added to a 2 mg/ml catalase (obtained from bovine liver) solution in phosphate-buffered saline (PBS; Gibco, USA), shaken for 2 h and washed three times with PBS. All procedures were performed at room temperature. MP were analyzed by FTIR spectroscopy (EQUINOX55, Germany) and scanning electron microscopy (SEM, TESCAN, Czech Republic). A fluorometric procedure (Invitrogen Qubit fluorometer, USA) was used to evaluate the efficiency of catalase immobilization by subtracting the amount of protein in the initial solution from the supernatant and washing solution.

2.2.3. Determination of catalase activity and kinetic parameters

Standard solutions of H₂O₂ (5-30 mM) were prepared and their absorbance at 240 nm determined. The ability of immobilized catalase to decompose the H₂O₂ solution was quantified by measuring the reduction in absorbance of H₂O₂. One unit of activity was considered as the decomposition of H₂O₂ at a rate of 1 μmol/min at 25°C and pH 7. Activity of immobilized catalase was measured by a UV-Vis spectrophotometer (Biochrom WPA Biowave II, United Kingdom). The influence of substrate concentration on the activity was verified by utilizing

different concentrations of H₂O₂ at constant temperature and pH. Kinetic parameters (K_m and V_{max}) of free and immobilized enzyme were determined by Lineweaver–Burk plots [22].

2.2.4. Measurement of generated oxygen

We measured the amount of oxygen produced over a 14-day period from MP (1 mg/ml) using a non-invasive, ruthenium-based oxygen sensor (PreSens, Germany). MP were in an unsealed vial that contained PBS in a 37°C humidified incubator at normal oxygen tension.

2.3. Cell culture studies

A total of 3×10^3 MIN6 cells (a generous gift from Professor Harry Heimberg; Virije Universiteit Brusse) were seeded on a 24-well plate (Nunc, Denmark) in Dulbecco's modified eagle medium (DMEM) that contained 10% fetal bovine serum (FBS; Gibco, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. The four experimental groups were defined as follows: two hypoxia groups that were incubated at 5% oxygen tension, one group with MP and one group without MP. The other two groups were incubated at normal oxygen tension in the presence or absence of MP. The two groups with MP received 3 mg/ml of MP. After 7 days of incubation, the supernatants were assessed for LDH by an enzyme-linked immunosorbent assay (ELISA) kit (Biorexfars, United Kingdom). In addition, seeded cells were analyzed for DNA content, the MTS (Promega, USA) assay and HIF1-α immunofluorescence staining (Biorbyt, orb69409, USA). All experiments were performed in triplicate.

2.4. Angiogenic scaffold

2.4.1. Preparation fibrinogen-conjugated heparin

Carbodiimide chemistry was used to create the fibrinogen-conjugated heparin structure. Carboxylic acid groups of heparin were activated in an EDC/NHS (1 mM) solution for 2 h at 25°C. The activated heparin was precipitated with excess anhydrous acetone and lyophilized. Fibrinogen was dissolved in PBS (5 mg/ml) and we added activated heparin (3 mg/ml) to this solution. Afterwards, in order to bond the amine groups of fibrinogen to activated carboxylic acid groups of heparin, the mixture was maintained for 3 h at 4°C with stirring. The fibrinogen-heparin solution was filtered through an Amicon Ultra-15 centrifugal filter (30 kDa; Millipore, USA) to remove any residual heparin. The filtered solution was lyophilized in liquid nitrogen [23]. FTIR spectroscopy was used to confirm the conjugation and dimethylmethylene blue (DMMB) staining was performed for evaluation of heparin conjugation efficiency.

2.4.2. Fabrication of collagen based scaffold

Type I collagen from rat tail tendon was prepared according to a previously published protocol [24]. The collagen solution (5 mg/ml) in acid acetic (50 mM) was neutralized by sodium hydroxide (1N), poured into a mold (diameter: 8 mm, depth: 3 mm), frozen at -20°C and freeze-dried for 24 h. Cross-linking of the collagen scaffold was carried out by EDC/NHS (50 mM) in an ethanol (70%) solution for 6 h. The scaffold was washed three times with distilled water and freeze-dried for 24 h. In order to fabricate a construct containing fibrin-heparin/VEGF collagen, fibrinogen (24 mg/ml, 60%), fibrinogen conjugated heparin (16 mg/ml, 40%) and VEGF (100 ng/ml, 37°C) were diluted in DMEM (1:1). In addition, thrombin (90 IU) was dissolved in a calcium chloride solution (6 mg/mL). Next, we injected the fibrinogen mixture solution into the collagen sponge after injection of the thrombin solution.

2.4.3. Determination of VEGF release kinetics

The release kinetics of VEGF from the scaffolds was evaluated for two weeks. Scaffolds in triplicate were soaked in PBS under continuous agitation at 37°C. The amount of released VEGF in the supernatant at specified intervals was measured by an ELISA kit (Invitrogen, USA) and expressed as cumulative percentage of the total amount of loaded growth factor.

2.4.4. Tube formation assay

Tube formation assay was performed by coating Matrigel (10 mg/ml) on a 96- well plate. Human umbilical vein endothelial cells (HUVECS) were isolated according to a previously reported protocol and cultured in endothelial growth medium (Lonza, Switzerland) [25]. Suspended HUVECs (25×10^3) accompanied by released VEGF (days 1, 4, 7), soluble VEGF at a concentration equal to day 1 released VEGF, and PBS as the negative control were added to each well. The plate was incubated for 18 h at 37°C after which acridine orange was used for staining viable cells. Tube formation was investigated with a fluorescent microscope (Olympus BX51, Olympus, USA) and quantified as tube area (μm^2) per field by image analysis software (Image J, USA) [26].

2.4.5. Subcutaneous transplantation and characterization

We compared the angiogenic potential of the fibrin/VEGF collagen scaffold with and without heparin *in vivo*. Scaffolds were transplanted subcutaneously in the flanks of C57BL/6J mice (n=3 mice per group). Subcutaneously implanted scaffolds were harvested after 2 weeks and stained according to standard procedures of the Royan Institute Histopathology Laboratory. The samples were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin blocks. The tissue

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4 blocks were sectioned into 5 μm sections and stained with haematoxylin and eosin (H&E).
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6 Scaffold vascularization was determined by calculating the area per vessel. We evaluated five
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8 tissue sections at 70 μm intervals and divided the total vessel area (measured by Image J) by the
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10 number of vessels per field ($40\times$).
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16 **2.5. Islet transplantation studies**

17 **2.5.1. Transplantation of the islet-containing scaffold**

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19 Wistar rats (Pasteur Institute, Iran) pancreatic islet isolation, assessment and T1D induction in
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21 nude mice (B6NU, provided from Pasteur Institute) were carried out according to our previously
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23 published protocols [11]. In order to place islets in the scaffold, 250 islet equivalents (IEQ) were
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25 suspended in DMEM and added to the fibrinogen mixture solution as previously described, then
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27 injected into five different points of the porous collagen scaffold. Transplantation was performed
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29 in the omental pouches of anesthetized recipient mice according to our previously published
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31 protocol [11]. Recipient mice were divided into four experimental groups based on the
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33 components which were accompanied by 250 IEQ within the scaffold: I) islet, II) islet/MP, III)
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35 islet/VEGF, and IV) islet/VEGF/MP. Prior to injection into the collagen sponge, MP were
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37 suspended in DMEM while VEGF was added to the fibrinogen-heparin solution in PBS. The
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39 remaining two groups comprised the: V) sham group which underwent transplantation and did
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41 not receive islets, and VI) normal group of mice with the same weights and normoglycemic
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43 conditions, but did not undergo any transplantation. All animal experiments were carried out
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45 according to the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication
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47 No. 85–23, revised 2010) after approval by the Royan Institute Ethics Committee.
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2.5.2. Evaluating function of the islet-containing scaffold

The function of transplanted islets was evaluated by measuring non-fasting blood glucose levels and body weight during four weeks after transplantation. The percentage and time to normoglycemia (blood glucose ≤ 250 mg/dl) in mice and other factors were considered. On day 30 post-alloxan injection (PAI) we measured serum C-peptide levels with an enzyme immunoassay kit (Alpco Diagnostics, USA) and performed the intraperitoneal glucose tolerance test (IP-GTT). IP-GTT was conducted according to the following steps: after a six-hour fast period, each mouse received an intraperitoneal injection of 50% dextrose in Ringer's solution at 2 g/kg body weight. Tail tip blood glucose levels were measured at 0, 15, 30, 60, 90, and 120 min post-injection by an Accu-Check glucometer (Roche Diagnostics, USA) and the area under the IP-GTT curve (AUC) was determined. In order to confirm the function of the transplanted islets, the scaffolds containing islets were harvested and blood glucose as an indicator of returning T1D was measured.

2.6. Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD). Statistical significance was tested by SPSS software (version 16) using one-way analysis of variance (ANOVA) followed by Bonferoni's post-hoc test. The trends of changes between groups during the measured time points were compared using the general linear model. Differences were considered significant at *P*-values less than 0.05.

Details of the methods can be found in the SI Materials and Methods.

3. Results

3.1. MP produced oxygen for two weeks

We used the coacervation method to prepare MP that consisted of a PVP/H₂O₂ core and PLGA shell (Fig. 1A). PLGA carboxylic acid end groups were used to immobilize catalase enzyme on the surface of the MP that would decompose the released H₂O₂. SEM analysis of their spherical morphology showed that the MP had a mean diameter of $3.5 \pm 1.9 \mu\text{m}$ (Fig. 1B and C). We performed potassium iodide staining to determine the efficiency of PVP/H₂O₂ entrapment, which showed $38 \pm 2\%$ efficiency. The carboxylic acid end groups of the PLGA chains were activated by EDC for bonding with the catalase tetramer structure and $229.3 \pm 26.6 \text{ mg catalase/g of MP was immobilized}$. Conjugation of catalase on the surface preserved enzyme functionality up to 40% for 14 days (Fig. 1D) and altered the enzyme kinetic parameters (K_m and V_{max}) compared to the free mode. Kinetic parameters at constant temperature, pH and various H₂O₂ concentrations were evaluated according to Lineweaver-Burk plots (Fig. 1E and F). Involvement of enzyme amine groups in covalent bonding altered K_m from 47.6 to 66 mM and V_{max} from 5882 to 250 $\mu\text{mole}/(\text{min mg protein})$. The Fourier transform infrared spectroscopy (FTIR) spectra of crashed MP showed two carbonyl bonds attributed to PLGA (1756 cm^{-1}) and the pyrrolidone ring of PVP/H₂O₂ (1656 cm^{-1}). After catalase immobilization, an amide II bond ($1500\text{-}1600 \text{ cm}^{-1}$) was observed (Fig. S1). The ability of MP (1 mg/ml) to generate oxygen over a two-week period fluctuated between 0.83 ± 0.15 and $0.06 \pm 0.05 \text{ mg/l}$. PBS was the control with 6.2 mg/l soluble oxygen under normal conditions (Fig. 1G).

3.2. Oxygen-generating MP reduced the effects of hypoxia on the MIN6 cell line and pancreatic islets

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4 The ability of synthesized MP to produce an adequate amount of oxygen was investigated using
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6 MIN6, a mouse beta-cell line, and pancreatic islets isolated from rats *in vitro*. Proliferation and
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8 alteration of the metabolic pathway in MIN6 cells were tested in the presence of MP under
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10 hypoxic conditions (5% oxygen tension) for 7 days. Cell survival was evaluated and compared
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12 with cells cultured at normal oxygen tension (Fig. 2).
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16 Hypoxia led to decreased viability of MIN6 cells as evidenced by decreased 3-(4,5-
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18 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) level concomitant with
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20 increased lactate dehydrogenase (LDH) release (Fig. 2A and B; $P<0.005$). However, MP (3
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22 mg/ml) co-cultured with hypoxic MIN6 cells showed significant improvement in cell loss
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24 compared to those cultured without MP based on increased MTS level concomitant with
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26 decreased LDH release (Fig. 2A and B; $P<0.05$). According to the MTS assay and based on the
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28 evaluation of LDH release, MIN6 cell survival under normoxic conditions was comparable with
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30 and without MP (Fig. 2A and B).
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36 Hypoxic conditions activate hypoxia-inducible factor (HIF) by stabilization of the HIF- α moiety
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38 [27, 28]. We observed cytoplasmic distribution of HIF- α in MIN6 cells cultured with MP under
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40 hypoxic conditions (Fig. 2C). Decreased cell metabolic activity and increased levels of LDH
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42 accompanied with nucleus translocation of HIF- α in cells cultured under hypoxic compared to
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44 normoxic conditions were symptoms of changing the metabolic pathway of MIN6 into anaerobic
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46 metabolism (Fig. 2).
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51 Isolated rat islets were also tested under hypoxic conditions in the presence and absence of MP.
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53 According to the results, not only did MP prevent cell lysis of islets under hypoxic conditions
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55 (Fig. S2A), they also changed gene expression of the islets (Fig. S2B). According to gene
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57 expression analysis, islets without MP compared to islets with MP under hypoxic conditions
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exhibited upregulation in LDH, monocarboxylate transporter 4 (MCT4) and Glucose transferase 1 (GLUT1) in addition to slight downregulation of glucokinase (GCK). These results showed the efficacy and utility of the oxygen-generating MP for hypoxia-induced cell dysfunction and cell-death for both cell-types.

3.3. The angiogenic scaffold promoted angiogenesis *in vivo*

A porous collagen scaffold frame was generated by the freeze-dry method and cross-linked to prevent fast *in vivo* degradation (Fig. 3A). For sustained release, fibrinogen was covalently bonded to heparin by carbodiimide chemistry and confirmed by FTIR. FTIR analysis of fibrinogen-heparin showed one broad hydroxyl peak (3200–3500 cm^{-1}) and a sulfone peak of heparin at 1050 cm^{-1} (Fig. S3). The amount of bonded heparin was determined by dimethyl methylene blue staining [29] and estimated to be 12%. VEGF was added to fibrinogen-heparin and loaded in the collagen scaffold in order to produce a sustained delivery system. The kinetics of VEGF release were determined by a VEGF enzyme-linked immunosorbent assay (ELISA) kit and expressed as cumulative percentage of the total amount of loaded growth factor. The results showed that 69% of the VEGF was released from the scaffold after 2 weeks (Fig. 3B). The bioactivity of released VEGF was evaluated *in vitro* using the tube formation assay at days 1, 4 and 7 compared to the soluble form of VEGF (with a concentration equal to the released VEGF on day 1) as the control. Tube formation was assessed by determination of the tube area per field. We observed no significant difference on days 4 and 7 compared to day 1 and the control (Fig. 3C-G). The requirement for the presence of heparin in the scaffold was determined by subcutaneous implantation of the scaffold for a two-week period. Hematoxylin and eosin (H&E) staining of harvested scaffold sections showed penetration of the cells and generation of new

capillaries in the fibrin-heparin/VEGF collagen scaffold. In the fibrin/VEGF collagen scaffold, this cell penetration was observed solely in the scaffold's margin (Fig. 3H and I). Immunohistochemistry analysis of CD31 demonstrated the presence of endothelial cells in harvested heparinized scaffold sections (Fig. 3J). We determined the area per vessel (μm^2), which showed that the presence of heparin in the construct resulted in a significantly higher rate of angiogenesis (Fig. 3K; $P<0.005$). Hence, we used the fibrin-heparin/collagen scaffold for the remainder of the experiments. Isolated islets, VEGF and MP, along with fibrinogen-heparin, were loaded into the collagen scaffold according to the four defined groups and used for further *in vivo* characterization (Fig. 3L and M).

3.4. Co-implantation of MP and islets within angiogenic scaffold improves graft function

Induction of T1D and qualification of isolated murine islets were performed as previously published [11]. The *in vivo* experiment was undertaken by transplantation of 250 IEQ as a minimal islet mass to the omental pouch of diabetic mice [11]. To determine the effect of oxygenation and induction of angiogenesis on the improvement of islet functional tests, islets were transplanted alone or in combination with MP, VEGF, or MP/VEGF ($n=6$ per group). The average blood glucose concentration in each group of mice was checked after transplantation. Blood glucose levels showed the preliminary event of mild hypoglycemia as a result of fasting and the surgical procedure of omental transplantation of islets. The average blood glucose concentration in the group that received islet/MP/VEGF was lower compared to the islet, islet/MP groups ($P<0.005$) and islet/VEGF group (Fig. 4A; $P<0.01$). There was a more rapid return to normal blood glucose levels in the islet/MP/VEGF group (7.6 ± 2.6 days) compared to the other groups (Fig. 4B; $P<0.05$). Mice that received islet/MP/VEGF had a positive trend

toward weight gain, which was the same as normal mice, and significantly different compared to the other groups (Fig. 4C; $P<0.005$). At four weeks after transplantation, 83% of mice in the islet/MP/VEGF group returned to the state of euglycemia compared to the other groups that had lower numbers of euglycemic mice ($P<0.005$). The IP-GTT, an index of glucose reactivity of transplanted islets, was performed after four weeks. The results indicated that this group had a higher rate of glucose clearance compared to the other groups (Fig. 4D; $P<0.005$). This finding was confirmed by calculating the area under the IP-GTT curve (AUC; Fig. 4E; $P<0.005$). Although the C-peptide level of this group was comparable with the normal group, this value was higher than the islet/VEGF, islet and islet/MP ($P<0.005$) groups (Fig. 4F). Induction of angiogenesis was evaluated by calculation of the area per vessel in the grafts that had been harvested four weeks after transplantation in order to determine the revascularization index. There was less revascularization in the grafts from the islet/MP/VEGF group compared to those from the islet/VEGF group. However, the grafts from the islet/MP/VEGF group had a higher amount of revascularization compared to the islet and islet/MP groups (Fig. 4G; $P<0.01$). Immunohistochemistry for insulin and H&E staining of the harvested grafts in this group showed a functional islet and mature vessels located nearby (Fig. 4H).

4. Discussion

We sought to provide oxygen supply within an entire scaffold during the early post-transplant period by using biodegradable oxygen-generating MP, accompanied by sustained delivery of VEGF in order to enhance angiogenesis. Previously, the effect of each approach, oxygenation [9, 15] and induction angiogenesis [30, 31], were separately reported. To our best knowledge, this is

first observation that the simultaneous exposure of oxygenation and angiogenesis induction have higher improvement in engraftment of transplanted islets than each one separately.

In order to provide oxygenation during the early post-transplant period, core-shell structural MP that contained immobilized catalase on its surface was prepared. These MP were made by the coacervation method and contained PLGA as the shell. PVP/H₂O₂ was used in the core of the MP due to the creation of a stable complex and slower H₂O₂ release rate [32, 33]. Embedding H₂O₂ or PVP/H₂O₂ within PLGA has previously been reported by other methods. However, in order to decompose H₂O₂, these studies used either the soluble form of catalase or its combination with hydrogel [32, 34]. Here, we immobilized catalase on the surface of MP to decompose H₂O₂ into water and oxygen. Catalase has a tetrameric structure and loses its activity at low concentrations due to dissociation of its subunits. In order to preserve catalase activity for a longer period, these quaternary structures were stabilized by covalent bonding to carboxyl groups of PLGA polymer chains on the surface of MP [35]. The immobilization procedure has been shown to change kinetic parameters due to alterations in enzyme structure, flexibility and steric hindrance of the active site [18]. Functional catalase was present during 14 days. The presence of catalase in the surface of MP led to the production of oxygen upon release of H₂O₂ and reduced the risk of cell-loss by released H₂O₂. It has been previously shown that increase in H₂O₂ concentration affects β -cell survival and function [36]. In the current system, we have measured the amount of generated oxygen per gram of MP. The fabricated MP generated oxygen in a tunable and uniform mode by using different quantities of MPs.

The active HIF transcriptional factor is a dimer that contains α and β subunits. HIF β is stable and capable of binding HIF α subunits. Stabilization of HIF α depends on hypoxic conditions due to proteasomal degradation of HIF α in the presence of oxygen. Therefore, decreased oxygen

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4 tension results in reduced HIF α proteasomal degradation, translocation into the nucleus, and
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6 enabling the stabilized form to dimerise with HIF β . It is followed by activation genes which
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8 impact glucose uptake, metabolism (β cell function) and induction of apoptosis [27, 28]. HIF
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10 activation has resulted in induction of LDH and monocarboxylate transporter-4 which convert
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12 pyruvate to lactate in an anaerobic metabolism pathway. Moreover, activated HIF altered
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14 expression of GLUT1 and GCK that changed rate of glucose uptake into the cell and its
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16 utilization [6, 37]. The capability of MP to improve cell survival and preserve cell functionality
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18 was observed *in vitro*. The presence of MP in the simulated hypoxic environment prevented the
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20 MIN6 cell line from stabilization and translocation of HIF α to the nucleus. We observed
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22 increased cell metabolic activity as well as decreased LDH release in cells cultured under
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24 hypoxic conditions in the presence of MP compared to those cultured without MP.
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28 Under hypoxic conditions, activation of HIF in islet cells induces the secretion of angiogenic
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30 factors such as VEGF [6]. However, it is not adequate to induce angiogenesis early after islet
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32 transplantation [8]. In order to promote angiogenesis, we have used a composite scaffold that
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34 consisted of a porous collagen sponge as a frame with fibrin-conjugated heparin for VEGF
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36 heparin-affinity based controlled release and maintenance of VEGF bioactivity [38, 39].
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38 Previously, the efficiency of VEGF delivery in improving engraftment of transplanted islets has
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40 been reported [30, 31]. Heparin was conjugated to fibrinogen via covalent bonding of the heparin
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42 carboxyl group to the fibrinogen amine group. This combination was reported to be an efficient
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44 method for slow passive release of heparin-binding growth factor [23, 40].
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49 To assess the impact of oxygenation, enhanced angiogenesis, or both, the outcome of grafted
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51 islets was investigated *in vivo*. Diabetic mice that received a scaffold which contained islet/MP
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53 compared with only islets showed reduction in euglycemia time and enhancement in percentage
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4 of euglycemic mice. According to the current study, the islet/VEGF group which received the
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6 inducing angiogenic scaffold has shown significantly improved results compared to the islet and
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8 islet/MP groups. When angiogenesis is enhanced without oxygenation by other external factors,
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10 the time that cells experience hypoxia is shortened. However, this short period of time
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12 irreversibly affects islet cell function [28, 39]. The combination of oxygen-generating MP and
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14 angiogenesis-promoting scaffold could be the ultimate goal due to maximum preservation of islet
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16 mass during the post-transplant period [9].
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23 **5. Conclusion**

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25 Collectively, our results showed that co-transplantation of oxygen-generating MP and minimal
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27 islet mass within fibrin-conjugated heparin/VEGF collagen scaffold has tremendous potential to
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29 improve islet revascularization, oxygenation and diabetes reversal. This improvement may be
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31 clinically useful by potential reduction of the amount of required islets and increasing the insulin
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33 independence following islet transplantation, which can be investigated in further studies. This
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35 may provide a promising avenue for other cell-based implants as well as ischemic tissues.
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Figure Legends

Fig. 1. Fabrication and characterization of microparticles (MP). (A) Schematic of production steps. Core-shell structural MP were produced by the coacervation method and catalase was immobilized on their surface. (B) Representative SEM micrograph of MP. (C) Size distribution histogram of MP. Diameters were calculated for 250 particles by ImageJ. Immobilization of catalase on the surface of MP maintained its activity up to 40% for 14 days and changed the kinetic parameters (K_m and V_{max}). (D) Catalase activity during 14 days. Lineweaver–Burk plots for determination of kinetic parameters: (E) free catalase and (F) immobilized catalase. MP produced oxygen by decomposition of released H_2O_2 by conjugated catalase on the surface. (G) Detection of oxygen concentration for 1mg/ml of microspheres in PBS at 37°C. Measurements were made in an open system incubator set at environmental oxygen by PreSens noninvasive, ruthenium-based oxygen sensors. Values represent mean $\pm$ SD, n = 3.

Fig. 2. Oxygenation by microparticles (MP) decreased the hypoxic effect on the MIN6 cell-line *in vitro*. Cells (3×10^3) were seeded on a 24-well plate and incubated under hypoxic (5% oxygen tension) and normoxic (normal oxygen tension) conditions in the presence or absence of MP for 7 days. Under hypoxic conditions, there was a decreased MTS level along with increased LDH release ($P < 0.005$). These hypoxic effects were reduced in the vicinity of MP ($P < 0.05$). (A) MTS level, (B) LDH release normalized by DNA content. (C) Representative immunofluorescence images of HIF1- α translocation during hypoxic conditions. Values represent mean $\pm$ SD, n = 3.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Fig. 3. Scaffold characterization for islets transplantation. (A) Representative SEM micrograph of the fibrin-heparin collagen scaffold. (B) Cumulative release (%) of VEGF by ELISA from the fibrin-heparin/VEGF collagen scaffold at a 100 ng/ml loading concentration. Human umbilical vein endothelial cells (HUVECs) stained with acridine orange for tube formation assay and in terms of tube area per field as analysed by Image J. (C) Quantitative results of tube formation assay. Stained HUVECS in the samples with: (D) soluble VEGF as a control, (E) VEGF released on days 1, (F) 4 and (G) 7. Presence of heparin in the scaffold promoted angiogenesis and cell migration. (H and I) Hematoxylin and eosin (H&E) staining of the harvested scaffold sections in the absence and presence of heparin. (J) CD31 immunohistochemistry of the harvested fibrin-heparin/VEGF collagen scaffold after subcutaneous implantation. Conjugated heparin increased scaffold vascularization ($P<0.005$). (K) Area/vessel (μm^2) calculated by dividing the total vessel area (measured by Image J) by the number of vessels per field (in five fields of five tissue sections). Presence of heparin in the scaffold promoted angiogenesis and cell migration. (L) Schematic of the scaffold preparation for islet transplantation. Islet and microparticles (MP) with fibrinogen-heparin were loaded into the collagen scaffold and fixed within the scaffold by fibrin formation. (M) Representative SEM micrographs that show MP surrounded by fibrin fibers in the collagen scaffold. Values represent mean $\pm$ SD, $n=3$, $*P<0.05$, $**P<0.01$, $***P<0.005$.

Fig. 4. Oxygenation and angiogenesis induction simultaneously improved function of the transplanted islets. (A) Blood glucose (mg/dl). Mice from the islet/MP/VEGF group had lower blood glucose levels compared to the islet and islet/MP groups ($P<0.005$) and islet/VEGF group ($P<0.01$). In this group, 83% of mice returned to euglycemia at 7.6 ± 2.6 days. (B) Percentage of euglycemic mice. (C) Body weight (g) during 30 days. (D) Intraperitoneal glucose tolerance test

(IP-GTT) curve. (E) Area under curve (AUC) of the IP-GTT on the 30th day post-alloxan injection (PAI). (F) Serum C-peptide (pM). The trend for weight gain, area under the curve (AUC) for IP-GTT and C-peptide concentration in islet/MP/VEGF group were similar to the normal group when compared with the other groups ($P<0.005$). Harvested grafts from the islets/MP/VEGF group showed more vascularization compared to grafts from the islet and islet/MP groups ($P<0.01$). (G) Area per vessel (μm^2) calculated by dividing the total vessel area (measured by Image J) to the number of vessels per field (in five fields of five tissue sections). (H) Representative images of hematoxylin and eosin (H&E) staining and insulin immunohistochemistry of the harvested islets/MP/VEGF scaffold. Staining of the graft from islet/MP/VEGF group showed functional islets close to the vessel. Values represent mean $\pm$ SD, n = 6 per group. * $P<0.05$, ** $P<0.01$, *** $P<0.005$. Tx: Transplantation.

Figure 1

Figure 2

Figure 3

Figure 4